Effect of Environmental and Occupational Pollutants on Health Status of Brick Kiln Workers: A Molecular and Cellular Based Study

By

Mehwish David

DEPARTMENT OF ZOOLOGY

FACULTY OF BIOLOGICAL SCIENCES

QUAID-I-AZAM UNIVERSITY

ISLAMABAD

2023

Effect of Environmental and Occupational Pollutants on Health Status of Brick Kiln Workers: A Molecular and Cellular Based Study

A dissertation submitted in the partial fulfillment of the requirements for the Degree of Doctorate of Philosophy

IN

ZOOLOGY

(REPRODUCTIVE PHYSIOLOGY)

By

Mehwish David

DEPARTMENT OF ZOOLOGY

FACULTY OF BIOLOGICAL SCIENCES

QUAID-I-AZAM UNIVERSITY

ISLAMABAD

2023

The fear of the LORD is the beginning of wisdom, and knowledge of the Holy One is understanding

Proverbs 9:10

Dedicated to My Parents

Who gave me the opportunity to

study from the best institutions and

supported me throughout my life

Author's Declaration

I **Ms. Mehwish David** hereby state that my PhD thesis titled "Effect of Environmental and Occupational Pollutants on Health Status of Brick Kiln workers: A Molecular and Cellular Based Study" is my own work and has not been submitted previously by me for taking any degree from Quaid-i-Azam University, Islamabad, Pakistan.

At any time if my statement is found to be incorrect even after my Graduate the University has the right to withdraw my Ph.D. degree.

Mehwish

Ms. Mehwish David

Date: 19.07.2023

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled "Effect of Environmental and Occupational Pollutants on Health Status of Brick Kiln workers: A Molecular and Cellular Based Study" was conducted by **Ms. Mehwish David** under the supervision of **Prof. Dr. Sarwat Jahan**. No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Zoology of Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Field of Reproductive Physiology.

Student Name: **Ms. Mehwish David** Signature: Mehwish

Examination Committee:

a) External Examiner 1: Signature:

Dr. Jahangir Arshad Khan
(Ex Chief Research Officer)
House No. 68, Street No.51,
F-11/3, Islamabad

b) External Examiner 2: Signature:

Prof. Dr. Shamim Akhtar
Chairperson
Department of Zoology
PMAS Arid Agriculture University,
Rawalpindi

Supervisor Name: **Prof. Dr. Sarwat Jahan** Signature: Sarwat Jahan

Name of HOD: **Prof. Dr. Amina Zuberi** Signature:

Date: 19.07.2023

Plagiarism Undertaking

I solemnly declare that research work presented in the thesis titled "Effect of Environmental and Occupational Pollutants on Health Status of Brick Kiln workers: A Molecular and Cellular Based Study" is solely my research work with no significant contribution from any other person. Small contribution/ help wherever taken has been duly acknowledged and that complete thesis has been written by me.

I understand the zero tolerance policy of the HEC and Quaid-i-Azam University towards plagiarism. Therefore I as an Author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/ cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of Ph.D degree, HEC and the University has the right to publish my name on the HEC/University Website on which names of students are placed who submitted plagiarized thesis.

Student / Author Signature: Mehwish

Name: **Ms. Mehwish David**

List of contents

List of Tables

List of Figures

List of Abbreviations

World Health Organization	WHO
Acquired immunodeficiency syndrome	AIDS
Coronavirus disease 2019	COVID-19
Environment protection agency	EPA
Particle induced Xray emission	PIXE
Atomic absorption spectroscopy	AAS
Scanning electron microscopy	SEM
Geographic information system	QGIS
Complete blood count	CBC
National Institute of Standards and Technology	NIST
Personal protective equipment	PPE
Standard reference material	SRM
Food and Agricultural Organization	FAO
Carbon monoxide	CO
Sulfur dioxide	SO_2
Nitrogen oxides	NOx
Volatile organic compounds	VOCs
Ozone	O_3
Particulate matter	PM
Gross domestic product	GDP
Demographic & Health surveys	DHS
Body mass index	BMI
Revolution per minute	rpm
Parts per million	ppm
Microgram per decilitre	µg/dL
White blood cell count	WBC
Red blood count	RBC

Platelet count	PLT
Haemoglobin	Hgb
Total haematocrit	HCT
Mean corpuscular volume	MCV
Mean corpuscular haemoglobin	MCH
Mean corpuscular haemoglobin concentration	MCHC
Red blood cell distribution width	RDW-CV
Red blood cell distribution width –standard deviation	RDW-SD
Platelet Distribution Width	PDW
Plateletcrit	PCT
Mean platelet volume	MPV
Sodium dismutase	SOD
Peroxidase	POD
Reactive oxygen species	ROS
Thiobarbituric acid reactive substances	TBARs
Malondialdehyde	MDA
Millilitre	ml
Millimole	mM
Micromole	µM
Nanomole per millilitre	nmol /ml
Umdrehungen pro Minute	U/min
Milli-international units per milliliter	mIU/ml
Nanometer	nm
Gram per decilitre	g/dl
Nanogram per millititre	ng/ml
Micromole per minute	µM/min
Nanomole	nmole
Absorbance	Abs

Number	n
Hours per day	h/day
Enzyme immune assay	EIA
Luteinizing hormone	LH
Follicle stimulating hormone	FSH
Testosterone	T
Mean and standard error of mean	Mean ± SEM
Pearson's correlation	r
Significance	p
Personal protective equipment	PPE
Hypothalamic pituitary gonadal	HPG
Lea	Pb
Chromium	Cr
Cadmium	Cd
Nickel	Ni
Zinc	Zn
International Centre for Integrated Mountain Development	ICIMOD

ACKNOWLEDGEMENTS

All praises to **God Almighty**, Who is powerful and worthy of all praises, Who galvanize this world into action; Who said and things came into being accordingly; He created man from the dust and gave him life, Who put his wisdom in mankind and gave him insight to differentiate right from wrong and explore hidden mysteries of this world.

It is a matter of great pleasure to express my sincere regards to my honorable and respectful Supervisor Professor **Dr. Sarwat Jahan**, Department of Zoology for her affectionate supervision, inspiring attitude, masterly advice, and encouragement. Without her useful intellectual suggestions, it would have been impossible for me to complete this tedious work. She has guided me throughout not only as a supervisor, but as a mother and friend as well. I would like to extend my thanks for providing me opportunity and making the department facilities available for me.

I am thankful to all my senior professors specially our chairperson **Dr. Amina Zuberi, Dr. Irfan Zia, Dr. Muhammad Shahab, Dr. Sajid Malik, and Dr Mariam Anees**, whose guidance and assistance in every research problem was remarkable.

I am also thankful to **Saeed bhai, Johnson and Maalik** for their support and cooperation during the laboratory work.

I would like to extend my humble thanks to **Dr. Muhammad Ishaq**, Director Experimental physics department (EPD, NCP), **Mr. Javaid Hussain** (SSO) and **Dr. Khurram** for their endless cooperation for using their PIXE machine and analysis of data. I would like to extend my humble thanks to Madam **Dr Karen Cloete** for her valuable suggestions and guidance throughout my PhD work.

I would like to specially appreciate my family members, my parents, brothers, and sister who always stood behind me like a shield and were there to support me endlessly. It was not possible to work alone without their involvement. I would like to extend my gratitude to God for blessing me with two angels in the form of my nieces Genesis Ayana and

Amaris Eva who are my ultimate source of happiness, energy and positivity and whose love, affection and kindness did let me survive in every challenging situation.

I am thankful to my senior, mentor and friend **Dr. Qurat ul Ain** who supported me like a sister and stood with me since day one till now in every problem. It would not have been possible without her sincere efforts. Her assistance, and help cannot be acknowledged in words. I would also like to extend my thanks to **Dr. Muhammad Afzal**, and **Dr. Suhail Razak** for their guidance and support.

I would like to thank everyone who became part of my journey from start to end. Their love, hatred, support, and criticism kept me going and motivated me to fulfill my dream of becoming a good researcher. I have learned from each and every one of you and there are certain names that I don't even remember but I am really thankful to all my juniors and lab fellows including **Ms. Faryal Tariq, Muhammad Shoaib, Faiza Aman, Naheed Turi, Mavra Abbas, Naham** and **Sania Ali** for their help during my whole research work. I wish to extend my greatest appreciation and thanks to my best friends **Zarba Dilbar** and **Syeda Tabinda Mariam** and my friend **Daud Danial,** who cheered and supported me and above all, trusted me. Their believe in me is something that helped me stand and face the hurdles of this journey.

I would like to extend my thanks to **Sir Naeem Masih** for his affectionate efforts, encouragement, and cooperation during my whole PhD.

I would specially like to pay my heartiest thanks to all my best juniors and fellows who made my last days of degree memorable with their care, respect, support, and positive attitude specially **Inam Ullah, Anosh Suhail, Rimsha Javaid, Monica Gill, Faiza Rafique, Kibria Hassan, Kashif Khan** for their constant respect, support and admiration.

GENERAL ABSTRACT

Background:

Environmental pollution is a matter of global concern. With the increasing need for industrialization, the rate of environmental degradation due to pollution is also increasing on daily basis. The brick kiln industry is the fastest growing industry in South Asia with an increased number of bonded laborers. This industry emits enormous amounts of poisonous gases and traces elements directly into the atmosphere that affect all life forms, especially brick kiln workers through external deposition (dermal) as well as through internal route (inhalation and ingestion). In Pakistan, the number of laborers associated with the brick kiln industry is quite high, which raises public health concerns. Several studies have reported that emitted metals are deposited in blood and affect multiple biochemical reactions in humans. Heavy metals are known to alter blood profile, increase production of reactive oxygen and acidic species, decrease antioxidants levels, alter hormone concentrations in blood, bring genotypic effects, as well as modifying gene expression. Heavy metals induce genetic changes by creating single nucleotide polymorphisms (SNPs) in important structural and functional genes, whose altered function might bring physiological conditions in body.

Objectives:

The present study aims to estimate the metal burden in blood and possible hazardous effects of heavy metals emission from coal clay brick kilns on human health regarding child growth, pubertal development, maternal health, and reproductive health in men. The objectives of the study include:

- The evaluation of the socio-demographic, work associated, and general health determinants among brick kiln workers and non-workers, along with an emphasis on the extrinsic and intrinsic deposition of heavy metal burden in soil and biological samples (blood and hair).

- Finding the comparative environmental health effects of heavy metal burden in blood among brick kilns workers and control individuals, by looking at blood parameters, oxidative stress markers, antioxidant enzymes concentrations, and reproductive hormones profile along with stress response on hypothalamic-pituitary-gonadal (HPG) axis.
- Measurement of possible deleterious effects of brick kilns emitted metals on female workers regarding reproductive health indicators, blood and lipid profile, antioxidant status, reproductive hormone concentrations, and direct effect on hypothalamic pituitary adrenal (HPA) axis.
- Identification of genetic mutations in the ABCG2 gene in brick kiln workers and control subjects from the Pakistani population.
- Investigation of lethal effects of kiln emitted heavy metals on pubertal development and child health by monitoring blood parameters, antioxidant enzyme status of the body, reproductive hormones, induced DNA damage, growth hormone and cortisol concentrations, that eventually may affect development and puberty.

Materials and methods:

The study involved a total of 1053 participants including men (n=346, n=200), women (n=118, n=114), and children (=n175, n=100 control) from Rawat, Punjab. Information regarding demographic data, personal health information, fertility indicators from women, and body mass index (BMI) was gathered. Hair and blood samples were collected for heavy metal determination through atomic absorption spectroscopy (AAS), external beam PIXE and scanning electron microscope (SEM/EDS). Further, blood was divided into two halves, one half was subjected to hematological and genotoxic analysis (comet assay), while the other half was centrifuged, plasma was obtained and stored at − 20° to study biochemical variables (sodium dismutase, peroxidase, reactive oxygen species, thiobarbituric acid

reactive species, protein estimation), lipid profile (HDL, LDL, TG, cholesterol), growth hormone (GH), cortisol and concentrations of reproductive hormone (testosterone, luteinizing hormone, follicle-stimulating hormone, oestradiol, progesterone, prolactin) concentrations by immunoassay.

To understand the functional changes in the ABCG2 gene, two exons (exons 3 and 5) were selected. The collected blood samples were subjected to DNA extraction, gel electrophoresis and nanodrop, DNA amplification, gel electrophoresis, gene sequencing, and post-sequencing analysis using BioEdit, Chromas, and mutation taster.

Results:

The results showed that about 10% of the adult kiln laborers were underweight and had multiple health issues including skin allergies (1%), asthma (10%), stomach and kidney disorder (5%), and other diseases. Almost 58% of brick workers were addicted to tobacco ($p = 0.363$) and were working in poor and unhygienic conditions without the use of any personal protective equipment. Examination of blood for heavy metals concentrations through AAS revealed significantly ($p<0.001$) higher levels of Cd, Cr, and Cr in the working group as compared to the control group. PIXE data further detected higher than permissible levels of Si, P, S, Fe, Co, Ni, Cu, Cl, K, Ca, Ti, Mn, and Zn, in the blood of kiln workers. The SEM/EDS analysis of hair depicted the presence of macro-elements with an average concentration in the order of K > S > Ca > P > Cl. Similarly, a micro-element profile with mean levels in the order of: Rb > Fe > Mn > Cu > Sr > Zn were seen.

Analysis of male samples showed a significant increase in platelet count ($p=0.010$); decreased antioxidant enzyme ($p<0.01$) and increased oxidants level ($p<0.001$); increased cortisol levels ($p<0.001$) were seen in workers in contrast to the control group. The inverse relationship was found between cortisol and pituitary gonadotropins (FSH $r = 0.676$, LH $r = 0.580$); cortisol and testosterone ($r = 0.832$).

Analysis of female samples showed increased platelet count; decreased antioxidant enzyme and increased oxidants level; amplified total cholesterol, low-density lipoprotein (LDL) and triglyceride (TG); reduced total protein and high-density lipoprotein (HDL); and increased cortisol levels among workers in contrast to control group. A significant decrease ($p<0.001$) in FSH, LH, estradiol, and progesterone concentration, while a significant increase in prolactin levels was seen among workers groups as compared with control. The findings of correlation studies revealed that blood plasma cortisol levels negatively correlate with FSH ($r=-0.872$), LH ($r=-0.856$), estradiol ($r=-0.923$) and progesterone ($r=-0.879$) concentrations with significance value of $p<0.001$. The blood plasma cortisol level positively correlated with prolactin concentration ($r=0.874$).

A total of twenty- eight genetic variations, including 25 novel ones were noted: 24 of them were present in the coding exons, and 1 in the intronic region. Results revealed that mutations were found in both worker and control samples. In addition to three previously reported nonsynonymous single nucleotide polymorphisms, g.91361G>A (rs2231137), g.91461G>A (rs142634180) and g.91521C>T (no rsID), the novel variations found in workers samples were present at genomic position g.91474G>A, g.91482delinsAA, g.91410T>A, g.91414G>A, g.91416C>A (rs142634180), g.91419T>A, g.91427G>A, g.91436T>A, g.91450C>T, g.91454T>A, g.91456C>A, g.91459T>A, g.91461G>A, g.91463G>A, g.91416C>A, g.91427G>C, g.91430G>C, g.91440_91441delinsAA, while those found in control are g.91474G>A, g.91487C>A, g.91507G>A, g.91521C>T, g.91346G>C, g.91416C>A, g.91430G>A, g.80676T>A, g.91446A>T. The V12M variant (rs2231137) has been previously reported and is associated with multiple metabolic diseases.

The demographic data for children demonstrated that 70% of boys and 68% of girls were illiterate and early marriage practice was common among them. The biochemical analysis

of children samples presented a significant decrease in percent hematocrit (HCT) in the brick kiln group in contrast to the control. Further, results showed a significant increase in white blood cells ($p<0.05$), reactive oxygen species ($p<0.001$), and decrease in catalase ($p<0.001$), peroxidase ($p<0.001$), and sodium dismutase ($p<0.01$). There was observed a drop in GH levels from 1.66±0.08 ng/mL in control to 0.87±0.13ng/mL in brick kiln children. Similarly, an increase in cortisol concentration from 0.83±0.14 ng/mL in control to 1.81 ± 0.05 ng/mL in brick kiln group was evident. The results of the comet assay showed a decrease in percent DNA in the head and an increase in the tail region among the exposed group in contrast to the control.

Conclusion:

A high concentration of trace elements and heavy metal burden in brick kiln workers induced multiple health disorders such as respiratory and stomach problems. Likewise, external, and internal deposition of heavy metal in soil and on hair and blood samples of kiln workers was quite elevated as compared to control subjects. Conclusively, it is stated that men, women and children subjected to brick kiln emissions experience heavy metal burden in blood; altered blood profile; decreased antioxidant levels, increased oxidant species production; disturbed lipid profile; and reduced production of LH, FSH, E, P and T, while increased production of cortisol and prolactin in women, respectively. Additionally, we found 28 variants of the ABCG2 gene (a placental gene) in females, indicating its possible altered function in humans. Moreover, we concluded that brick kiln children experience poor hygiene, poor health conditions, and growth spurt in growing boys and girl children that puts them at high risk for the development of metabolic and reproductive disorders.

GENERAL INTRODUCTION

Introduction

In developed countries, understanding and protection of a healthy environment are subjects of interest. Whereas, developing countries including Pakistan and other Asian countries lack such attitude. The environment is comprised of three major elements, including soil, air, and water, and thus, environmental pollution is defined as the pollution of physical components of the environment. It is a result of rapid urbanization and industrialization, burning of fossil fuels, mining, man-made activities, and the release of hazardous compounds and gases directly into the environment (Ukaogo *et al*., 2020). These compounds become part of air and are deposited in soil and water bodies and are consumed by living organisms. They might be present in bulk quantities than permissible amounts in the form of solid, liquid, or gases, compromising the quality of our surroundings (Manisalidis *et al*., 2020). In recent years, it has been noticed that the problem of environmental pollution is not only restricted to developing countries, but it has also become a global issue. Due to risks associated with the prevalence of adverse health effects pertaining to environmental pollution, now efforts are being made on an international level to limit/regulate environmental degradation either through redevelopment or via the introduction of eco-friendly technologies/products (Luka *et al*., 2018). However, it is observed that the increasing need for industrialization is responsible for a day-to-day increase in the rate of environmental degradation owing to pollution (Ismail *et al*. 2012).

Air pollution

Air pollution is a global problem for developed and developing countries. Rapid industrialization and urbanization, on-road vehicles, burning of fuels, emission of industrial waste, industrial accidents, human activities, and overpopulation are some of the contributing factors that have caused poor air quality on a global scale (He *et al*., 2020;

Manisalidis *et al*., 2020; Silver *et al*., 2018). Air pollution can be described as the "*occurrence of specific concentrations of one to many contaminants such as smoke, gases, steam, particles, mist, disgusting odor present in the open air"* (Mostafavi *et al*., 2021). It serves as one of the environmental hazards to public health (Tan *et al*., 2021). It is known to be responsible for more deaths worldwide than acquired immunodeficiency syndrome (AIDS) (Rohde & Muller, 2015). According to the world health organization (WHO), it is estimated that air pollution is accountable for approximately one million premature deaths globally each year (Shah *et al*., 2013). A recent report by WHO Global Conference on Air Pollution and Health (WHO, 2018), indicated that household and outdoor air pollution has caused 7 million deaths each year (Ouyang *et al*., 2018). It has also been reported that in 2012 components of air pollution i.e, airborne particulate matter (PM) killed almost 93,000 people in the European region (Ortiz *et al*., 2017). Similarly, among Asian countries, China has been badly affected by air pollution, with 17% of all deaths due to air pollution causing 1.6 million casualties/year (Rohde and Muller, 2015). Globally, each year, approximately two million deaths occur due to respiratory disorders and lung failure-as a direct consequence of air pollution (Shah *et al*., 2013). A recent study reports that 91% of the world's population currently resides in areas with poor air quality than permissible conditions as determined by WHO (Mostafavi *et al*., 2021). Therefore, it is inferred that with rapid industrialization and population spread, the quality of air has been compromised which can be seen by statistical reports from the literature.

Components of air pollution and their effects

Since olden times, air pollution is known to exert detrimental effects on human health due to multiple air pollutants. These include oxides of carbon (CO), sulfur (SO_2), nitrogen (NOx), and other components (Kampa *et al*., 2008; Woodruff *et al*., 2008). These air pollutants are introduced into the air via vehicle smoke, industrial smog, hospital wastes

and industrial emissions (Ismail *et al*., 2012). Once they become part of environment, they are either inhaled or ingested via food among human and animals. Short term and long-term exposure to air pollutants impart public health concerns (Ortiz *et al*., 2017). These are responsible for causing large number of respiratory disorders as suggested by different study groups (Hauser *et al*., 2015; Kim *et al*., 2015; Raza *et al*., 2014; Woodruff *et al*., 2008). Recent studies reported that there occurs direct correlation of some air pollutants with the prevalence of human respiratory viruses such as coronavirus disease 2019 (COVID-19), severely affecting the respiratory system (Domingo & Rovira, 2020). Along with respiratory diseases, the risk of myocardial infarction and several types of cancers have also increased in individuals exposed to different types of air pollutants (Kampa *et al*., 2008; Raaschou *et al*., 2011; Shah *et al*., 2013). Studies suggest that air pollution adversely affect different body organs and disturb normal functioning of cardiovascular, developmental, respiratory, reproductive, and neurological systems (Curtis *et al*., 2006). Further, it has been reported by different researchers that air pollution not only raise public health concerns, but also compromises reproductive health (Bhatt, 2000; Eljarrat *et al*., 2020; Kumar, 2018). Normal physiological roles of male and female reproductive systems are altered/compromised by exposure to different kinds of pollutants, that might result in infertility (Alaee, 2018). For example, exposure to oil related environmental contaminants such as benzene and toluene are known to exert toxic effects on female reproduction by interfering hypothalamic pituitary ovarian (HPO) axis and fertility outcomes (Sirotkin & Harrath, 2017). Similarly, other studies report that air pollution is a major cause of multiple reproductive adversities in males and may cause infertility (Jurewicz *et al*., 2018). Thus, it is suggested that different kinds of air pollutants exert multiple health hazards and reproductive anomalies among animals and human.

Causes of air pollution- Role of industries

Large number of industries contribute to air pollution especially heavy polluting industries (Lin *et al*., 2021). These include petrochemical, textile, mining, construction, oil refinery, vehicle industries, and brick kiln industries (Khan *et al*., 2019). The consumption, burning and emission of fossil fuels such as coal, petroleum and factory combustibles is responsible for polluting the environment. United States Environmental Protection Agency (EPA) has suggested that 21% of the global emission of greenhouse gases by burning of fossil fuel is contributed by different industries across the globe (Intergovernmental Panel on Climate Change, 2014). The key source of air pollution is brick kiln industry especially in under developing countries including Indo-Pak region. Brick kiln industry is the rapidly growing industry with constant increase in population growth (Bhat *et al*., 2014). Clay bricks formed through firing are the most commonly used bricks in construction, that use burning of fossil fuels (Sain & Meena, 2018). Combustion of low-quality fuels like rubber tires, motor oils and coal emits large amount of hazardous and greenhouse gases from these traditional brick kiln industries (Achakzai *et al*., 2015, 2017).

Brick kiln industry- A major source of air pollution

According to ICIMOD, the brick industry is responsible for 20–25% of air pollution, worldwide and therefore, is a major cause of global warming (https://www.icimod.org/success-stories/chapter-5/pakistans-brick-kiln-makeover/). The traditionally running brick kiln industries in suburban areas in under-developing countries are polluting environment as they serve as a source of hazardous emission (Gómez *et al*., 2012; Shahid *et al*., 2017). This suggests that heavy emitting industry such as brick kiln industry is a major contributor of air pollution worldwide, but specially in South and Southeast Asia. The figure 1 shows the global production of brick kiln according to the

International Centre for Integrated Mountain Development (ICIMOD) (https://www.countercurrents.org/nazareth011215.htm).

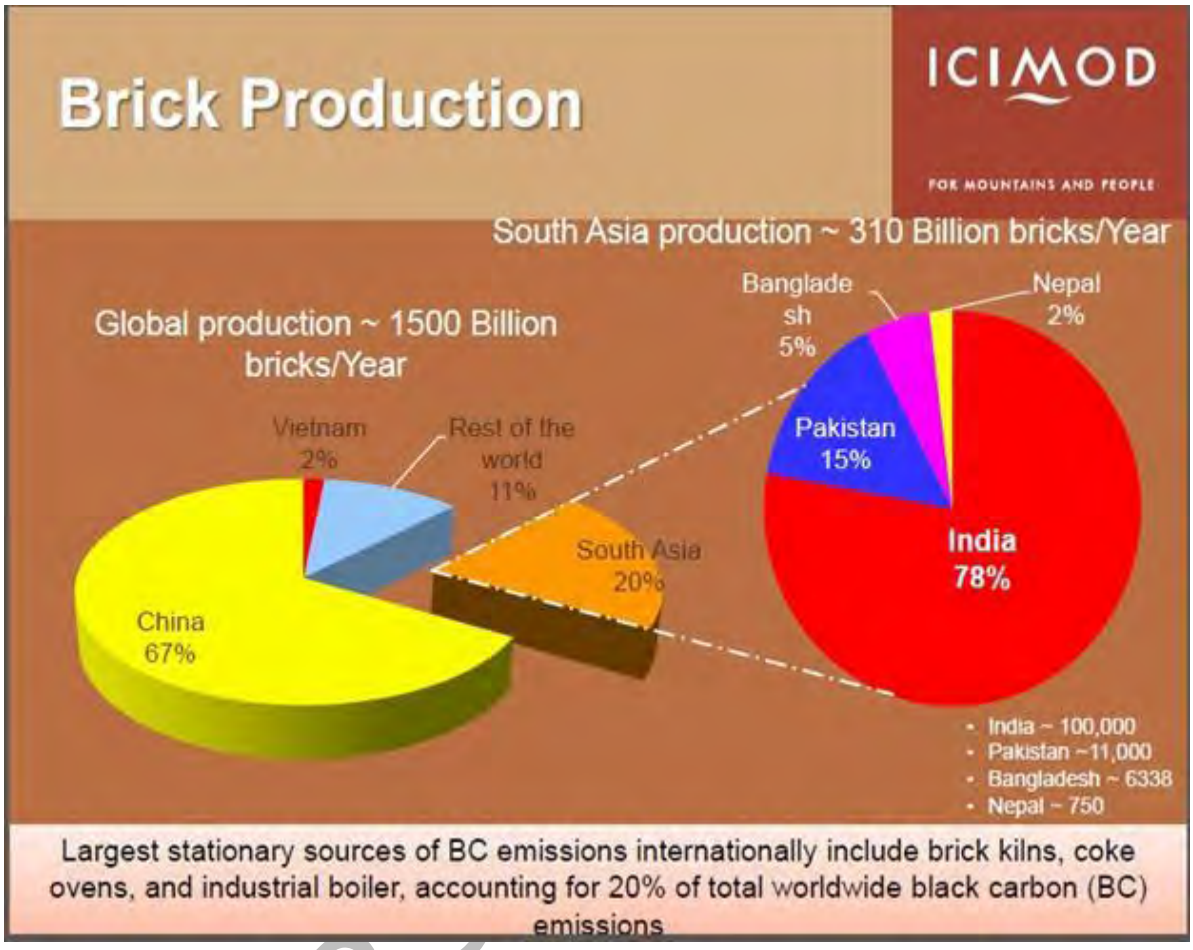


Figure 1. Brick production rate around the globe with focus on South Asian countries (ICIMOD, 2015).

It is estimated that in Asia, yearly greater than 1.2 trillion bricks are produced (Lopez *et al*., 2012). Studies report that China (54%,) India (11%), Pakistan (8%) and Bangladesh (4%) produces major bulk of the world's bricks (Baum, 2010). These countries are using different technologies for brick manufacturing. Saeed (2017) have reported that Pakistan stands 3rd in production of bricks after China and India in South Asia (Saeed, 2017). According to ICIMOD survey of 2018, the annual production rate of bricks in Pakistan is 82.5 billion while annual brick demand is 112 billion bricks (Sheet, 2018). Considering

the fact that Pakistan is a developing country, it is facing many issues of land and air pollution, public health and population progression, thus, needs for shelter, food and energy are rising, burdening natural resources as well as country's economy (Kamal *et al*., 2014). This has in turn increased the demand for construction of buildings, houses, and residential flats. A remarkable role has been played by brick kiln industries in this regard. Pakistan has large number of brick kilns in different cities (Bales, 2012). Studies have reported that approximately 20,000 brick kilns are present across the country, while 10,000 kilns are present only in Punjab province producing 45 billion bricks per year (Sharif & Nasim, 2018). With rising demands for bricks required for construction, expansion of brick kiln industries has also been increased. However mostly, these are developed on the roadsides or near agricultural land due to lack of proper land allotment (Ercelawn & Nauman, 2004). The pollution resistant plants are seen, grown near the brick kiln sites. The monitoring systems for controlling and checking pollution index in developing countries especially in Pakistan are underprivileged. No strict rules and regulations are followed for development and implementation of industrial projects that are adversely affecting not only the air quality of specific area, but also, are responsible for causing various respiratory and other health diseases in humans. Previously, Kamal *et al*. (2014) reported in their study that number of brick kiln workers in Pakistan is approximately 1.8 million (Kamal *et al*., 2014). These include men (77%), women (23%), and children of all age groups (Sheet, 2018). The survey report by Labor and Human Resource Department, Government of the Punjab, presents that currently, 87,000 families are associated with brick kiln industry in the Punjab province only, with another study reporting 249, 682 brick kiln workers including men and women (David *et al*., 2020). With such a large number of brick kilns across the country and associated labor, it is suggested that a serious

public health concern is raised and thus, there is a need to regularly monitor the efficiency of environmental and occupational safety for workers.

Brick kiln pollutants

Brick kilns release large amounts of hazardous gases in the form of black smoke directly into the environment. These include oxides of carbon, nitrogen, sulfur, ozone, dust, heavy metals (arsenic, cadmium, chromium, nickel), and particulate matter (PM). These pollutants are accumulated in the environment in soil, water and air and become part of them. Long term deposition of these metals in physical components of environment also affect the biological components. As brick kilns are constructed outside urban areas and are mostly associated with agricultural land, therefore brick kiln emissions are deposited on plants as well. Large number of heavy metals such as arsenic (As), cadmium (Cd), nickel (Ni), zinc (Zn), chromium (Cr), lead (Pb), and iron (Fe) are present in the environment either in air, soil, or water. Animals and humans are exposed to these pollutants/metals directly via inhalation or through ingestion of contaminated plant parts. Human exposure to brick kiln emission raise serious public health issues and is known to impart deleterious effects on overall physiology of body (Begum *et al*., 2011; Shaikh *et al*., 2012).

Impact of kiln emitted gases on environment and health

The brick kiln emitted gases are known to impart hazardous effects on the surrounding environment. Oxides of carbon i.e Carbon monoxide (CO) and dioxide CO_2 are released from brick kilns in large quantities. On a global scale, brick kiln industry is a major source of CO_2 and black carbon (BC) production (Lopez *et al*., 2012). It is reported that in South Asia, brick kiln yearly emits tons of CO (3.9 million tons) and CO_2 (127 million tons) (Rajarathnam *et al*., 2014). Another studies by Skinder, (2014) showed that only in Dhaka, Bangladesh, brick kiln industry emits these oxides in abundance with CO (302,000 tons),

BC (6,000 tons) and CO_2 (1.8 million tons) (Skinder, 2014). CO is generated by the combustion of carbonaceous fuels. Exposure to CO is shown to induce adverse neurodevelopmental disorders (Levy, 2015). Further studies report that each year, black carbon is accountable for causing approximately 2.4 million premature deaths (Lopez *et al*., 2012). Another important oxide released by brick kilns is sulfur dioxide (SO_2). It is estimated that SO_2 is present in large quantities in atmosphere as a key airborne pollutant and the burning of fossils fuels releases 75-85% of SO_2 worldwide (Skinder, 2014; Zhang *et al*., 2016). SO_2 can cause various respiratory and optic disorders (Geravandi, Goudarzi, & Mohammadi, 2015). It is also reported to impart harmful effects on male reproduction among humans and different animals (Zhang *et al*., 2016). Nitrogen dioxide (NO_2) is another toxic gas found in the atmosphere, known to induce neurotoxic effects in rodents through its oxidant potential (Li *et al*., 2012). In humans, short term exposure to NO_2 is shown to be a contributing factor causing conjunctivitis (Lu *et al*., 2019). NO_2 induces inflammatory actions in lungs, and cause respiratory syndromes (Ogen, 2020). Recent studies have suggested that NO_2 exposure is also responsible for accelerating the Covid-19 relevant death ratio in various countries (Pacheco *et al*., 2020; Zoran *et al*., 2020). Therefore, it can be suggested that exposure to toxic gases emitted from brick kilns impart multiple health conditions and have deleterious effects on public health.

Presence of volatile organic compounds (VOCs) present another hazardous component of polluted air. VOCs are produced because of natural sources and anthropogenic activities such as the combustion of fossil fuels and industrial wastes (Bozkurt *et al*., 2018). Biogenic VOCs release also cause the formation of fine $P.M_{2.5}$ and ground level ozone (O_3) pollution (Ren *et al*., 2017). VOCs exposure is known for causing respiratory syndromes, DNA damage, reproductive, developmental dysfunctions and deficient immune responses in children (Montero *et al*., 2018). In 2015, it is reported that

VOCs were responsible for causing more than 179 million deaths worldwide (Ren *et al*., 2017). Oxides of nitrogen and hydrocarbons in the atmosphere result in the formation of airborne ozone, which is considered as environmental oxidant (Bromberg, 2016). Exposure to airborne ozone pollution is associated with premature mortality (Madaniyazi *et al*., 2016). O_3 is also responsible for causing 153 million deaths worldwide (Ren *et al*., 2017). It causes pulmonary inflammation by macrophage activation and epithelial receptors (Bromberg, 2016). It mediates its toxic effects in body through its ability to produce free radicals through multiple mechanisms (Mauro *et al*., 2019). Burning of fossil fuels release enormous amounts of fine particulate matter (PM) into the environment. About 0.94 million tons of PM is produced only in South Asia (Rajarathnam *et al*., 2014). This airborne PM is responsible for 3-7 million deaths per year and in 2015, was considered as fifth ranking mortality risk factor (Cohen *et al*., 2017; Rohde & Muller, 2015). It is known to impart hazardous effects on lungs, kidneys, heart, inducing all-cause mortality (Andreão *et al*., 2018; Guttikunda *et al*., 2013; Khan *et al*., 2019a; Li *et al*., 2018). $PM_{2.5}$ and PM_{10} is associated with multiple health and eyes problems (Lu *et al*., 2019). A study reported that $PM_{2.5}$ caused 26 million deaths in 2015 (Ren *et al*., 2017). Recent studies also indicated that positive correlation is found between $PM_{2.5}$ concentrations and corona virus transmission in Islamabad, Pakistan (Aslam *et al*., 2021). Thus, it is concluded that the industrial release of biogenic VOCs with associated P.M and O_3 into the environment raises serious health concerns for public.

Release and deposition of emitted metals on the surrounding environment

Soil and water constitute an important component of environment, that deposit large amounts of heavy metals. Since the last few decades, it is evident that with population explosion, the pattern of land use has changed, making soil the most important source of pollution in developing countries (Achakzai *et al*., 2017). Heavy metals released as a result of industrial and anthropogenic activities are deposited in the soil surrounding kiln areas, where vegetation is grown (Achakzai *et al*., 2015; Shen *et al*., 2019). The presence of these metals in the soil is the major cause of soil degradation. These metals in the soil are absorbed by the plants and are deposited on edible parts of plants (Jaiswal *et al*., 2018). Animals and humans living in the vicinity of brick kilns consume these crops and vegetables and therefore, are directly exposed to metals (Khan *et al*., 2019). Similarly, drinking water, and natural water resources also deposit metals from the environment. Studies have suggested that nearly 700 organic and inorganic compounds are known to be present in water, of which heavy metals are considered as the most toxic component due to their deleterious effects at the cellular level (Ali, 2010; Järup, 2003). These metals exert toxic effects on aquatic life forms by causing metabolic or reproductive disorders (Paschoalini *et al*., 2019; Sfakianakis *et al*., 2015). Following WHO and the Federal Environmental Protection Agency (FEPA) guidelines, the concentration of metals in water is more than the permissible limits, thus posing health risks in plants and aquatic species (Jaiswal *et al*., 2018). Recent studies indicate that a variety of metals impart cytotoxic effects on biochemical processes and physiology of plants depending upon their type, concentration, chemical form, and duration of exposure (Ackova, 2018). Therefore, it is concluded that brick kiln surroundings where, soil, water, air, and even food components are polluted with metals, the increased incidence of public health concerns is definite that needs monitoring.

Heavy metal emission from brick kiln

Brick kilns release enormous amounts of heavy metals into the atmosphere. These include cadmium, chromium, nickel, arsenic and lead (Achakzai *et al*., 2015; Ismail *et al*., 2020). These heavy metals are deposited in the environment and affect surrounding life forms. Some of the most common metals and heavy metals that are known to impart hazardous effects on human health are Cd, Cr, Ni, As, mercury (Hg), Zn, Pb, and thallium (Tl) (Ruano *et al*., 2016). Figure 2 summarized the path of metals and PM through the respiratory tracts and their ultimate effects on body systems.

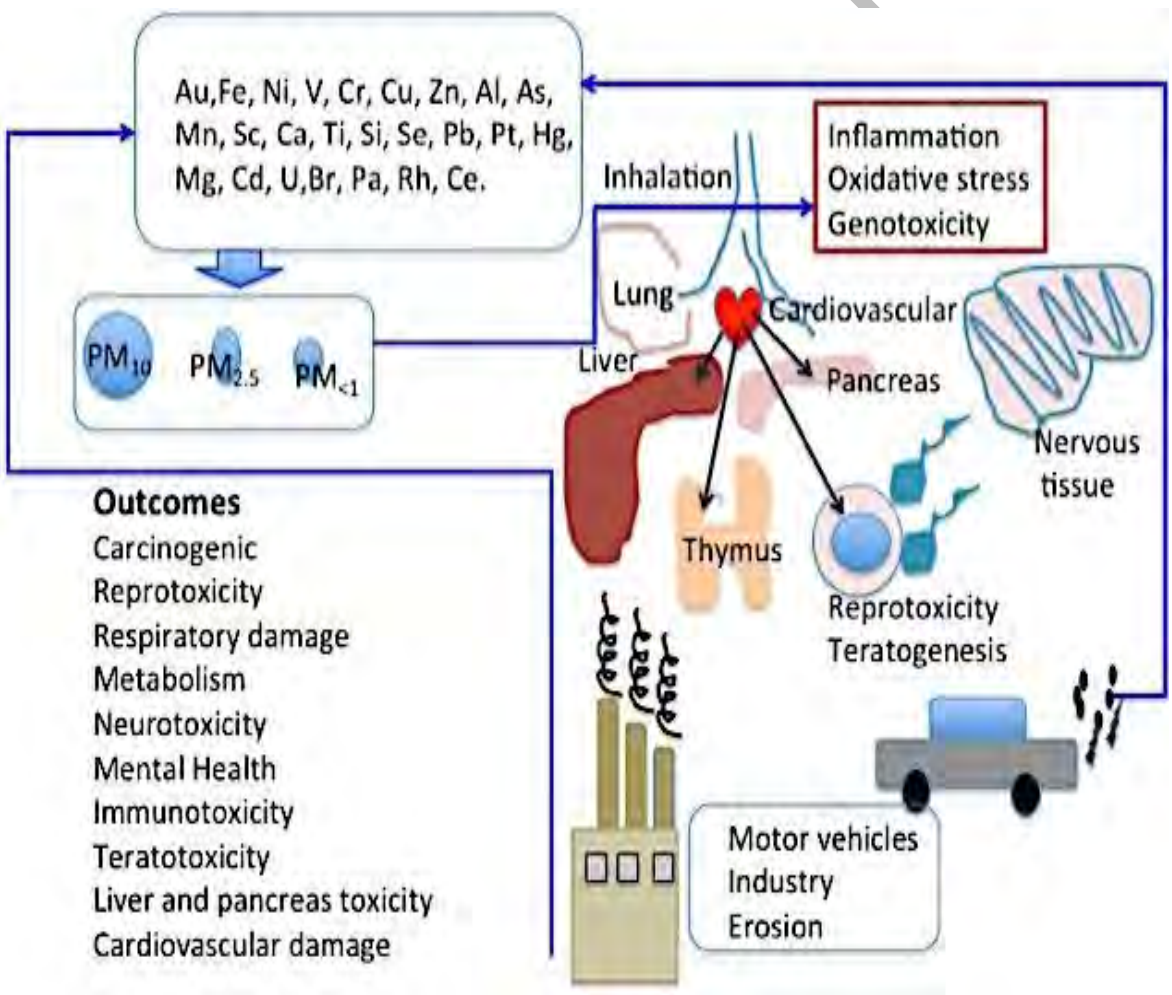


Figure 2. Route of inhalation of different environmental pollutants (heavy metals and PM) and their target tissues (Ruano *et al*., 2016).

Cadmium toxicity in cells

Among industrialized countries, increased rate of industrial and anthropogenic activities since the last few decades have resulted in cadmium (Cd) pollution, thus, raising Cd related health concerns (de-Angelis *et al*., 2017). Cd exert its actions in organisms at cellular level by affecting signaling cascades (Thévenod, 2009). Cd toxicity not only affects general health, but also reduces the reproductive potential among species. Figure 3 summarizes the overview of possible mechanism of actions through which cadmium toxicity is mediated (de-Angelis *et al*., 2017). Cd is known to induce toxicity in reproductive tissues of males through multiple mechanisms (de-Angelis *et al*., 2017). Cd exposure is reported to downregulate the expression of genes responsible for sperm motility; Cd along with nickel also decrease the sperm efficiency by reducing percent sperm count, motility and altering sperm morphology (Mohammadi *et al*., 2018a). In females, Cd exposure result in multiple pathological conditions such as abnormal oocyte maturation, and reduced steroidogenesis (Thompson & Bannigan, 2008).

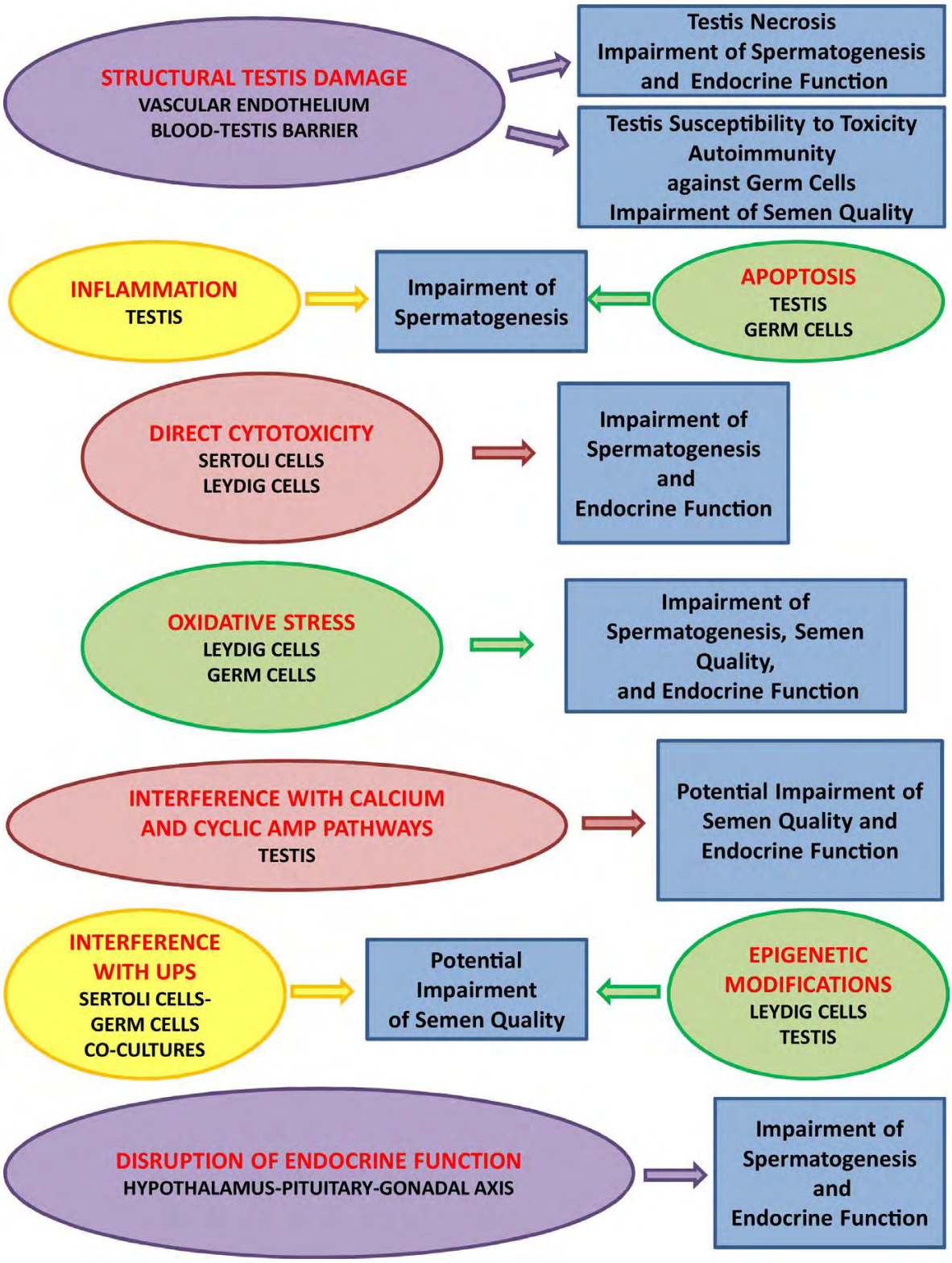


Figure 3. Mechanistic summary of cadmium mediated toxic effects on male reproduction (de-Angelis *et al.*, 2017).

Nickel induced cellular toxicity

Nickel (Ni) is another transition metal known to exhibit carcinogenic potential. It is known to have a genotoxic, hematotoxic, neurotoxic, immunotoxic, reprotoxic, nephrotoxic, and hepatotoxic potential (Das & Das, 2008; Mohammadi *et al*., 2018). It has gained attention as an environmental pollutant since it is used in industrial processes and has shown prominent concerns regarding public health (Song *et al*., 2017). The route of exposure to Ni determine its toxic effects such as inhalation, ingestion or through skin (Das & Das, 2008; Zambelli *et al*., 2016). Nickel mediates its effects through its oxidant potential as it causes oxidative stress (Das *et al*., 2019). Besides raising public health concerns, Ni is associated with reproductive problems as well (Chen *et al*., 2018). Figure 4 presents an overview of oxidative stress responses generated due to Ni exposure.

Chromium induced cellular toxicity

Different industrial processes release enormous amounts of chromium in the environment, which is ingested/inhaled by human in occupational setups (Tseng *et al*., 2018). It occurs usually in two forms Cr (III) and Cr (IV), both of which are known to cause pathologies in human (Cherfi *et al*., 2014). Cr ions mediate its genotoxic action within the cells either via the formation of reactive oxygen species (ROS), initiation of apoptotic pathways, and DNA damage (Annangi *et al*., 2016). A summarized view of chromium mediated toxic effects in cells has been shown in figure 5. Cr is known to possess carcinogenic, genotoxic, neurotoxic, and oxidant potential and therefore, it affects multiple organs and organ systems (Pal & Shil, 2019).

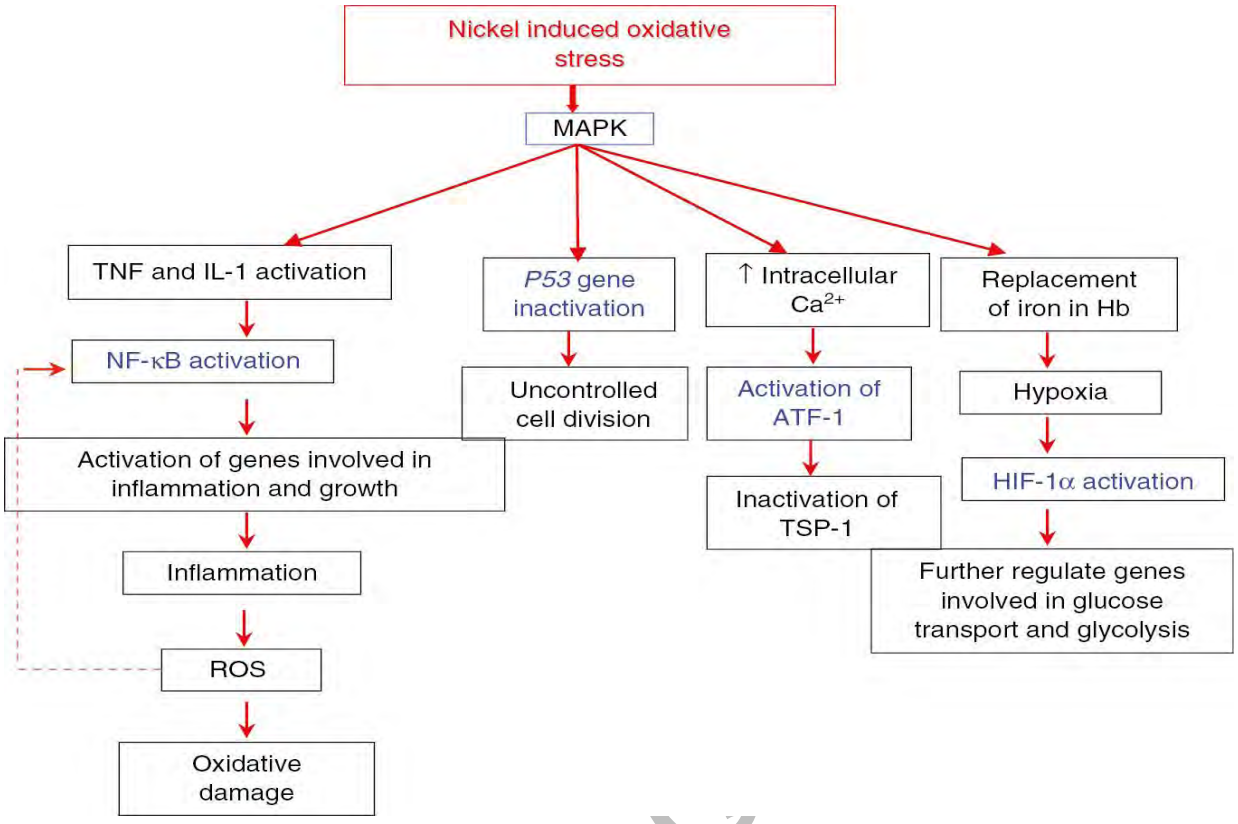


Figure 4. Overview of nickel-stimulated oxidative stress (Das *et al.*, 2019).

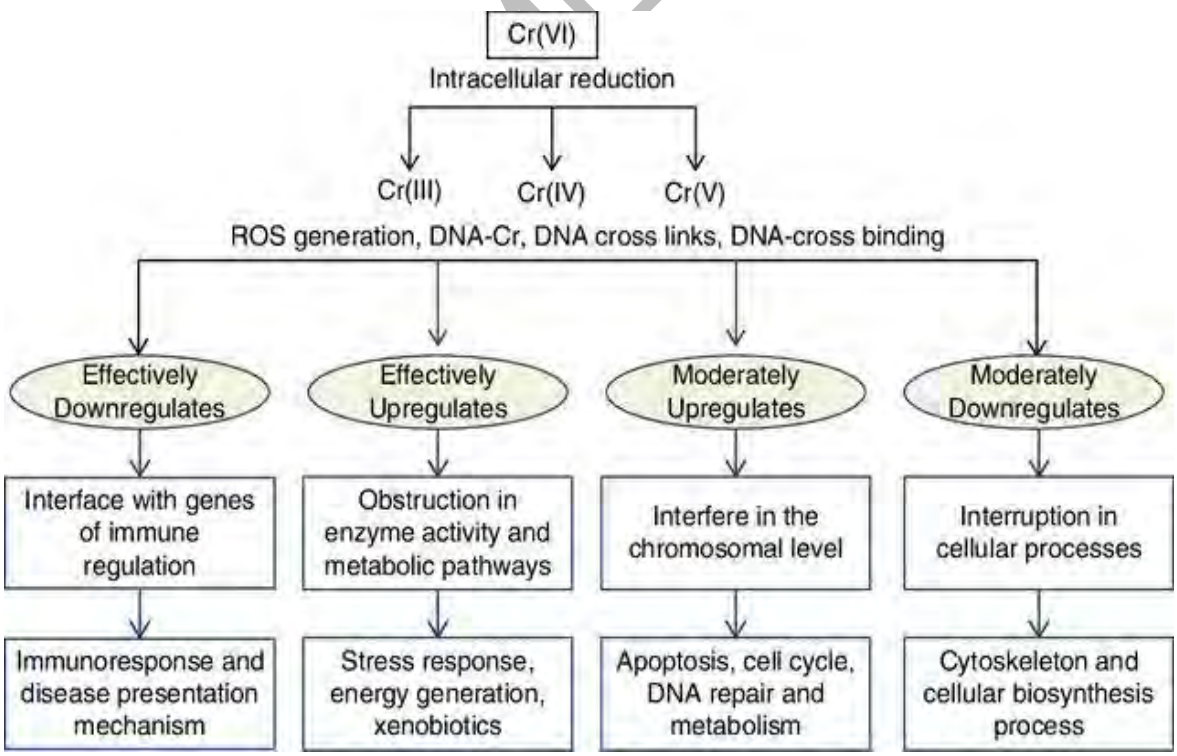


Figure 5. A quick overview of chromium mediated toxic effects in cells (Pal & Shil, 2019).

Occupational factors- a threat to public health

Apart from environmental exposure to heavy metals, industry workers are also exposed to large number of occupational factors. These include exposure to heavy metals in soil, poisonous gases, diesel exhaust, smoke, heat and radiation in the atmosphere also impose damaging effects on human health (Kaushik *et al*., 2012). Smoking presents another attribute that has deleterious effects on occupant's health. The burning of cigarette smoke release heavy metals and other toxic compounds not only in the atmosphere, but they are also deposited (absorbed) within the body. It has been speculated that a daily consumption of one packet of cigarette cause smokers to absorb around 1–3 µg of Cd that enhances the chance for the development of cancers and respiratory disorders (U.S. Department of Health and Human Services, 2010). Therefore, it is established that among brick kiln workers, consumption smoke along with other occupation factors serves as a greater risk for the development of respiratory and other metabolic diseases.

Reproductive issues associated with environmental exposure to brick kiln emission

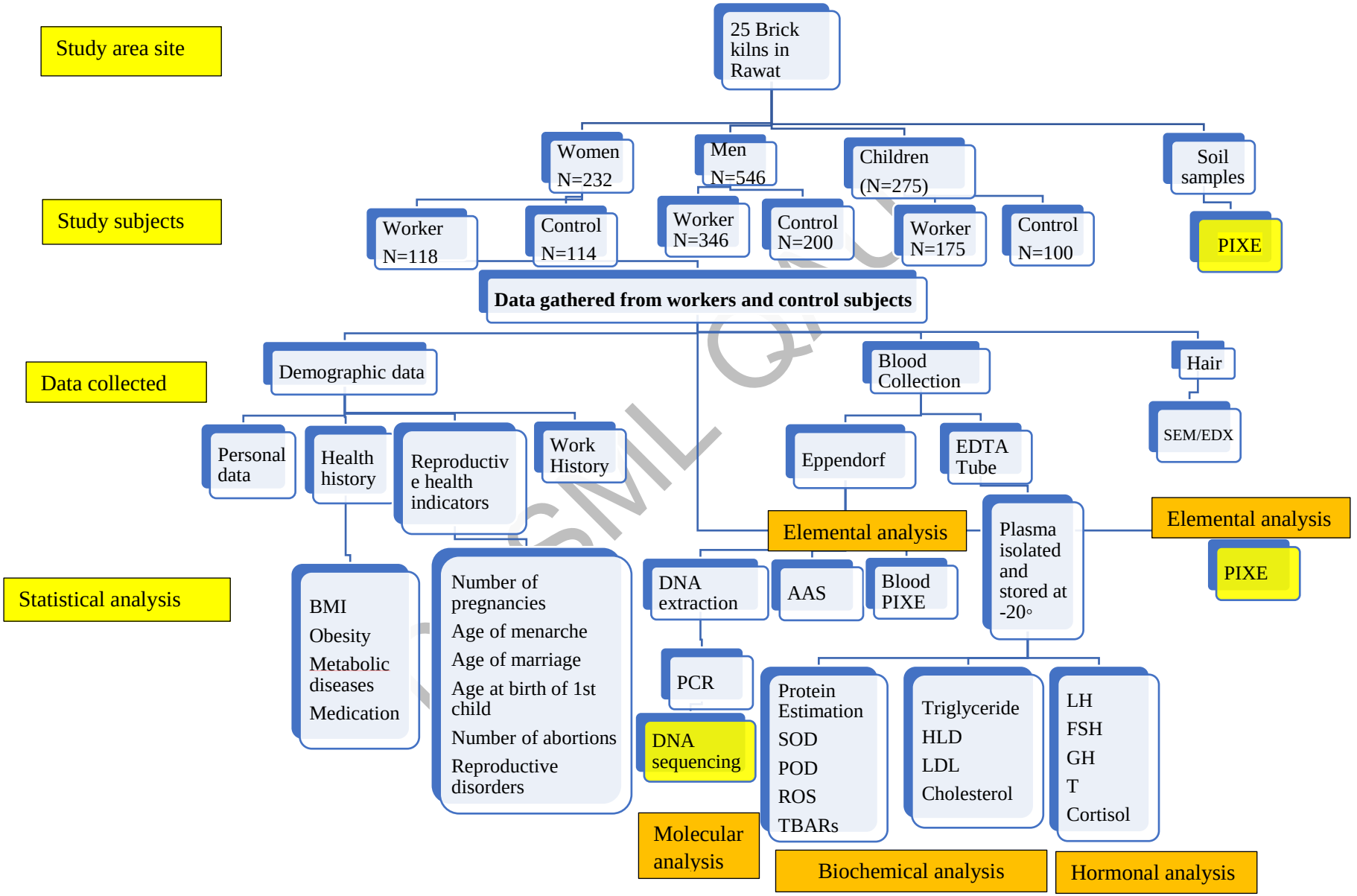
It is documented that exposure of kiln workers and people living nearby to brick kiln emitted particles raise reproductive health issues (Jahan *et al*., 2016). In another study, it is evident that exposure to these coal clay brick kiln emitted pollutants disrupts hypothalamic pituitary testicular axis (HPT) axis by lowering the serum concentrations of reproductive hormones LH and testosterone as well as BMI in children and adult male workers (Shahid *et al*., 2017). Therefore, the current study is designed to find out the heavy metal burden in body tissues of brick kiln workers to assess the intrinsic and extrinsic exposure of metals. Further, the study is planned to see the possible environmental health effects by assessment of BMI, blood profiles, lipid profile, oxidative stress markers, reproductive health functions, and genetic changes induced.

AIMS AND OBJECTIVES OF THE STUDY

The present study aims to deal with Sustainable Development Goal (SDG) No. 3 that states "*Ensure healthy lives and promote well-being for all at all ages*" with focus on different persistent and emerging reproductive health issues. The study is designed to estimate the hazardous effects of heavy metals emission from coal clay brick kilns on human health regarding child growth, pubertal development, fetal and maternal health and reproductive health in men. To address reproductive health in individuals of different age groups working in brick kilns, the study aims to

- Find the association of brick kiln emissions with parity, socio-demographic and occupational factors among brick kiln workers
- Conduct qualitative and quantitative estimation of metals and other elements using soil, blood and hair samples through advanced techniques.
- Assess body mass index, hematological parameters, biochemical indicators, oxidative stress markers and reproductive hormone concentrations in male and female workers and control
- Find out the correlation of stress response (HPA axis) on the reproductive axis (HPG axis) among males and females
- Investigate the association of fertility indicators on maternal and reproductive health
- Study the genetic variations in ABCG-2 gene by looking at single nucleotide polymorphisms (SNPs) and disease-causing variants among the two groups
- Understand the growth, developmental and health risks among boys and girls of different age groups
- Evaluate the possible biochemical alterations and DNA damage caused by of heavy metal burden in blood using children's samples among control and worker groups.

EXPERIMENTAL DESIGN



Chapter 1

Estimation of socio-demographic parameters associated with brick kiln industry and investigation of heavy metal burden and elemental analysis of soil, hair, and blood samples

ABSTRACT

Brick kiln industry emits enormous amounts of poisonous gases and trace elements directly into the atmosphere that affect all life forms specially brick kiln workers through external deposition (dermal) as well as through internal route (inhalation and ingestion). The present study aims to evaluate the socio-demographic, work associated, and general health determinants among brick kiln workers and non-workers, along with emphasis on extrinsic and intrinsic deposition of heavy metal burden in soil, blood and hair samples. Therefore, blood, and hair samples along with demographic data was collected from a total of 778 participants including men (n=546), and women (n=232) from Rawat, Punjab. Information regarding body mass index (BMI) was gathered. An elemental analysis for collected samples was done using atomic absorption spectroscopy (AAS) and external beam PIXE. Results showed that about 10% of the kiln labors were underweight and had multiple health problems including skin allergies (1%), asthma (10%), stomach and kidney disorder (5%), and other diseases. Almost 58% of brick workers were addicted to tobacco ($p=0.363$) and were working in poor and unhygienic conditions. Examination of blood for heavy metals concentrations through AAS revealed significantly ($p<0.001$) higher levels of Cd, Cr and Ni in worker group as compared to the control group. PIXE further detected higher than permissible levels of Si, P, S, Fe, Co, Ni, Cu, Cl, K, Ca, Ti, Mn, and Zn, in blood of kiln workers. The SEM/EDS analysis of hair depicted the presence of macro-element with an average concentration in the order of: K > S > Ca > P > Cl. Similarly, a micro-element profile with mean levels in the order of: Rb > Fe > Mn > Cu > Sr > Zn were seen. The study concluded that multiple health disorders were evident in brick kiln workers; soil contained greater deposits of toxic elements, external and internal deposition of heavy metal was quite elevated in hair and blood of workers; which poses serious health risks to occupational individuals.

INTRODUCTION

Brick kilns work at increased temperatures, emitting masses of black smoke containing harmful gases that become part of the environment and affect human health (Kaushik *et al*., 2012; Khan *et al*., 2019). Smoke and dust from brick kilns causes air pollution (Shaikh *et al*., 2012). The emitted smoke from kilns release oxides of different elements (sulfur, nitrogen, carbon) and heavy metals including Cd, Cr, As and Ni (Ismail *et al*., 2012). These emitted elements ultimately become part of the environment and are deposited in soil, water, vegetation, and atmosphere in the form of poisonous gases, smoke, heat and radiation affecting all life forms (Kaushik *et al*., 2012). The deposition of air pollutants on the water or land surfaces makes atmosphere harmful for living (Saju *et al*., 2020). The gases combine with the rainwater and flow into the river/sea affecting major component of ecosystem (Saju *et al*., 2020). Ishaq *et al*., (2010) suggested that Cd, Zn, Cr, Ni are repetitively accumulated in the soil irrigated by polluted water, which release them directly into groundwater making them available for plant uptake; transform metals from soil to plants and affecting whole food web (Ishaq *et al*., 2010). Elements including Ni, Cd, Hg, and Pb induce toxicity in the plants as they are classified as non-essential elements and may impact the pH of the soil (Ishaq *et al*., 2010). Other studies suggested the burning of different carbon-based fuels at brick kilns releases CO_2 which serves as a major contributor of global warming (Saju *et al*., 2020). Further, it is suggested that PM along with oxides of carbon and Sulphur are responsible for acid rain and reduction of ozone layer. Thus, it can be concluded from previous studies that brick kiln produced CO,CO_2, and SO_2 have detrimental impact on the environment, whereas others pollutants including carcinogenic dioxin, and PM impart harmful actions on the human health (Khan *et al*., 2019).

Heavy metals and gases released from brick kiln industries induce toxic effects on human and animal health (Hanif *et al*., 2020; Hu, 2002; Mehra & Thakur, 2010). A number of studies have shown that brick kiln released elements are responsible for causing various diseases such as respiratory symptoms, illness, cancers, musculoskeletal disorders and reproductive malfunctions among humans which acquire them by ingestion or inhalation (Inbaraj *et al*., 2013; Jahan *et al*., 2016; Kamal *et al*., 2014; Rajesh & Niraj, 2010; Sanjel *et al*., 2016; Shaikh *et al*., 2012). Literature study reports that exposure to elevated levels of Cr, Zn, Ni, and Cd impart lethal actions on human and organisms health, mediated through their hepatotoxic, reprotoxic and genotoxic potential (de Angelis *et al*., 2017; Jahan *et al*., 2016; Lu *et al*., 2005; Wicke *et al*., 2012). Heavy metals are the principal cause of oxidative deterioration of biological macromolecules and work by attaching to the nuclear proteins and DNA (Flora *et al*., 2008; Satoh *et al*., 2008). For instance, environmental exposure of cadmium in humans induces harmful health effects on hepatic, renal hematological, pulmonary, cardiovascular, musculoskeletal, and reproductive functions (Bhattacharya, 2020). Similarly, exposure to chromium, another heavy metal present in soil and ground water is carcinogenic in nature and is known to impart damaging effects on liver, kidney and lungs of human (Cherfi *et al*., 2014). Jahan *et al*. (2016) reported previously that exposure to heavy metals (Cd, Cr, and Ni) coming from brick kilns induces homeostatic imbalances among male brick kiln workers via decrease in antioxidant concentration and reproductive enzymes (testosterone) concentrations (Jahan *et al*., 2016). Therefore, it can be concluded that exposure to heavy metals from brick kilns raise health and reproductive health issues not only among brick kiln workers but also among residents in the vicinity of these kilns.

About 4.5 million people in Pakistan are associated with brick kiln industries. Across the country, more than 20,000 kiln are present in four provinces (CCAC secretariat, 2018). Due to such a large a number of associated labors, several researchers have conducted studies to understand the impact of brick kiln industry on socio-economic, demographic, public health and environmental determinants. Different studies from Pakistan have reported that brick kiln emissions affect physical aspects of environment in multiple ways. Ishaq *et al*. (2010) conducted a study in Peshawar, Khyber Pakhtun Khwa to determine the heavy metal concentration in soil and plant samples collected from kiln sites (Ishaq *et al*., 2010). Another study from Peshawar was done by Jan *et al*., (2014) on plants present in the vicinity of brick kiln sites (Jan *et al*., 2014). The study concluded that aerial parts of plants (i.e., leaves) accumulate more PAHs content as compared to underground parts (stem, roots); suggesting that consumption of these plants raise public health concerns. Recently, studies conducted from different areas of Punjab and Khyber Pakhtunkhwa indicated that among brick kiln emissions, the oxides of carbon and sulfur bring detrimental consequences on the atmosphere, while human health is severely affected by PM and SO_2 (Khan *et al*., 2019). A study from District Rawalpindi was performed using plants present near brick kilns to measure their air pollution tolerance index and heavy metal determination (Achakzai *et al*., 2015, 2017). Thus, it is concluded that in Pakistan, air pollution due to brick kiln emitted particles is a threat for physical and biological components of environment.

Emissions from Pakistani brick kilns affect health outcomes in brick kiln workers and non-workers as indicated by multiple researchers. Studies conducted by Kamal *et al*. (2014) assessed cancer risk evaluation among brick kiln workers from different cities of Punjab province (Lahore, Gujranwala and nearby villages) (Kamal *et al*., 2014). It was inferred that adults and child labor at kilns are extremely exposed to potent cancerous

agents that might be ingested or inhaled. Another study reported from Gujranwala, Punjab indicated that brick kiln workers are exposed to polycyclic aromatic hydrocarbons (PAH) that are known to cause metabolic and cardiovascular diseases (Kamal *et al*., 2014; Kim *et al*., 2015). Further, study from Sindh province reported that occurrence of illness and prevalence of respiratory diseases is more common among brick kiln workers as compared to non-workers (Shaikh *et al*., 2012). A study conducted at KPK suggested that release of toxic gases and metals from brick kiln induce genotoxic effects in brick kiln workers (Khisroon *et al*., 2018). Another report from Baluchistan evaluated the health effects of fluoride exposure in brick kiln workers (Khalid, 2019). Recent research by Nasir *et al*. (2021) conducted at Peshawar, KPK stated harmful effects of polluted air due to brick kiln emitted particles on physical and mental health of children (Nasir *et al*., 2021). Previous studies also indicated that heavy metal exposure in kiln workers might change the biochemical and hormonal responses in kiln workers, affecting their reproductive potential (Jahan *et al*., 2016; Shahid *et al*., 2017). Considering the metabolic deposition, fate, and effects of trace elements in the biological samples of organism with a malignancy, it might help the researchers to further investigate about the disease indication (Karimi *et al*., 2012; Resistance, 2020).

A wide range of occupational risk factors related to public health, are reported to be faced by many workers around the globe. These includes atmospheric pollution, chemical exposure and injury risks, infections and bodily/skeletal damage, and mental vulnerabilities (Sanjel *et al*., 2016). Brick kiln emitted heavy metals induce reproductive disturbances among brick kiln workers and affect whole ecosystem (Jahan *et al*., 2016; Shahid *et al*., 2017). Occupational exposure to heavy metals serves as a risk factor for female fertility (Amadi *et al*., 2017). Studies suggest that some heavy metals (As, Cd, Pb,

Hg) may act as endocrine disruptors and their exposure during pregnancy may adversely affect mother and the fetus, concerning low birth weight (Georgescu *et al*., 2011; A. Rahman *et al*., 2016). Previous epidemiological studies have reported that occupational exposure to Cr increases carcinogenic risk in the respiratory tract (Cohen *et al*., 1993). Recent studies report that female workers, occupationally exposed to Cr experience high threat for abortion and miscarriages (Amadi *et al*., 2017). Additionally, occupational workers are also exposed to occupational factors, which play key role in shaping the employees' health as previous studies have suggested (Patil, 2017; Shaikh *et al*., 2012). Hence, it is endorsed that occupational risk factors affect the public health and reproductive outcomes of brick kiln workers by various mechanisms.

Human Biomonitoring (HBM) is of great interest in risk assessment and population/occupational health studies because they can be used for the characterization of exposure, biochemical and biological effects. The present study concerns the detection of trace elements and heavy metals in hair and blood tissues of brick kiln workers and non-workers. Both samples are analyzed in combination to evaluate the internal as well as external route of deposition of heavy metals in the body. Various studies have confirmed that heavy metals deposit in different body tissues such as blood, plasma, urine, hair etc (Engwa *et al*., 2019). The heavy metal burden within the human body can be measured through different body tissues/fluids such as hair, blood and urine (Jan *et al*., 2015). Keratinous structure of hair make it suitable for better preservation and provide information regarding one's health and diet, also allows the deposition of heavy metals (Davis & Briggs, 1995; Kempson *et al*., 2010). Previous studies have suggested that deposition of heavy metals and trace elements in body tissues such as nail and hair is associated with certain diseases such as prostate cancer (Karimi *et al*., 2012). Thus, hair

serves as a good biological tissue for studying environmental variable such as heavy metal burden; because it is easy to collect, store (at room temperature), transport, is non-invasive and requires minimum training for its sampling as previous studies have suggested (Gerber *et al*., 2012). Additionally, segmental analysis of hair for the drug/chemical exposure is possible (Humbert *et al*., 2014). However, apart from using hair as a good study tissue, the disadvantage of using hair is that it is exposed to environmental chemicals, oils, pollutants and artificial products, that may give false results (Jenkins, 1979). However, previous study reported by Li *et al*. (2014) also used human hair and blood tissues to determine the heavy metal content and its correlation with sociodemographic factors (Li *et al*., 2014). Therefore, to strengthen our studies and to investigate both extrinsic (such as atmosphere) and intrinsic factors (via food and water) that might have contributed to develop heavy metal burden on biological tissues, both hair and blood samples were analyzed in our study.

Different techniques have been established so far to conduct the qualitative and quantitative analysis of metals and trace elements in blood. These include atomic absorption spectroscopy (AAS), dry ashing of whole blood, flame atomic absorption, Zeeman background correction, inductively coupled plasma–mass spectrometry and isotopic fingerprinting (Rao, 2005). However, all these methods require laborious and time taking procedures for sample preparation and analysis. In current decade, another useful technique known as proton induced X-ray emission (PIXE) is being introduced that was initially proposed by Johansson and his co-workers ((Johansson *et al*., 1970). PIXE is a non-destructive, and sensitive tool that can be used to conduct quantitative and qualitative analysis of trace elements present in minute quantities (parts per million) as suggested by literature review (Lahiry *et al*., 2017; Naga & Pradeep, 2012; Naga & Ravi, 2013; Vijayan & Marg, 2002). In present study, PIXE is used for the determination of heavy metal burden

in blood of brick kiln occupants. The technique has not been previously performed on blood for the determination of trace and heavy metal load in Pakistan. Also, scanning electron microscopy (SEM) was used in present study to accurately determine the heavy metal distribution on hair samples. It is a semi-quantitative method and is capable to detect elements deposited on the hair surface even in the minute quantities (Ha, 2011).

Aims and objectives of the study:

The present study was designed to monitor the health condition of brick kiln workers through demographic data and blood sample collection. The study also aims to screen the levels of trace elements in soil and blood indicating ingestion or other exposure routes to elements and metals; and to monitor the surface of hair for heavy metals to assess exposure to toxic elements from the external environment (dust, air) over time that might be deposited on the hair or inhaled. Further, the study is intended to report socio-demographic and work-related determinants along with reproductive indicators in men and women subjects belonging to different age group. To address demographic parameters and public health in study subjects, the objectives of study are to:

- Find the association of brick kiln emissions with parity and socio-demographic factors in brick kiln workers
- Understand the morbidity profile and occupational conditions of the workers
- Calculate and compare the body mass index (BMI) among workers and control subjects
- Determine the heavy metal and trace element burden in soil collected from brick kiln site
- Measure the heavy metal burden in blood through AAS
- Carry out the qualitative/quantitative scrutiny of heavy and other elements in whole blood through PIXE
- Perform the qualitative/quantitative analysis of heavy and trace elements in hair samples through micro-PIXE

MATERIALS AND METHODS

Study plan

The current study was performed at Reproductive Physiology Laboratory, Department of Zoology, Quaid-i-Azam University Islamabad. In order to investigate the toxic effects of brick kiln emissions different brick kilns from Rawalpindi district, Punjab province were selected, and sampling was done from March 2018 to March 2019. The approval to conduct this study was obtained by Ethical Committee of the Department of Zoology, Quaid-i-Azam University (QAU) Islamabad, Pakistan and was assigned protocol # **BEC-FBS-QAU2018-97**.

Selection of brick kiln sites

Different brick kiln sites from Rawat (Phambli town, District Rawalpindi) were selected for present study (figure 6). Due to presence of several unregistered brick kilns, the data was collected from those kilns where owners were willing to participate. Approximately 50 brick kilns were visited out of which 25 brick kiln workers agreed to participate in the present study.

Figure 6. Figure of the brick kiln site (Rawat) showing type of kilns and drying of nascent bricks in sunlight.

Description of study area

The District Rawalpindi lies on the Potowar Plateau, 9 miles (14 km) southwest of Islamabad. Rawat is a village in the Rawalpindi District of Punjab, Pakistan and is in the hilly part of Punjab province, near North-West Frontier Province (NWFP) and Kashmir at 33.4951°N, 73.1969°E. The average annual temperature is 21.5°C or 70.8°F in Rawalpindi. The average annual rainfall is abundant at 1346.8mm (53.02 inch), most of which falls in the monsoon season. The geographic information system (QGIS) software was used for the development of sampling site on map. Figure 7 shows the brick kiln sites, from where sampling was done.

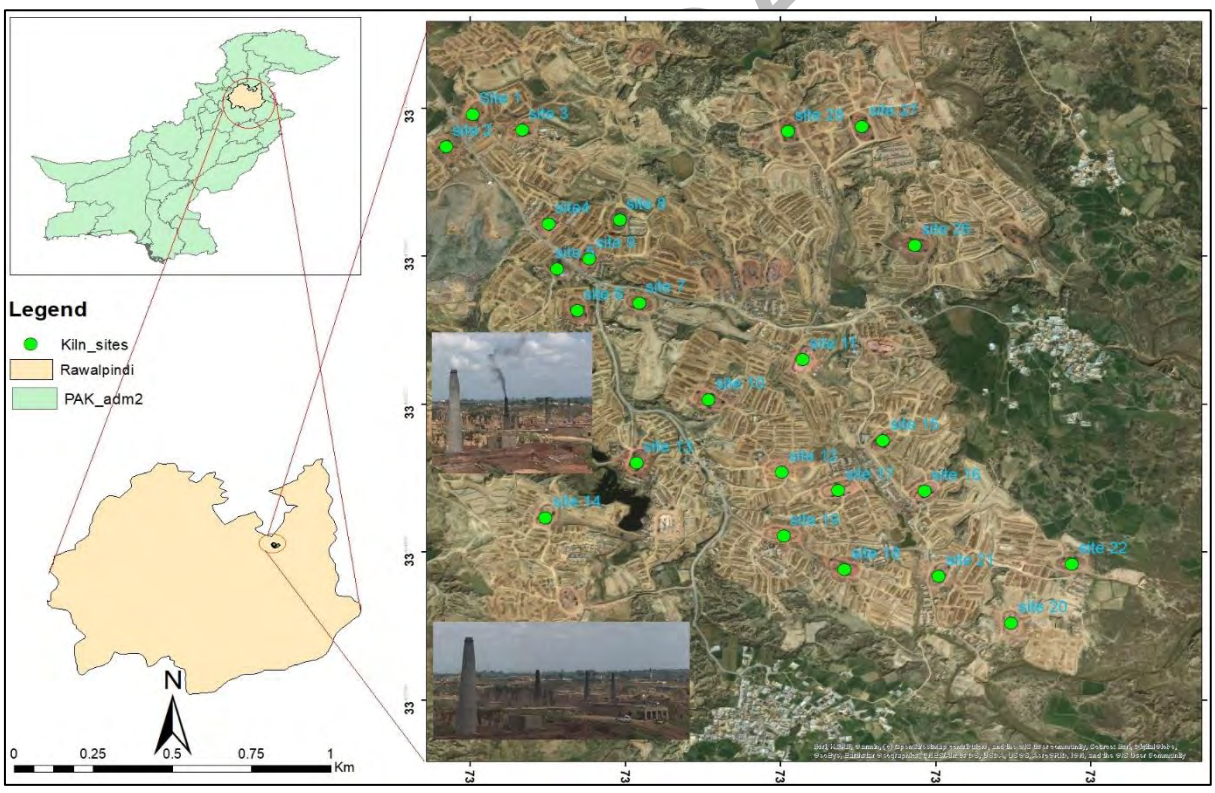


Figure 7. Area map of sampling sites near Rawat city (District Rawalpindi, Pakistan).

Questionnaire design

A detailed literature review was done, and a questionnaire was designed according to the variables and indicators of Demographic & Health Surveys (DHS) (Rutstein & Rojas, 2006). The questionnaire was designed as a "mixed" type questionnaire with open and close ended questions keeping in view the nature of questions, and the data obtained was both qualitative and quantitative type. The questionnaire was divided into following parts. The first part comprised of socio-demographic factors such as age, education, height, weight, employment status, marital status, earlier history, family history and wealth quintile. The next part included the variables and their indicators related to their occupation and health, which included work history, disease and reproductive history, fertility issues (if any), smoking status/ habits, job nature and exposure duration and knowledge of other associated diseases.

Subject selection

Subjects of different age groups of men and women working/living at brick kilns were considered along with control subjects living in the same district but away from kiln sites. A total number of 464 individuals working for varied years at brick kilns and non-workers/control (n=314) living in the vicinity, aged 19–60years including males and females were considered; where male workers (n= 346), control males (n=200), female workers (n=118), control females (n=114) were randomly selected for inclusion in this study, with each individual representing a population. Most of these participants were housewives or unemployed women. All samples were anonymized before being processed.

Inclusion and exclusion criteria

All the individuals either healthy or unfit and despite of their ethnicity were involved in the study group. The individuals above 80yrs of age were not included in present study.

Grouping of subjects

Based on the obtained information, males and females were divided into following groups. Group I was considered as Control males (Not exposed to kiln emissions), while Group II included kiln male workers, Group III has brick kiln female workers, Group IV was composed of control females that were not exposed to kiln emissions.

Collection of demographic data

After having interview session using self-reported questionnaire (SRQ) that contained the information concerning the demographic details such as age, gender, ethnicity, height, weight, education, diet, work duration, data regarding daily working hours and family disease history was gathered (figure 8).

Soil sample collection

Soil samples were collected from five different sites of brick kiln area and at 0-15 cm depths from each of the selected sites in paper bags.

Blood samples collection

After having written information, blood samples were collected by venipuncture in a 5ml syringe by a registered female/male nurse (figure 8). Half of the blood was then kept at 4 °C for the analysis of complete blood count (CBC) and heavy metal detection. The other half was centrifuged at 13000 revolution per minute (rpm) for 10 minutes to separate plasma, later, transferred and stored at −20 °C until further analysis.

Hair sample collection

Hair samples were collected from the base of the head, 4 cm or more usually from the tips at the back of the ear (figure 8). Hair samples were collected using fine edged sterilized rubber scissor and samples were stored in paper bags with title of the participant. The samples were kept at room temperature of 25°C for further processing.

Figure 8. Figure showing the collection of demographic data through questionnaire filling, followed by soil, blood and hair sampling at brick kiln sites.

Body mass index (BMI)

BMI for all the subjects was calculated by the online BMI calculator centered by Centers for Disease Control and Prevention (https://www.cdc.gov/healthyweight/assessing/bmi/adult_bmi/index.html).

Following formula was used for determination of BMI:

$$\text{BMI} = \frac{Weight\ (Kg)}{Height\ (m2)}$$

Atomic absorption spectroscopy

For the analysis of heavy metals, blood samples were digested in acid following the protocol previously used with little modifications (Ishaq *et al*., 2010; Tripathi *et al*., 1997).

Acid digestion

Acid digestion for the samples was performed through four steps. Firstly, all the equipment to be used were dipped with 10% nitric acid for overnight, followed by washing with 69% nitric acid and dried over absorbent paper and kept it overnight. Secondly, in 0.5 ml whole blood, 5ml of 69% HNO_3 was added till it became clear. This step is called pre-digestion. Manual digestion was then performed through boiling of whole blood in HNO_3 at 400° C till solution becomes half. Later, filtration was done for these samples using Whatman filter paper. Lastly, distilled water was added to make the final volume of filtrate up to 15 ml, that was used for AAS.

Formulation of metal standards (Cd, Ni and Cr)

Following formula was used for the preparation of standard solution for each metal salt (1000ppm concentration):

$$\text{Molecular weight of salt} = \frac{\text{X (Molecular weight of sample)}}{0.1}$$

To make 1000 ppm solution, X grams of salt was mixed in 100ml of distilled water. Later, the instrument was calibrated by using multiple concentrations from 1-50 ppm and calibrations were done using the formula:

$$C_1V_1=C_2V_2$$

Where, C_1= concentration, V_1=vol. of standard's stock solution, C_2= required concentration, V_2=Required volume

Heavy metal concentrations were determined and analyzed using AA 40 FS Fast Sequential Atomic Absorption Spectrometer. For each metal, a series of standards were run, and calibration curve was formed. Concentrations in ppm were measured and following formula was used to present them in µg/dL.

$$Concentration = \frac{AA\ reading \times dilution}{Weight\ of\ sample}$$

PIXE estimation

Soil sample preparation

Soil samples were processed by pressing 1-2 grams of soil at the pressure of 7-8 metric tons and making hard pellets with a diameter of 13mm and 4mm thickness using bench top pellet press as shown in figure 9 (Carver 4350 Manual Bench Top Pellet Press, CARVER *INC*). These pellets were then subjected to attach with the sample holder using carbon tape and later, placed in PIXE machine present at National center of physics (NCP), Islamabad.

Blood samples preparation

Blood samples collected previously kept at 4°C temperature was thawed and kept at room temperature of 25°C. Using 99.99% pure copper strips, blood samples were prepared for PIXE analysis. About 20-30µl of blood volume was drawn using a micropipette and was placed on the copper strips coated with black tape. No binding agent was used for

binding purpose. The blood films were air dried for about 30-40 minutes at room temperature and humidity of 40% (figure 9). The strips were then placed in the PIXE machine with holder.

For PIXE analysis of blood for metals (Cr, Ni, Cu and Zn), we used 5 MV Pelletron Tandem Accelerator, previously used by Nadeem *et al*., 2019 in their respective study (Nadeem *et al*., 2019). Same machine was used and all the laboratory conditions were also kept similar as that of Nisar *et al*., 2019 study design (Ahmad *et al*., 2020). Each copper strip containing dried blood sample was placed in vacuum chamber at pressure of 10–7 torr and was irradiated with 3.0 MeV proton beam energy having 2 mm^2 collimator, followed by placement in vacuum chamber at 90º with respect to incident beam. The current beam was positioned at 3–5 nA and the integrated charges of beam were investigated up to 2μC. The emitted X-rays from the blood samples were detected by Sirius SD detector with resolution of 129 eV which was adjusted at an angle of 45º with respect to incident beam. An absorbing foil of Mylar having thickness of 40 μm was placed in the front of detector in order to minimize the intensity of low energy X-rays emitting from matrix elements. GUPIXWIN software was used to analyze the data obtained from PIXE technique (Nadeem *et al*., 2019). The PIXE of workers and control blood samples produced data with accuracy of 15% ± 5% within standard tolerance limit. For better absolute results, detector was well calibrated with K-shell x-rays of standard 99.99% pure Cooper (Japan) with 20 um thickness and TiV standard (NIST) before analyzing the blood samples as shown in figure 10. The system constant or H-values, including all electronics and system error was corrected to 0.001531 steradian. Recently, using standard reference material of Bovine Lever SRM 1577 c, the reproducibility of the PIXE analysis of our system, was of the order of < 5.5% or better (figure 11).

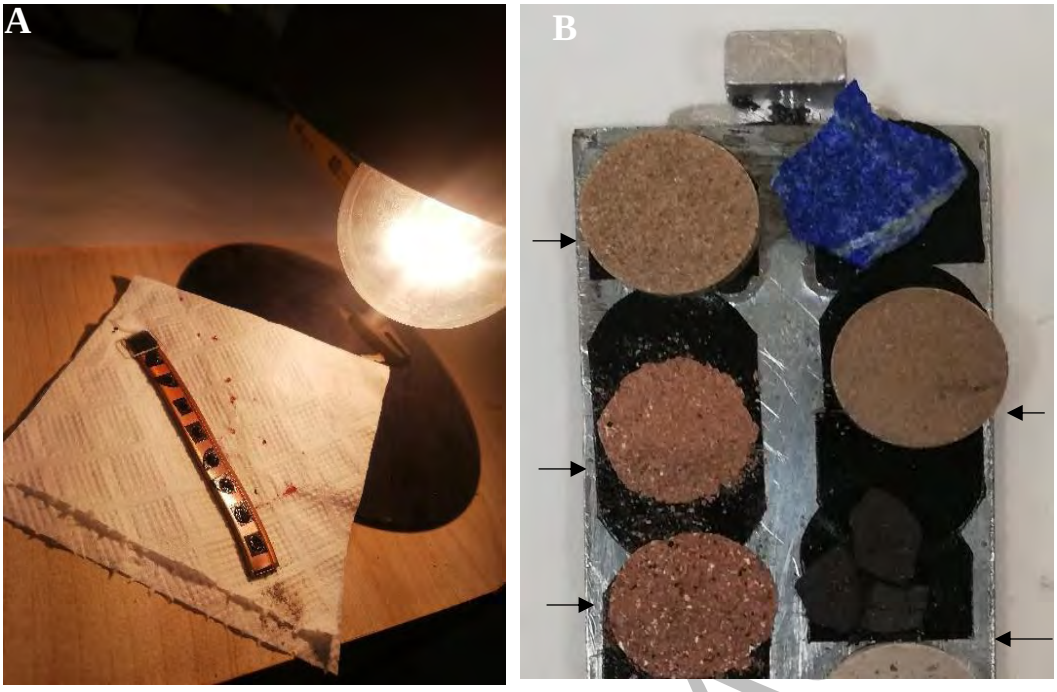


Figure 9. Figure showing sample preparation for PIXE of blood and soil samples. (A) Copper strip with coated black carbon tape having blood samples. (B) Sample holder coated with black carbon tape containing soil pellets. The arrows show the five samples of soils collected from kiln sites and processed for PIXE analysis.

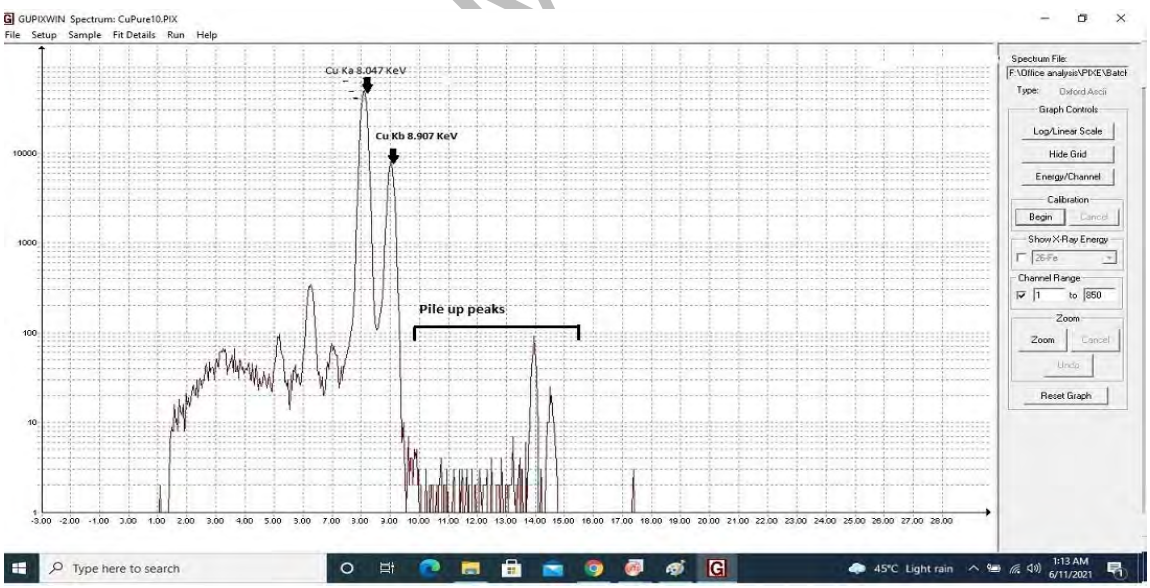


Figure 10. GUPIX PIXE System was calibrated using pure Cu standard. Channel to energy conversion at 285/Cu Ka: 8.047keV and 311/Cu Kb: 8.905 keV was performed to calibrate the PIXE setup. H- value was fixed to 0.001531 Str. Si-Escape peak was found at channel no/energy 234/6.4 keV.

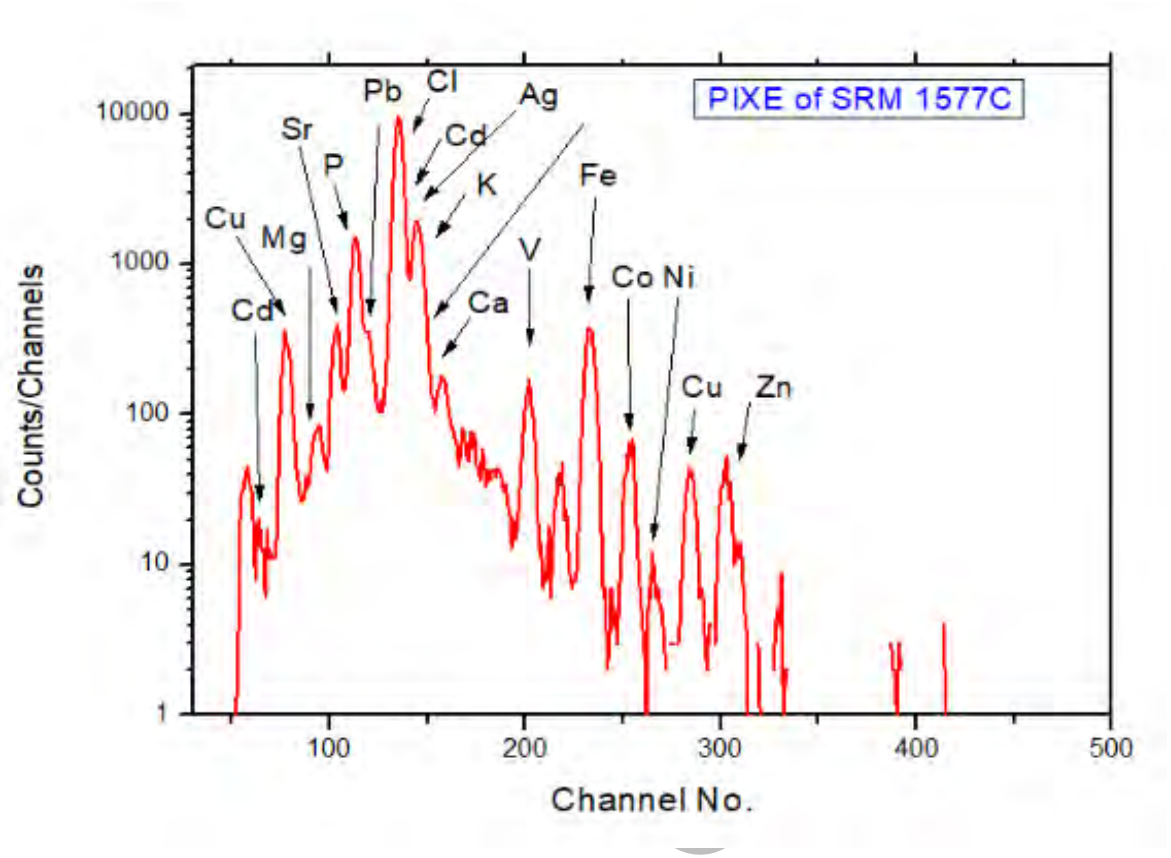


Figure 11. PIXE analysis of SRM 1577c of bovine liver using 3MeV proton energy at NCP, Islamabad using 5 MV tandem Accelerator.

Statistical analysis

The data is represented as mean ± SEM and the significance level was set at $p<0.05$. Using statistical tools, Graph pad prism and SPSS 17, the demographic data was analyzed using student's unpaired t-test. The concentration of heavy metals measured through AAS and PIXE among control and workers was statistically analyzed by applying unpaired t-test using computer software SPSS 17. GUPIXWIN software was used to analyze the data obtained from PIXE technique.

RESULTS

Area distribution of subjects

The subjects living at brick kiln sites belonged to different cities across Pakistan, and kilns had inequal number of males and female workers, therefore area distribution for study subjects have been shown separately as shown in figures 12 and 13. The number of local residents working at brick kiln was quite less than the labors who came from different cities across Pakistan.

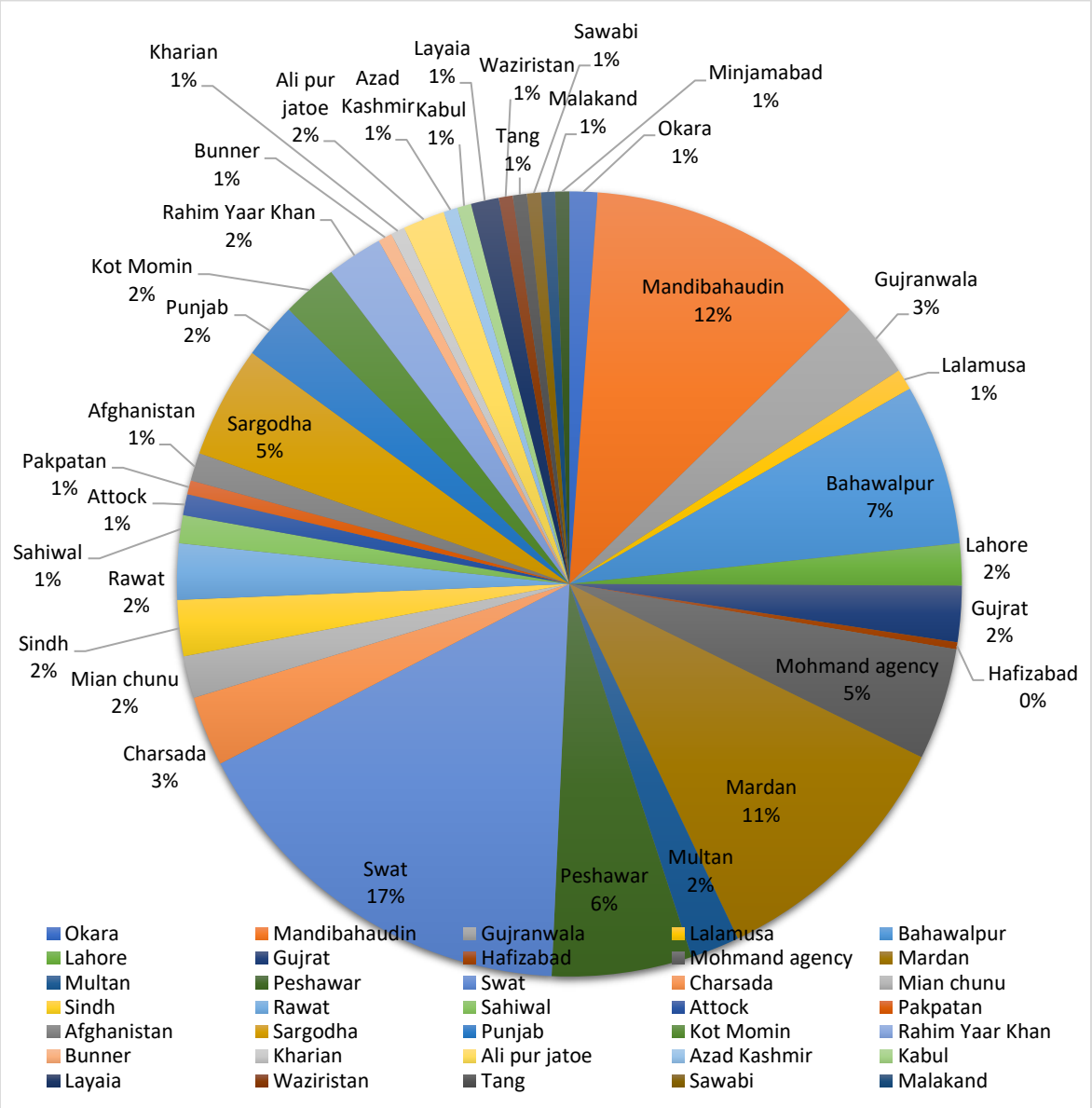


Figure 12. Area distribution of male brick kiln workers.

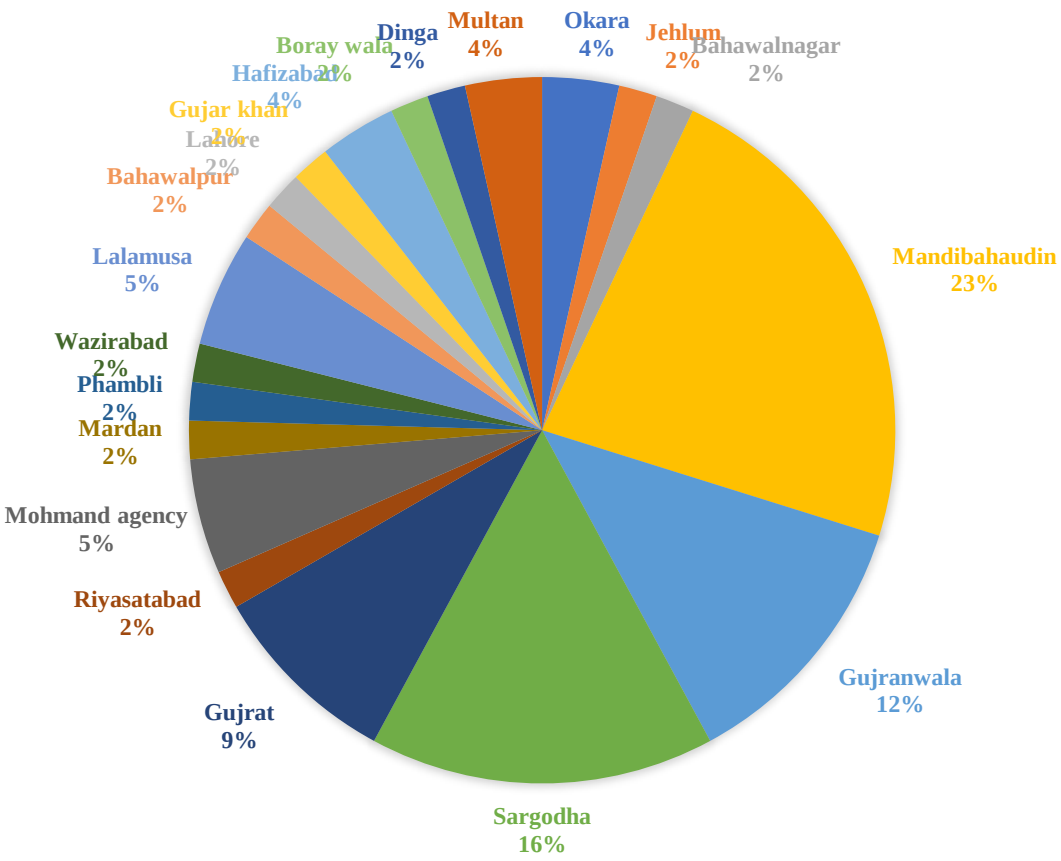


Figure 13. Area distribution of female brick kiln workers.

Sociodemographic data

A total of 546 men were examined for the study, out of which men workers (n=346) and control subjects (n=200) were included with mean age of 23.01±0.23 and 26.91±0.77 (years), respectively. Our results showed that most of the men working at brick kiln sites were having normal weight, while 10% of them were obese, 18% overweight and another 10% underweight. Individuals exposed to brick emitted smoke were facing various health issues including asthma (8%) stomach problems (10%), kidney disorders (5%), skin allergies, tuberculosis and other mild symptoms of flue, cold and headache (table 1). Majority of brick workers were married (77%) and their sleep duration was between 8-12 hours (71%). Approximately 84 percent of the studied subjects were illiterate and therefore, the trend for the use of personal protective equipment (PPE) at workplace was

not practiced among visited kilns. Almost 21 percent of the labor was on medication of headache or stomach issues as suggested by local physician such as Panadol and Paracetamol.

The individuals were working at very low wages ranging from PKR 10,000-20,000 per month only. While majority (n=178) of them were having monthly salary of PKR 10000-15000/- only. Further categorization for the work history has also been done as shown in table 2. Mean years for these workers living near brick production site was 14 years, and mean duration of their job was 12 years.

The study also included brick kiln female workers (n=118) and control subjects (n=114) with mean age of 35.76±1.88 and 27.68±1.36 (years), respectively. Results showed that 16% of the exposed women were underweight, and were facing metabolic disorders such as stomach problems, kidney disorders, skin allergies and asthma (table 1). Mean years for female workers living near brick production site was 19 years, and mean duration of their job was 14 years. The mean exposure time to kiln emissions was 7 hour per day.

Table 1. Table showing demographic data of men and women working in brick kilns at Rawat.

Socio-demographic parameters								
	Females				Males			
Parameters	**Control**		**Workers**		**Control**		**Workers**	
Age	**n**	**Percentage**	**n**	**Percentage**	**n**	**Percentage**	**n**	**Percentage**
18-28	84	73.68	46	38.66	114	57.00	186	53.76
29-38	14	12.28	30	25.21	32	16.00	66	19.08
39-45	4	3.51	18	15.13	36	18.00	34	9.83
45+	12	10.53	24	20.17	18	9.00	60	17.34
BMI								
Normal weight	54	47.37	54	45.38	42	21.00	206	59.54
Obesity	34	29.82	12	10.08	68	34.00	38	10.98
Overweight	20	17.54	32	26.89	78	39.00	64	18.50
Underweight	6	5.26	20	16.81	12	6.00	38	10.98

Marital Status								
Married	36	31.58	118	99.16	98	48.48	268	77.46
Unmarried	78	68.42	0	0.00	102	51.52	78	22.54
Sleep Duration								
less than 8	48	42.11	6	5.04	56	28.00	96	27.75
8-12	56	49.12	108	90.76	126	63.00	248	71.68
12+	10	8.77	4	3.36	18	9.00	2	0.58
Education Status								
Illiterate	8	7.02	116	97.48	2	1.00	292	84.39
Primary	2	1.75	2	1.68	0	0.00	40	11.56
Matric	10	8.77	0	0.00	54	27.00	6	1.73
Intermediate	2	1.75	0	0.00	60	30.00	8	2.31
Bachelors	8	7.02	0	0.00	36	18.00	0	0.00
Masters	52	45.61	0	0.00	36	18.00	0	0.00

Higher studies	32	28.07	0	0.00	12	6.00	0	0.00
Health History								
Allergy	4	3.51	2	1.68	6	3.00	6	1.73
Asthma	2	1.75	2	1.68	0	0.00	28	8.09
Diabetes	2	1.75	0	0.00	6	3.00	2	0.58
Obesity	2	1.75	0	0.00	0	0.00	2	0.58
Stomach issue	2	1.75	14	11.76	0	0.00	36	10.40
Hyperandrogenisn	2	1.75	0	0.00	0	0.00	0	0.00
Kidney issue	2	1.75	10	8.40	0	0.00	18	5.20
Tuberculosis (TB)	0	0.00	0	0.00	8	4.00	12	3.47
Hepatitis	0	0.00	0	0.00	0	0.00	6	1.73
Other	2	0.00	56	47.06	30	15.00		0.00
None	98	85.96	34	28.57	150	75.00	104	30.06
Medication								
Yes	16	14.04	16	13.45	14	7.00	76	21.97
No	6	5.26	70	58.82	42	21.00	174	50.29

No need	92	80.70	32	26.89	144	72.00	96	27.75
Tobacco consumption status								
Addicts	0	0.00	32	0.135	44	22	202	58.3
Non-addicts	114	100	86	0.728	156	78	144	41.6

Table 2. Table showing work history of men and women working in brick kilns at Rawat.

Work History								
	Females				Males			
Parameters	**Control**		**Workers**		**Control**		**Workers**	
Income	**n**	**Percentage**	**n**	**Percentage**	**n**	**Percentage**	**n**	**Percentage**
None	76	66.67	26	22.03	0	0.00	0	0.00
<10000	6	5.26	38	32.20	74	36.36	52	15.03
10000-15000	14	12.28	34	28.81	18	9.09	178	51.45
16000-20000	2	1.75	18	15.25	18	9.09	98	28.32
20000+	16	14.04	2	1.69	90	45.45	18	5.20
Work Type								
Carriage & placement	0	0.00	2	1.69	0	0.00	88	25.43
Bakers	0	0.00	0	0.00	0	0.00	78	22.54
Molders	0	0.00	98	83.05	0	0.00	124	35.84
Non-workers	0	0.00	18	15.25	26	13.00	8	2.31
Others	114	100.00	0	0.00	174	87.00	48	13.87

Years of Living								
<1	0	0.00	8	6.78	0	0.00	94	27.17
1-5	46	40.35	36	30.51	68	34.00	96	27.75
6-10	12	10.53	20	16.95	30	15.00	52	15.03
11-20	12	10.53	20	16.95	24	12.00	56	16.18
20+	44	38.60	34	28.81	78	39.00	48	13.87
Work Duration(years)								
1-5	NA	NA	28	23.73	NA	NA	56	16.18
6-10	NA	NA	14	11.86	NA	NA	72	20.81
11-20	NA	NA	36	30.51	NA	NA	108	31.21
20+	NA	NA	40	33.90	NA	NA	110	31.79
Exposure time (h/day)								
<1	NA	NA	12	10.17	0	0.00	4	1.16
1-5	NA	NA	40	33.90	104	52.00	24	6.94
6-10	NA	NA	30	25.42	66	33.00	132	38.15
11-15	NA	NA	32	27.12	30	15.00	178	51.45
15+	NA	NA	4	3.39	0	0.00	8	2.31

Body mass index

There was observed a little change on BMI of control and kiln workers as shown in table 3. A significant decrease ($p<0.001$) in BMI of male workers was observed as compared to control men (figure 14) while the average BMI for female workers was 23.20±0.63 as compared to 24.63±0.76 among control with a significance value of $p<0.01$ (figure 15).

Table 3. The comparison of BMI between control and brick kiln workers.

	BMI (Kg/m^2)		
Subjects	**Control**	**Workers**	**P value (correlation)**
Males	26.91±0.77	23.01±0.23***	p<0.001
Females	24.63±0.76	23.20±0.63**	p<0.001

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control

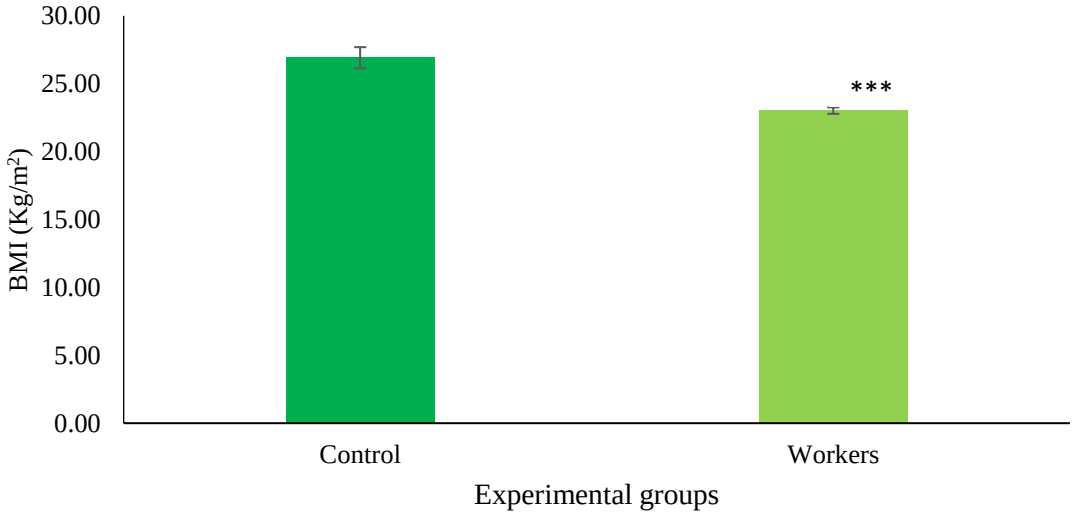


Figure 14. Figure shows the body mass index (BMI) of control and workers males.

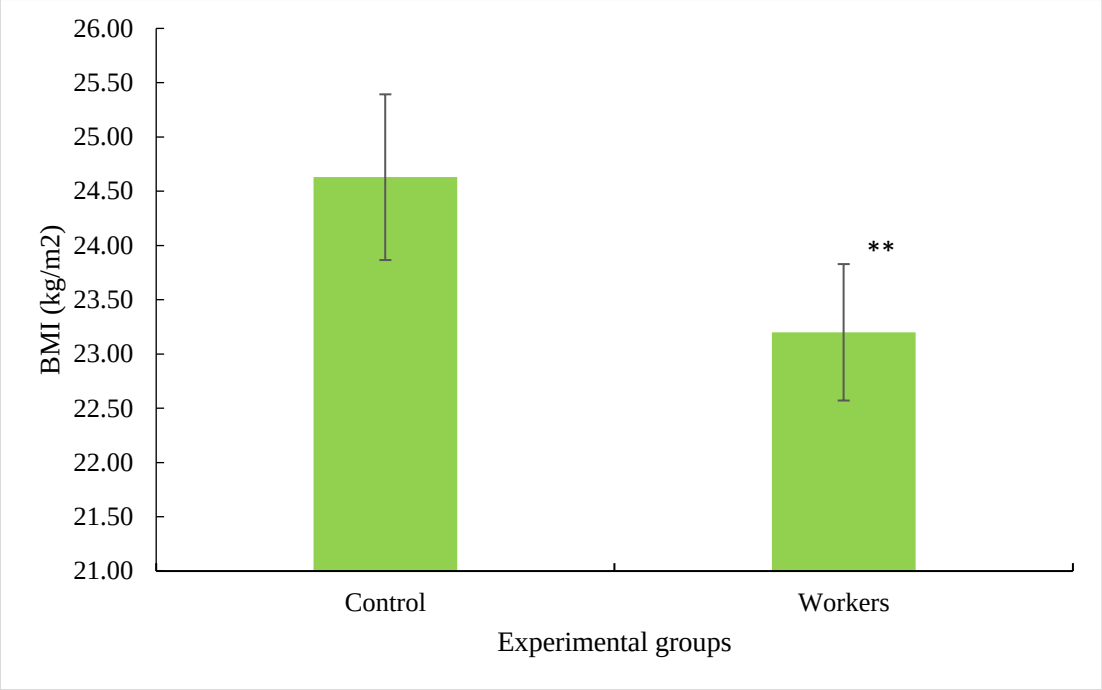


Figure 15. Figure shows the body mass index (BMI) of control and female workers.

Atomic absorption spectroscopy

Cd, Ni, and Cr were detected in increased concentrations among male and female workers in comparison with control participants (Table 4). Cd levels were highly significant ($p<0.001$) in male and female workers as equated to non-workers. Ni concentrations measured in blood of control female was 3.24±0.02µg/dl, while among workers, its levels were 5.59±0.03µg/dl. Similarly, in males, the Ni levels seen were 6.40±0.02µg/dl as compared to 3.88±0.01µg/dl in control. Cr levels were 2.81±0.02µg/dl in control group, while 4.20±0.02µg/dl in workers group of females (Fig 16). Highly significant rise ($p<0.001$) in levels of Cr were evident in male worker group (5.27±0.02µg/dl) in contrast to control group (2.02±0.01µg/dl) as shown in figure 17. No comparable change in levels of zinc were found among the all the groups.

Table 4. Mean ± SEM heavy metals concentration in whole blood of control and brick kiln workers measured through AAS.

Heavy metals	Males		Females	
	Control	**Workers**	**Control**	**Workers**
Cd (µg/dl)	2.37±0.01	5.14±0.02***	1.19±0.01	3.09±0.01***
Ni (µg/dl)	3.88±0.01	6.40±0.02***	3.24±0.02	5.59±0.03***
Zn (µg/dl)	1.24±0.01	1.10±0.01	--	--
Cr (µg/dl)	2.02±0.01	5.27±0.02***	2.81±0.02	4.20±0.02***

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ as compared to control.

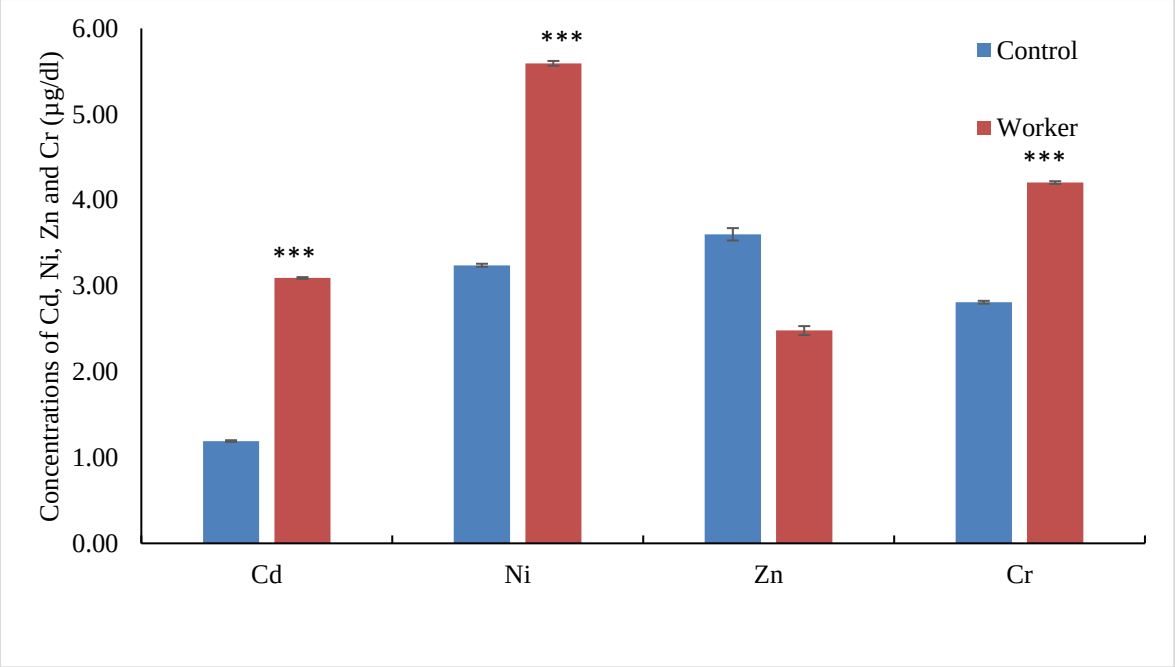


Figure 16. The comparison of heavy metal burden in blood plasma among control and female workers.

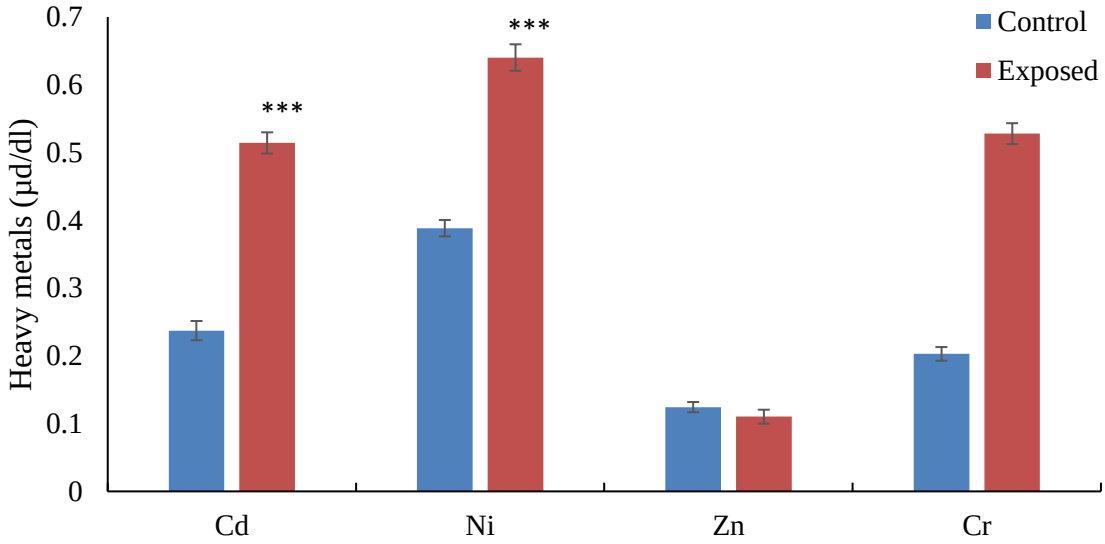


Figure 17. The comparison of heavy metal burden in blood plasma among control and male workers.

Soil analysis

The soil analysis of PIXE showed the presence of elements and metals including silicon (Si), Sulphur (S), Chlorine (Cl), Argon (Ar), Potassium (K), Calcium (Ca), Titanium (Ti), Vanadium (V), Chromium (Cr), Manganese (Mn), Iron (Fe), Cobalt (Co), Nickel (Ni), Copper (Cu), and Zinc (Zn) as presented in table 5. Metals such as Cr, Mn, Fe, Co, Ni, Cu, and Zn were also detected in soil samples collected from Rawalpindi as reported by previous studies (Iqbal *et al*., 2012).

According to standard regulatory bodies such as World Health Organization (WHO), Food and Agricultural Organization (FAO) and Ewers U, Standard Guidelines in Europe, the maximum permissible limits of heavy metals in soils have been established and are mentioned here as well (table 5) (Chiroma & Ebewele, 2014). The results of present study showed that metal levels were lying within the recommended limits.

Table 5. Table showing the normal range, obtained average concentration, and permissible limits of elements and heavy metals in soil.

Elements/metals	**Normal Range in soil (ug/g)**	**Average concentration (ug/g) obtained**	**Earlier values from Rawalpindi (ug/g)** (Iqbal *et al*., 2012)	**Permissible limits** (Chiroma & Ebewele, 2014)
Silicon (Si)	1663.3-1955	1839.14±50.29	-	-
Sulphur(S)	85.6-935	473.52±155.43	-	-
Chlorine (Cl)	30.3-78.5	42.8±9.07	-	-
Argon (Ar)	3.5-12.9	3.28±2.50	-	-
Potassium (K)	406-618.3	516.94±34.03	15,104±2,539	-
Calcium (Ca)	866.4-3050.2	1971.92±413.14	-	-
Titanium (Ti)	181.6-1041.5	392.3±160	4,550±691	-
Vanadium (V)	5.2-11.2	5.8±1.79	79.9±20.2	-
Chromium (Cr)	5.9-16.8	11.02±1.80	115±20	100
Manganese (Mn)	13.4-34.6	25.26±4.25	586±70	2000
Iron (Fe)	1659.4-4369.7	2469.6±495.66	30,262±2,692	50,000
Cobalt (Co)	14.7-17.9	9.94±4.09	11.69±1.36	50
Nickel (Ni)	1.9-7.2	4.58±1.07	135±18	50
Copper (Cu)	2-6.4	4.3±0.77	45±8.2	100
Zinc (Zn)	5.1-38.5	21.96±5.69	70.6±21.3	300

*NG= Data not given

External beam PIXE analysis

Table 6 shows the elements detected in the blood sampled from brick kiln workers and control participants. These elements included Si, P, S, Cl, K, Ca, Ti, Cr, Mn, Fe, Co, Ni, Cu, and Zn. Cr was only detected in the blood samples of brick kiln workers, whilst control participants had significantly higher levels of Co ($p=0.011$), Mn ($p=0.017$), Ni ($p=0.04$), and Ti ($p=0.05$). Although preparation and calibration were completed in such a manner to negate the effect of using 99.99% pure Cu strips as sample holders, Cu levels were significantly higher ($p=0.003$) in the control group (11883.11 ± 3701.31) than the brick kiln group (6296.72 ± 1966.0). No significant differences were observed between the blood samples retrieved from brick kiln workers and controls for Si ($p=0.408$), P ($p=0.331$), S ($p=0.518$), Cl ($p=0.394$), K ($p=0.596$) and Ca ($p=0.334$). The PIXE spectra for the elements detected has been shown in figure 18.

Table 7 shows previously published metal levels in participants exposed to industrial pollutants compared to metals levels detected in Pakistani brick kiln workers from the Rawalpindi district.

Table 6. Elemental levels detected in blood samples from the brick kiln and control group.

Metals (µg/mL)	Control	Workers	P-value statistics
Silicon (Si)	1323.65±238.96	1183.63±222.33	p=0.408
Phosphorus (P)	592.70±143.47	608.38±83.6	p=0.331
Sulphur (S)	709.94±225.27	786.01±213.31	p=0.518
Chlorine (Cl)	396.87±111.75	556.15±120.21	p=0.394
Potassium (K)	471.53±161.19	495.72±96.88	p=0.596
Calcium (Ca)	94.33±35.93	92.33±65.50	p=0.334
Titanium (Ti)	3271.80±3152.50	1345.58±634.8	p=0.052
Chromium (Cr)	--	23.9±4.01	p<0.001
Manganese (Mn)	979.24±577.92	64.44±39.42	p=0.017
Iron (Fe)	138.22±66.46	144.05±20.52	p=0.055
Cobalt (Co)	2243.26±1381.42	209.41±105.47	p=0.011
Nickel (Ni)	1818.41±974.09	1212.98±147.40	p=0.045
Copper (Cu)	11883.11±3701.31	6296.72±1966.0	p=0.003
Zinc (Zn)	225.94±18.73	179.10±33.92	p=0.580

Values are expressed as mean± SEM. P<0.05 shows the significant difference.

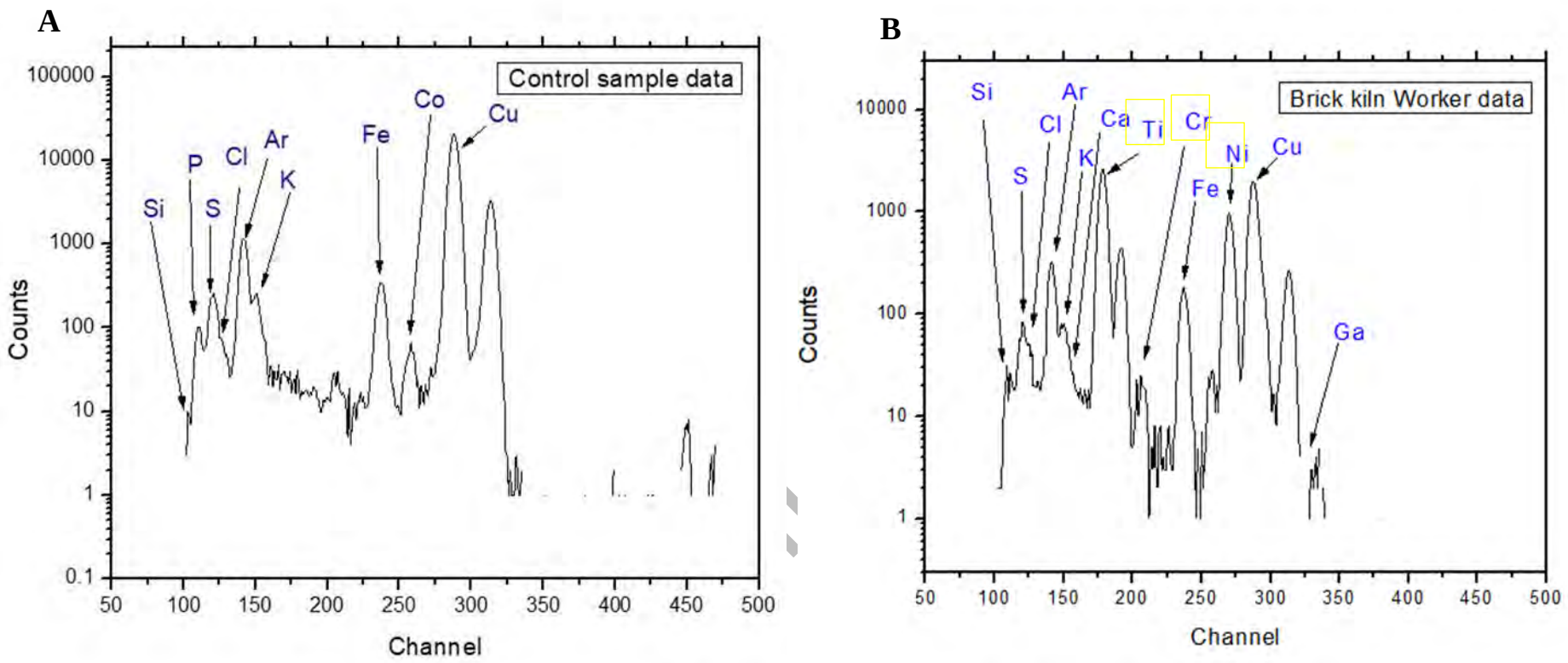


Figure 18. PIXE analysis of blood samples from (A) control subjects and brick kiln occupants (B) using 3MeV proton energy at NCP, Islamabad using 5 MV tandem Accelerator. Note the difference between the peaks of certain heavy metals that are absent in control data. The concentration of titanium (Ti), chromium (Cr), nickel (Ni) and gallium (Ga) are most evident in brick kiln emission exposed group.

Table 7. Previously published metal levels in participants exposed to industrial pollutants versus metals levels detected in Pakistani brick kiln workers from the Rawalpindi district.

Countries	Cr	Ni	Mn	Cu	Zn	References
China (AAS)	--	--	0.37±0.05 (µg/mL)	1.34±0.07 (µg/mL)	14.82±0.8 (µg/mL)	(X. Shen *et al*., 2019)
Pakistan (FAAS)	16.30±4.37 (µg/L)	4.66±1.73 (µg/L)	--	1.52±1.33 (µg/L)	13.50±2.44 (µg/L)	(Junaid *et al*., 2016)
Nigeria	--	0.07±0.00 ((µg/L)	--	19.72±0.21 (µg/L)	17.11±0.46 (µg/L)	(Ogbodo *et al*., 2020)
Pakistan (PIXE)	23.9±4.01 (µg/mL)	1212.98±147.40 (µg/mL)	64.44±39.42 (µg/mL)	6296.72±1966.0 (µg/mL)	179.1±33.92 (µg/mL)	Present study

*the results of present study (brick kiln industrial workers) are being compared with occupants of other industries.

SEM data

Figure 19 shows the cuticle morphology of the hair shaft of both control and brick kiln worker samples, which clearly shows raised and shredded cuticles on the hair surface of both the samples. The cuticle scales of the brick kiln worker harbored more surface deposits in the form of particulate matter (in the size range of <2.5 µm) than that of the control participant.

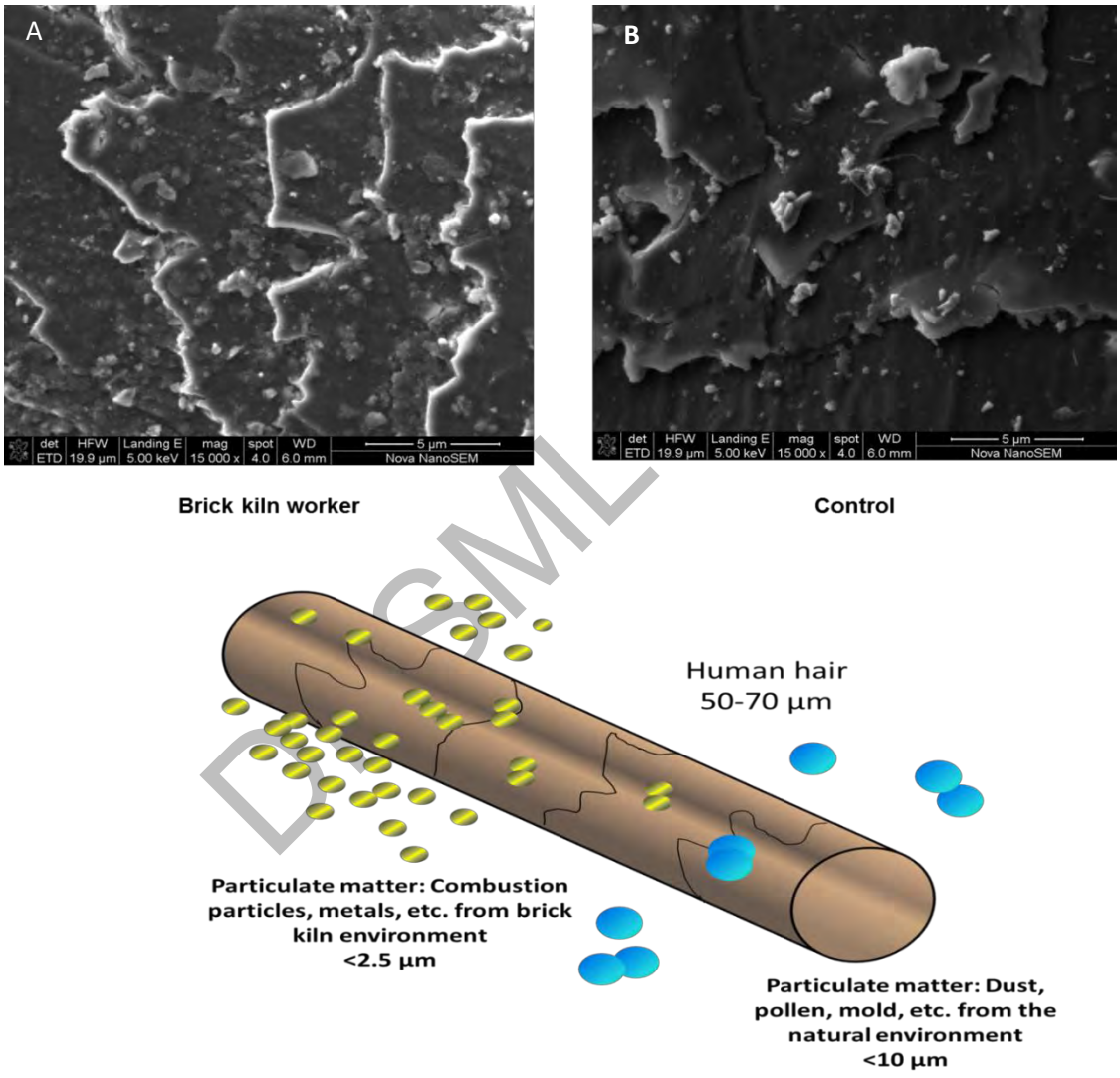


Figure 19. Scanning electron micrographs taken at 15000X magnification showing the hair surface of a male brick kiln worker (A) and a control participant (B). Fewer particulate matter (bottom image) is visible on the hair surface of the control sample (right image) than that of the brick kiln worker (left image).

DISCUSSION

The study monitored and reported the socio-demographic trends and brick kiln occupant's health status from Rawat, Punjab. Our findings suggested prevalence of multiple health issues among workers including allergies, kidney, stomach disorder as well as respiratory illness and tuberculosis. The use of dust protective equipment (masks or gloves) was not found among workers and moulding of bricks using bare hands was practiced, this suggests exposure to various environmental pollutants through respiratory, digestive or dermal route that further increases health risks among them. As occupational workers are exposed to atmospheric pollutants such as poisonous gases, metals, trace element and particulate matter (P.M), occupational factors (such as job length, lack of PPE, work type) also play a key role in affecting the employees' physical and mental health as previous studies have suggested (Patil, 2017; Shaikh *et al*., 2012). It is known that work-related risk factors are a key source of respiratory illness (Shaikh *et al*., 2012). The findings of present study highlighted that no health or welfare facilities were provided to labour and working environment was open and poor. Likewise, findings have been reported by Patil. (2017) in their respective study, where cross sectional-observational study was conducted on brick workers from village Karad Taluka, India and similar conditions at brick kiln sites were noticed (Patil, 2017). Of 346 men who participated in present study, 58% of them were addicted towards various drugs such as cigarette, naswaar (amalgamation of tobacco leaves, calcium oxide and wood ash) and charras (hashish form of cannabis) as well as hukka (tobacco mixture). Direct exposure to these toxic chemicals induce various types of musculoskeletal symptoms along with different types of cancers and therefore, raise serious public health concerns among occupational workers (Rajesh & Niraj, 2010; Rzymski *et al*., 2014; Sanjel *et al*., 2016; Shaikh *et al*., 2012).

A significant decreased in BMI of male workers was observed. The low BMI values of workers indicate generally poor health conditions of workers with poor immune system, making them prone to various allergies, musculoskeletal problems, respiratory disorders, and viral diseases (Shahid *et al*., 2017). Environmental exposure to metals such as cadmium contribute to negative correlation with BMI as suggested by previous studies (Padilla *et al*., 2010). Another study findings suggested that exposure to even low levels of Cd has significantly negative effect on body weight (Shirai *et al*., 2010). Similarly, long term exposure to lead (Pb) can result in build-up in the body and may cause anemia, abdominal pain, nausea, and kidney damage and weight loss (Ishaq *et al*., 2010). Our results also suggest that health risks associated with body fat are more increased in male brick kiln workers as compared to control subjects.

Maternal pre-pregnancy BMI genetic risk and gestational weight gain are an important determinant of fetal weight (Shrestha *et al*., 2019). In our study, most of the studied married female workers were either overweight or underweight despite of their BMI, which is near to normal (i,e app. 24). The number of underweight women working in brick kilns was found quite high as compared to control women that might be due to promising concentration of Cd circulating in blood. Previous studies have found that environmental exposure to low levels of Cd has significantly negative effect on body weight (Shirai *et al*., 2010). Recently, the generational effect of BMI has been reported, where it is established that even the BMI of grandmother can indirectly increase the grandchild's body weight via maternal body weight and BMI (Shen *et al.,* 2020). Another study suggested the negative correlation between neonatal outcomes and pregnant women with an inadequate BMI (Papazian *et al*., 2017). As most of the brick kiln workers are bonded labor and thus, they continue to work at kiln sites generation after generation.

Therefore, among occupational women exposed to various environmental pollutants, it can be speculated that metals exposure through atmosphere may accord alterations in human weight (Padilla *et al*., 2010). However, ethnic differences have been noticed among Asian and European peoples (Barba *et al*., 2004). Similarly, our results have shown that health risks associated with body fat are more increased in brick kiln female workers as contrasted with control subjects. Therefore, when planning a pregnancy, it is important to reach an optimal BMI before conception along with proper nutritional counseling by health care professionals (Papazian *et al*., 2017).

In present study, analysis of heavy metals through AAS in whole blood revealed a remarkable increase in concentrations of Cd, Ni and Cr in both male and female workers. Brick kilns emit large amount of greenhouse gases as well as heavy metals (Rajonee & Uddin, 2018) which become part of the environment through air, water and soil, eventually, imposing health risks among people living in/around kilns (Mohammadi *et al*., 2018). Multiple studies across Pakistan have reported that brick kiln emissions prove to be an environmental pollutants affecting many life forms (David *et al*., 2020; Khan *et al*., 2019; Nasir *et al*., 2021; Shaikh *et al*., 2012). Exposure to the detected metals may impart adverse health risks by triggering various disorders of the heart, liver, skin, brain, kidney, liver, and respiratory disorders (Ghosh *et al*., 2020). Studies reported that increased levels of Cd were seen in males and females. Ingestion of contaminated food or water as well as smoke inhalation makes human susceptible to Cd^{2+} ion toxicity. Thevenod (2009) reported that exposure to Cd^{2+} imparts adverse effects on multiple organs including liver, lungs, testis, placenta, and bone (Mohammadi *et al*., 2018; Thévenod, 2009). Cd is known to exert reprotoxic effects in males, through multiple processes (de-Angelis *et al*., 2017a). In females, Cd concentration increases in ovaries usually with age, and hinders oocyte

development. Gradual Cd accumulation in embryos prevents development to the blastocyst stage causing degeneration and decompaction with apoptosis and breakdown in cell adhesion (Thompson & Bannigan, 2008). The increased abortion rate in brick kiln workers observed in current study is correlated with previously reported literature (Thompson & Bannigan, 2008). Nickel is another metal present in food, water and soil and is known to possess neurotoxic, immunotoxic, hematotoxic, genotoxic and reprotoxic potential. Ni is reported to accumulate in various tissues such as liver, lungs and kidneys of workers, who are exposed to nickel (Das & Das, 2008). The findings of current study showed that women and men working at brick kilns have metabolic and reproductive disorders that might be associated with Ni toxicity. It is known to induce cancers, respiratory disorders, neurotoxicity, epigenetic changes and reproductive diseases among males and females by various mechanisms (Rizvi *et al*., 2020). Growth and reproduction is found to be affected by Ni exposure that influence the health and survival of individual (Furness & Rainbow, 2018). The results of AAS revealed elevated levels of Cr in present study, which is thought to be associated with multiple pathologies in humans such as carcinogenicity (Pavesi & Moreira, 2020). It is also known to induce cellular toxicity via production of ROS with subsequent cellular damage (Seydi *et al*., 2020). Cr species are another major environment pollutants due to its extensive use in various industrial applications (Duran *et al*., 2011). The chronic exposure to Cr induces toxicity in liver and kidney (Cherfi *et al*., 2014). Thus, it is inferred that the presence of higher levels of these hazardous metals in blood serum may have detrimental effects on the health outcomes of kiln workers and participants residing in the close proximity.

This is to our knowledge the first report combining external beam PIXE to screen elemental and most importantly, metal levels in blood combined with the use of SEM to

screen pollutant and more precisely, PM deposition on the hair surface sampled from brick kiln workers and control participants. Both techniques contributed to the non-destructive analysis of small sample sizes of blood and hair. The elements detected included Si, Ti, Mn, Fe, P, S, Cl, K, Ca, Co, Ni, Cu, and Zn. Cr was only found in the blood samples retrieved from brick kiln workers and not in control participants, whilst control participants had significantly higher levels of Cu, Co, Mn, Ni, and Ti. No significant differences were detected between the control and brick kiln samples for essential (P, S, Cl, K, Ca) and non-essential elements (Si).

In human body, Cu plays an important role in enzymatic reactions, however, Cu toxicity may occur resulting in multiple cellular changes (Zhou *et al*., 2018). Similarly, the presence of Co at elevated levels in blood serum causes erythrocytosis, and poses a risk to the development of heart and thyroid gland pathologies, and is responsible for occupational asthma and dermatitis (Banza *et al*., 2009). Although manganese and Fe are naturally occurring metals which usually coexist in the environment such as in ground water, elevated levels of Mn in blood are usually most evident in Fe deficient children, causing anemia, and may compromise mental health (Rahman *et al*., 2013). Ni is another metal that exerts a negative effect on human health and is responsible for triggering the development of cancer and dermatitis (Zambelli *et al*., 2016). Similarly, even minute amounts of Ti in the body may exert toxic effects through altering cell cycle events and constriction of nuclear membranes inducing cell death (Baranowska *et al*., 2020). Interestingly, Cr was detected in the blood of brick kiln workers group and not in that of control participants. Previous work has also shown elevated blood levels of Cr in kiln workers that was associated with multiple health effects, including lung cancer, an altered immune response, and allergic reactions (Achmad *et al*., 2017; Paustenbach *et al*., 2003; Shrivastava *et al*., 2002).

Interestingly, the non-destructive analysis of hair using SEM showed that scalp hair retrieved from brick kiln workers visually showed a higher and also homogeneous density of particulate matter of various sizes either trapped or deposited on the hair surface compared to control participant hair samples. The particulate matter sized <2.5µm ($PM_{2.5}$) deposited on the hair of brick kiln workers may contain metals or other inorganic/organic solids, combustion particulate matter, or other aerial particulate matter (Galliano *et al*., 2017a; Qu *et al*., 2018). They are released during the activities performed at the brick kiln including the carriage, molding, and baking of bricks. SEM-EDX it is a semi-quantitative technique compared to PIXE, whilst the irregular surface of the hair may also negatively impact the data obtained using this technique or the operational, instrumental, and specimen errors (Rendón *et al*., 2017).

In general, the deposition and adherence of particulate matter from aerial pollution on the hair surface is poorly studied. Previous research has shown that a large number of particulate matter of various sizes can be deposited onto hair cuticle scales of individuals living in aerially polluted (e.g. brick kiln) environments, a process that may be mediated by the presence of sebum on the hair surface as well as the hair's physio-chemical properties including a multitude of environmental factors such as humidity (Galliano *et al*., 2017; Qu *et al*., 2018). The hair's physio-chemical properties may also be significantly altered by frictional forces (brushing and shampooing), solar UV ray exposure, and the extent of exposure to air pollutants that may contain oxidizing and per-oxidizing compounds that breaks down keratin and render the hair surface more amenable to particulate matter deposits increasing anionic binding sites by and lifting the hair cuticle (Galliano *et al*., 2017b; Qu *et al*., 2018; Rendón *et al*., 2017).

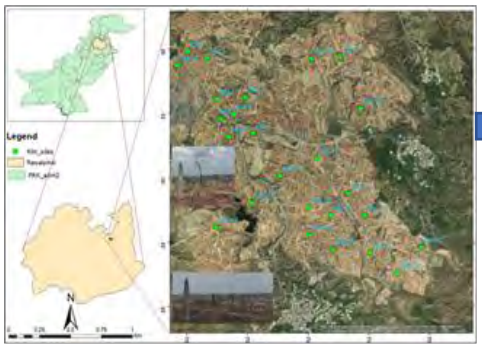
CONCLUSION

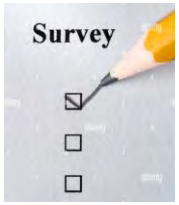
- The study concluded that brick kiln individuals experienced lowered body weight, disturbed BMI, where 10% of them were underweight and had multiple health issues including skin allergies, asthma, stomach and kidney disorder, and other diseases as well, where occupational factors played their role as well.
- The PIXE analysis of soil showed the presence of trace elements and heavy metals including Si, S, Cl, Ar, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, and Zn.
- Further, we found that the viability of external beam PIXE and SEM-EDX to provide elemental data on hair and blood samples serves as biomarkers for exposure to toxic elements. The average values of Co, Ni, Ti, Cr, Mn and Fe in blood were found higher than permissible limits recommended by FDA.
- SEM/EDS analysis of hair depicted the presence of macro-element with average levels in the order of: K > S > Ca > P > Cl and a micro-element profile in the order of: Rb > Fe > Mn > Cu > Sr > Zn. The results here confirmed that blood and human scalp hair represent an excellent screening biomatrices, in which xenobiotic compounds such as metals and airborne particulate matter can be incorporated. Using non-invasive techniques such as PIXE and SEM, blood and hair may well serve as pollutant probes for both aerial and ingested pollutants.
- Conclusively, it can be stated that external and internal deposition of heavy metal on hair and blood of brick kiln workers is much higher than that of normal population that poses health risks among occupational individuals and may raise health problems and diseases.

SUMMARY

Area- Rawat, Pakistan

n=546 n=232





SAMPLING

Questionnaire

Blood

Hair

Soil

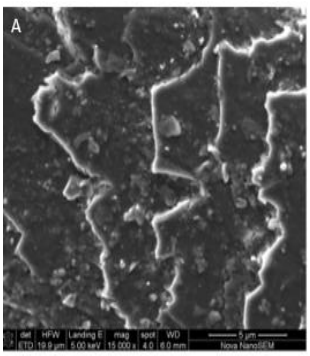
Atomic absorption spectroscopy

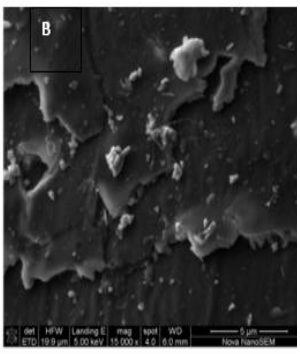
Elemental analysis

Proton induced X ray emission

Increased levels of heavy metals Cd, Cr, Ni and Zn were evidenced in brick kiln workers samples as compared with control group.

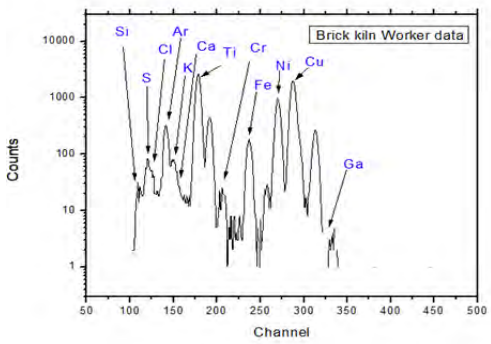
Scanning electron microscopy (SEM/EDS)





Brick kiln worker Control

Concentration of Ti, Cr, Ni, and Ga are most prominent in worker group



More visibility and deposition of particulate matter (PM) on hair surface of brick kiln worker sample

Chapter 2

Assessment of effects of heavy metal burden on body mass index, hematology, oxidative stress, and hormonal profile of male brick kiln workers

ABSTRACT

Due to increased number of labors working at brick kiln industry, the concerns for public and reproductive health risks are much increased among them. Thus, the present study design aims to explore the possible properties of heavy metal concentration in blood and its potential impact on blood parameters, production of antioxidants, and hormonal profile of adult male workers from brick kilns. The study involved a total of 546 men, of which 200 served as control group. Blood was collected and processed for determination of hematological profile, oxidant/antioxidant enzymes levels and concentrations of reproductive hormones. The results showed that a significant increase in platelet count ($p=0.010$); notable reduction ($p<0.001$) in hemoglobin, red blood cells number and percent hematocrit; decreased antioxidant enzyme ($p<0.01$) and amplified oxidants levels ($p<0.001$) in brick kiln workers as compared to control. Intensified cortisol levels ($p<0.001$) were also seen in workers in contrast to control group. The correlation of hormonal data indicated an inverse relationship between cortisol and pituitary gonadotropins (FSH $r = 0.676$, LH $r = 0.580$); and cortisol and testosterone ($r = 0.832$). Therefore, the study concluded that as a consequence of increased heavy metal burden in male worker's blood, they are at the risk for the development of public and reproductive health problems due to long-term compromised antioxidant enzyme levels, increased oxidative stress conditions and disturbed production of gonadotropins and sex steroids that ultimately affect their reproductive outcomes.

INTRODUCTION

Various large-scale industries release air pollutants directly in the atmosphere such as plastic industry, brick kiln industry and many others. One of the key source of air pollution is brick kiln industry especially in under developing countries including Indo-Pak region (Gómez *et al*., 2012). These air pollutants are introduced into the air via vehicle smoke, industrial smog, hospital wastes and brick kiln emissions and may pose a threat to all life forms (Ismail *et al*., 2012). Thus, brick kiln emitted pollutants affect all the components of the environment, including soil, water, air, plants and animals (Khan *et al*., 2019a). The brick kiln industry also emits large amount of hazardous and greenhouse gases which result from the combustion of low quality fuels such as rubber tires, motor oils and coal (Achakzai *et al*., 2017). These emissions are responsible for the climate change resulting in depletion of ozone layer as well as acid rain. The resulting acidic raindrops may harm the plant parts and animal skin (Khan *et al*., 2019). Eventually, the aquatic life may also get harm when these toxic gases flow with water into the larger water bodies such as rivers/sea. The absorption of these chemicals by the soil may alter its pH and may affect the growth of plants (Fatima *et al.*, 2011). As most important component of the clay brick is the soil, different environmental problems of soil degradation may cause (Khan & Vyas, 2008). Thus, it is inferred that multiple industries contribute to air pollution which affects all components of the environment.

Apart from the adverse effects of brick kiln emitted pollutants on physical components of environment, these pollutants also exert hazardous effects on human health and reproductive well-being (Kampa *et al*., 2008; Woodruff *et al*., 2008). Some of the released gases include oxides of sulfur, nitrogen and carbon as previously discussed. SO_2 can cause various respiratory disorders and eyes irritation (Geravandi *et al*., 2015). It is also reported to have toxic effects on humans and animal reproduction in males (Zhang *et*

al., 2016). Exposure to CO is shown to induce adverse neurodevelopmental and developmental disorders (Levy, 2015). However, brick kiln workers are exposed to other toxic pollutants as well, including heavy metal (in the soil), diesel exhaust, smoke, heat and radiation in the atmosphere (Kaushik *et al*., 2012). The gases and pollutants which become part of the environment via activities of brick kilns remain a hazardous threat to the overall environment as well as the well-being of kiln workers and people residing in the nearby areas (Kaushik *et al*., 2012).

Different study groups have reported that exposure to elevated levels of heavy metals such as lead, cadmium or chromium in brick kiln soil is found to induce neurological, hematological, gastrointestinal and immunological pathologies and even neoplasms, in which chief target is heme synthesis enzymes, thiol-containing antioxidants and enzymes (Flora *et al*., 2008; M. Ismail *et al*., 2012; Kaushik *et al*., 2012). Usually, diesel is used for combustion in kilns for baking of bricks. The products and byproducts formed by complete/incomplete combustion of diesel are known to have genotoxic, cytotoxic, carcinogenic, free radical generating and DNA damaging effects (Kaushik *et al., 2012)*. Exposure to heat and radiation can cause genetic mutations, DNA strand breaks, DNA protein cross-links, radiation pneumonitis, fibrosis and diffuse alveolitis (Kaushik *et al*., 2012).

Aims and objectives

Large number of people are associated with brick kiln industry. Due to problems of life debt and slavery in Pakistan, we reported that approximately 249, 682 people including men and women are associated with this profession, only in Punjab (David *et al*., 2020). Due to such an increased number of labors associated with brick kiln industry, the concerns for public and reproductive health risks are much increased among them. Therefore, the present study find its roots from the findings of previous studies, which depicted the deleterious actions of brick kiln emitted pollutants on biochemical profile of industry workers, exposed to chimney emission of heavy metals, oxides and smoke (Bonde, 2013; Jahan *et al*., 2016). Hence, the current study intends to find out the comparative environmental health effects of heavy metal burden in blood among brick kilns workers and unexposed individuals, by looking at blood parameters, oxidative stress markers, antioxidant enzymes concentrations, and reproductive hormones profile along with stress response on hypothalamic pituitary gonadal (HPG) axis.

To address reproductive health in individuals of different age groups working in brick kilns, the study aims to

- Investigate the possible effect of brick kiln emitted heavy metals on blood parameters
- Measure and compare the antioxidant levels in blood plasma samples of brick kiln workers and control
- Evaluation of oxidative stress markers among brick kiln workers and control subjects
- Address possible effects of heavy metals on reproductive hormones concentrations
- Assessment of hypothalamic pituitary adrenal (HPA) axis via monitoring stress hormone concentrations in blood
- Find out the association of stress response (HPA axis) with the reproductive axis (HPG axis) by correlation analysis

MATERIALS AND METHODS

Study population

Adult males employed for a varied number of years at the brick kiln sites were selected for this study. Random selection was done for participants willing to be included in this study, with everyone representing a population. After data collection, the data was sorted, analyzed and subsequently only those participants were included who fulfilled our study objectives. Because the study focused on the health status of individuals, the sample size was calculated with 5% level of significance and 10% minimum error based on the number of healthy and diseased workers. The sample size was calculated as previously described by Patil *et al*., (2017) using given formula which provided a calculated sample size of 336.

$$n = 4pq/L^2$$

where n = Sample size, p = Workers who had some health problems (%), q = Workers who had no health problems (%), L = Level of significance.

The final total number of n = 346 adult male workers aged 18–55 years and working for at least 1–5 years and more (< 1yrs- > 20yrs) at brick kiln sites were included in the study. Control samples included adult males (aged 18–58 years; n = 200) who lived in the same district, but at least 40 km away from the kilns. Control participants' least exposed to environmental pollutants such as vehicle and industrial smoke were selected.

Hematology analysis

Hematology analysis was performed on an automated hematology analyzer at MultiLinks Laboratory, Rawalpindi. The data collected included mean corpuscular hemoglobin concentration (MCHC), platelets count (PLT), mean corpuscular volume (MCV), hemoglobin (HGB), mean corpuscular hemoglobin (MCH), white blood cell count (WBC), red blood count (RBC), and hematocrit (HCT).

Biochemical studies

The blood plasma previously kept at -20°C (as mentioned in chapter 1) was utilized for the biochemical analysis; for determination of antioxidant enzymes activity of sodium dismutase (SOD), peroxidase (POD), lipid peroxidation (MDA) and total protein using UV Spectrophotometer (Agilent 8453).

Superoxide dismutase activity

Used Reagents:

1. Homogenate 0.3ml
2. Sodium pyrophosphate 1.2 ml (0.052mM; pH=7)
3. NADH 0.2 ml (780µM)
4. Phenazine methosulphate 0.1 ml (186µM)
5. Glacial Acetic Acid 1 ml

Procedure:

To measure SOD activity, 0.3 ml of blood plasma sample was taken and was supplemented with sodium pyrophosphate buffer and phenazine methosulphate followed by gentle mixing. Later, NADH was administered for initiation of processing and left for 1 minute (Kakkar *et al*., 1995). Lastly, glacial acetic acid was dispensed for stoppage of reaction. At 560nm, absorbance was measured, and results are given as Units/mg of protein.

Guaiacol peroxidase activity

POD levels in blood serum were defined by spectrophotometric method previously given (Chance & Maehly, 1955). 0.1ml of blood plasma was taken and the reaction mixture was prepared by the addition of 0.1ml guaiacol (20mM), 0.3ml H_2O_2 (40mM) and 2.5ml phosphate buffer (50 mM (PH 5.0)) and kept for one minute. At 470nm, difference

in absorbance was noted and 1 unit of pod action was described as an absorbance change (ΔA) of 0.01 as unit/min.

Estimation of ROS

The estimated number of reactive oxygen species was determined by protocol previously devised (Hayashi *et al*., 2007). Initially, R1 solution was prepared by dissolving 100g/ml DEPPD in sodium acetate buffer (0.1M) whose pH was maintained at 4.8. Secondly, 4.37M R2 solution was prepared by dissolving ferrous sulphate in sodium acetate buffer. The calibration curve was constructed by using H_2O_2 as a standard solution. The reading was taken UV Spectrophotometer (Agilent 8453).

Lipid peroxidation (MDA)

Thiobarbituric acid (TBA) was used for determination of malondialdehyde (MDA) in blood plasma. In 0.1ml plasma sample, 0.1 M of 0.29ml phosphate buffer (pH 7.4), and 0.1ml ascorbic acid (100 Mm) were supplemented and kept for incubation of one hour at 37° C in shaking water bath (Wright *et al*., 1981). After 60 minutes, 0.5ml of 10% trichloroacetic acid was added to prevent the further reaction; 1ml of 0.67% trichlorobarbituric acid was also dispensed, and reaction mixture was maintained for 20min at 95° C in boiling water bath. The samples were removed from water bath and moved to the crushed ice bath. Later, centrifugation was done at 2500X g for 10min, and supernatant was collected. Optical density was noted for all the samples and the reagent blank to measure the TBARs concentration at 535nm using spectrophotometer (Agilent 8453). The results were stated as TBARS/min/ml plasma at 37° C using following formula.

$$\text{Concentration of the test} = \frac{\text{Abs (test)} - \text{Abs (blank)}}{1.56} \times 1000000$$

Total protein measurement

Total protein content in blood plasma was quantitatively determined by using kit provided by AMEDA Labor diagonstik GmbH (Austria).

Principle of Assay:

In alkaline solution, Cu^{2+} ions and the peptide bonds of protein form a chelate (biuret reaction). The intense the color, the more concentration of protein is depicted in the sample (pH≥12).

Cu^{2+} + sample protein complex Copper-protein

Reagents used

Potassium sodium tartrate	21 mM/L
Potassium iodide	6 mM/L
Copper sulphate	6 mM/L
Sodium hydroxide	0.75 M/L

Standard:

Bovine serum albumin	7 g/dL

Procedure

Standards were prepared by adding 20µl of standard provided with the kit along with the reagent (1.0 ml). Samples and reagent blank were prepared by adding 20 µl of plasma and 20µl of distilled water with reagent (1.0 ml) and mixed gently. All the samples were incubated for 10min at 37°C. Change in absorbance of standard and samples was measured at 546 nm using chemistry analyzer.

Enzyme Immunoassay

Hormonal concentrations for testosterone (T), luteinizing hormone (LH), cortisol, and follicle stimulating hormone (FSH) were calculated by different commercially available enzyme immune assay (EIA) tests kits.

Luteinizing hormone (LH)

The concentration of LH in plasma was measured quantitatively using LH ELISA kit bought from Reddot biotech INC.

Procedure:

For the determination of LH, the LH antibody coated wells were filled with 50µl of 7 standards, 1 blank and plasma samples to be tested, followed by immediate supplementation of 50µl detection reagent A; stirred gently and covered with the provided plate sealer. Micro plate was then incubated at 37˚for 60 min. Cloudiness of detection reagent A was cleared by gentle shaking of plate. After incubation, wells were cleaned with 350µl of wash solution (3X) using auto washer and dried by inverting on porous paper. Later, 100µl detection reagent B was administered in all wells; sealed with plate sealer and kept for 60min at 37˚C. Washing was repeated for 5 times. After that, 90µl of Horseradish Peroxidase (HRP) was dispensed in all wells. Again, plate was kept for 15-25mins at 37˚after covering with new plate sealer to prevent from light during the process. HRP addition rendered the wells blue in color. The quantity of bounded HRP conjugate was in contrast to sample LH concentration, so do the color intensity. At last, 50µl stop solution was augmented to all wells and the liquid in wells turned yellow. The plate was tapped gently for thorough mixing and uniform appearance of color. Values were recorded at 450nm respectively. Minimum detection limit of the kit was 0.11ng/ml.

Follicle stimulating Hormone (FSH)

FSH concentration in plasma was measured quantitatively by Follicle stimulating Hormone ELISA kit bought from Reddot biotech INC.

Procedure:

For the determination of FSH, the FSH antibody coated wells were filled with 50µl of 7 standards, 1 blank and plasma samples to be tested, followed by immediate

supplementation of 50µl detection reagent A; stirred gently and covered with the provided plate sealer. Micro plate was then incubated at 37˚for 60 min. Cloudiness of detection reagent A was cleared by gentle shaking of plate. After incubation, wells were cleaned with 350µl of wash solution (3X) using auto washer and dried by inverting on porous paper. Later, 100µl detection reagent B was administered in all wells; sealed with plate sealer and kept for 60min at 37˚C. Washing was repeated for 5 times. After that 90µl of Horseradish Peroxidase (HRP) was dispensed in all wells. Again, plate was kept for 15-25mins at 37˚after covering with new plate sealer to prevent from light during the process. HRP addition rendered the wells blue in color. The quantity of bounded HRP conjugate was in contrast to sample FSH concentration, so do the color intensity. At last, 50µl stop solution was augmented to all wells and the liquid in wells turned yellow. The plate was tapped gently for thorough mixing and uniform appearance of color. Before taking measurements in micro plate reader, plate was cleaned to remove any water drops or fingerprints to remove any bubbles if present. Values were recorded at 450nm respectively. Minimum detection limit of the kit was 0.11 ng/ml.

Determination of serum testosterone (T)

Testosterone concentration was measured using purchased EIA test kits (Amgenix Inc, USA) following protocol provided with the kit. The assay sensitivity was 0.05ng/ml.

Procedure

To measure T levels in blood plasma, the required number of coated wells were secured in the holder. Standards, specimen, and controls (19µl) were dispensed, followed by addition of Testosterone-HRP conjugate Reagent (100µl) and Rabbit anti-Testosterone Reagent (50µl) and a thorough mixing for 30sec. The microtiter well plate was incubated at 37˚C for 90min, followed by cleaning 5 times with distilled, and administration of TMB Reagent (100µl). Later, after gentle mixing for 10sec, the micro well plate was incubated

at 18-25° C for 20min. Lastly, stop solution (100µl) was augmented to stop the reaction and was mixed gently for 30sec. Within 15min, the absorbance was read at 450nm using microtiter well reader and expressed in ng/ml.

Determination of serum Cortisol hormone

Using Cortisol hormone ELISA test kit (The Calbiotech, Inc. USA), cortisol levels in blood plasma were quantified using protocol provided with the kit. The sensitivity of the assay was 1.16ng/ml.

Assay Procedure

In order to measure cortisol levels in human plasma, the required number of coated wells were secured in the holder. Standards, specimen, and controls (25µl) were dispensed, followed by addition of Biotin reagents (50µl) and Cortisol Enzyme Conjugate Reagent (100µl) and a thorough mixing for 10sec. The microtiter well plate was incubated at room temperature (20-25°C) for 60min, followed by removal and washing the wells 3 times with 300µl of 1X wash buffer, followed by administration of TMB substrate (100µl). Later, the micro well plate was incubated at 20-25°C for 15min. Lastly, stop solution (50µl) was augmented to stop the reaction and was mixed gently. Later, within 20min, the absorbance was read at 450nm using microtiter well reader.

Statistical analysis

The data was represented as mean and standard error of mean (Mean ± SEM). Data was analyzed by applying student's unpaired t-test, carried by computer software SPSS 17 for blood parameters, antioxidant enzymes profile and for change in hormonal concentrations. Correlation was determined among cortisol and other reproductive hormones such as FSH, LH and testosterone in pairwise fashion using Pearson's correlation (r) and significance value (p) was measured. $p<0.05$ was considered significant.

RESULTS

Blood parameters

The results of complete blood showed no significant change in number of white blood cells control and worker subjects (table 8). A significant decrease ($p<0.001$) in HGB level was evident in workers as compared with control. Highly significant decline in number of RBC was observed in brick kiln workers with $p<0.001$. The total hematocrit (HCT) concentration reduced from 43.70±0.84 in control group to 38.69±1.12 in workers with $p<0.001$. The normal range of all the blood variables have also been given in table 8. A significant lowering in MCV and MCH with $p=0.007$ and 0.012, while significant surge ($p<0.001$) in RDW-CV was notable in worker men when comparison was made with the control group. A momentous reduction in MCHC with a significance value of $p=0.088$ was found in worker group. Increase in RWD-SD and PCT was noted in brick kiln group with significance value of $p=0.001$ and $p=0.029$. Significant boost in PLT ($p=0.01$) was seen in workers group as compared to control. No comparable change in MPV ($p=0.898$), and PDW ($p=0.194$), was experienced among the two experimental groups.

Table 8. Effects of heavy metal burden on blood profile of male workers and control.

*, **, *** indicates significant difference at probability p<0.05, p<0.01 and p<0.001

Blood parameters	Control	Worker	Normal range	P value statistics
WBC (1×10^3)	8.17±0.40	8.84±0.25	4-11	p=0.163
HGB (g/dL)	14.11±0.26	12.5±0.41***	13-18	p=0.001
RBC (1×10^3)	5081.87±63.73	4295.41±89.24***	4500-6500	p<0.001
HCT (%)	43.70±0.84	38.69±1.12***	40-54	p=0.001
MCV (fL)	87.54±0.92	82.56±1.52**	79-96	p=0.007
MCH (pg)	28.43±0.36	26.53±0.63*	27-32	p=0.012
MCHC (g/dL)	32.56±0.18	32.01±0.25*	30-36	p=0.088
RDW-CV (%)	13.68±0.17	15.79±0.49***	11.8-14.5	p<0.001
RWD-SD (fL)	42.29±0.46	46.30±0.95***	40.0 - 55.0	p=0.001
PLT (1×10^3)	246.93±11.68	293.12±12.95**	140-450	p=0.010
MPV (fL)	8.99±0.12	9.02±0.12	7-12	p=0.898
PDW (%)	15.46±0.04	15.54±0.03	10.0-17.9	p=0.194

compared to control

Biochemical analysis

The biochemical studies showed a significant decrease (p=0.006) in levels of SOD from 16.96±0.72 U/min in control group to 14.49±0.49 U/min in workers group (figure 20). No considerable change in POD levels among control and worker subjects was evident (p=0.393). A highly significant rise (p<0.001) in reactive oxygen species number was evident in workers group as compared to control. The mean serum MDA levels for workers group was 5.28±0.40 nmol /ml with significance level of p=0.279, as compared to control group (4.69±0.36 nmol /ml). There is a significant difference at p value of less than 0.05 between workers and control group for the protein estimation (table 9).

Table 9. Effects of heavy metal burden on biochemical profile of male workers and control.

Biochemical parameters	Control	Exposed	Statistics
SOD (U/min)	16.96±0.72	14.49±0.49**	p=0.006
POD (nmole)	21.72±2.22	19.23±1.44	p=0.393
ROS (µmol/min)	1.17±0.06	1.50±0.07***	p=0.001
MDA (nmol /ml)	4.69±0.36	5.28±0.40*	p=0.279
Protein estimation (g/dl)	11.65	8.95*	p=0.013

*, **, *** indicates significant difference at probability p<0.05, p<0.01 and p<0.001 compared to control

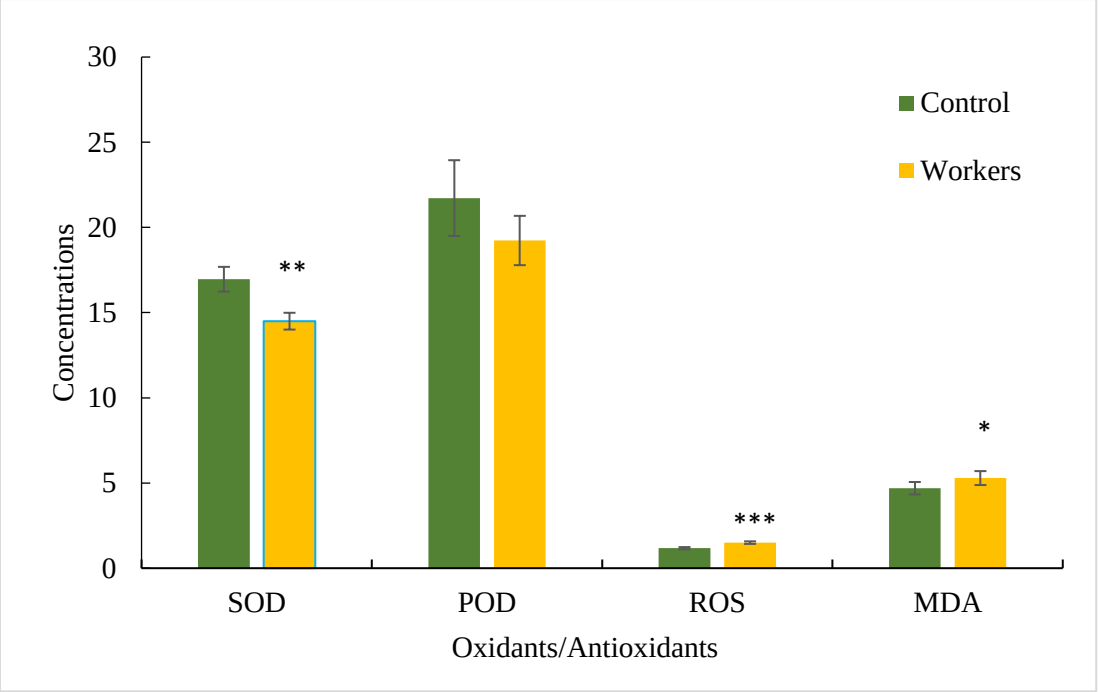


Figure 20. Effect of heavy metal burden on activity of sodium dismutase (U/min), peroxidases (nmole), reactive oxygen species (µm/min), and malondialdehyde (nmole/ml) in blood plasma among male workers and control.

Hormonal analysis

Table 10 shows the hormone levels in control and worker samples as well as their normal ranges in adult males. A highly significant change in FSH levels was observed between control and workers group with a significant value of p<0.001 (table 10). Significant rise (p<0.001) in LH level was experienced in workers group as given 10.80±0.19 mIU/ml, compared to control group 7.86±0.12 mIU/ml. The concentration of testosterone in serum plasma of workers group presented by mean value of 2.13±0.12 ng/ml was significantly different (p= 0.000) from that of control group 5.80±0.06 ng/ml (figure 21). Significant increase (p<0.001) in cortisol concentration of workers group was seen in contrast to control.

Table 10. Effects of heavy metal burden on hormone concentration of male workers and control.

Hormones	Control	Workers	Normal ranges	P value (Correlation)
FSH (mIU/ml)	12.19±0.21	17.91±0.44***	1.5-12.4[a]	p<0.001
LH (mIU/ml)	7.86±0.12	10.80±0.19***	1.7-8.6 [b]	p<0.001
Testosterone (ng/ml)	5.80±0.06	2.13±0.12***	2.8-11[c]	p<0.001
Cortisol (µg/dl)	18.31±0.41	30.46 ±0.52***	-	p<0.001

*, **, *** indicates significant difference at probability p<0.05, p<0.01 and p<0.001 compared to control

[a](Release, Set, This, Lbxfsh, *et al*., 2002),[b] (Release, Set, This, Lbxlh, *et al*., 2002), [c] (Unicel, 2012)

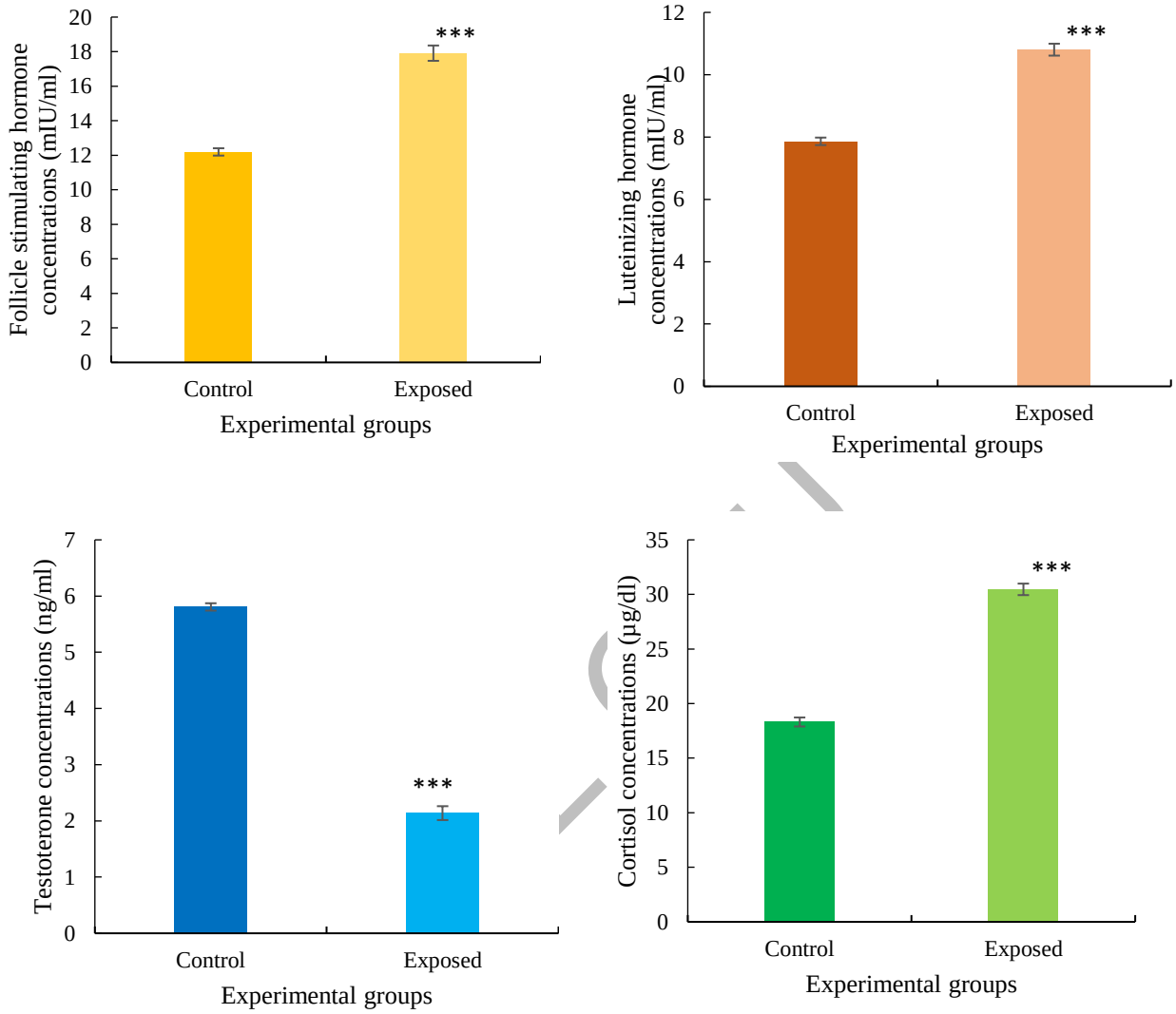


Figure 21. Comparison of Follicle stimulating hormone (mIU/mL), Luteinizing hormone (mIU/ml), testosterone (ng/ml), and cortisol (µg/dl) concentration in blood plasma among male workers and control.

Correlation analysis

The findings of correlation studies showed that blood plasma cortisol levels negatively correlate with FSH (r=-0.580), LH (r=-0.676) and T (r=-0.832) concentrations with significance value of p<0.001 (table 11, figure 22). Further correlation analysis showed a positive correlation between FSH and LH concentrations (r=0.675) as well as among FSH and testosterone (r=0.749, p<0.001). Moreover, a direct correlation was also evident between plasma concentrations of LH and testosterone (r=0.623, p<0.001). Figure 23 summarizes the correlation of cortisol with LH, FSH and testosterone.

Table 11. A precise table showing Pearson's correlations among plasma LH, FSH, testosterone and cortisol in male workers and control.

Parameters	**Correlation**			
	Cortisol (µg/dl)	**FSH (mIU/ml)**	**LH (mIU/ml)**	**T (ng/ml)**
Cortisol (µg/dl)	r=1			
FSH (mIU/ml)	r=-.676** p<0.001	r=1		
LH (mIU/ml)	r=-.580** p<0.001	r=0.675** p<0.001	r=1	
T (ng/ml)	r=-.832** p<0.001	r=0.749** p<0.001	r=.623** p<0.001	r=1

** Correlation (r) is significant at the 0.01 level (2-tailed).

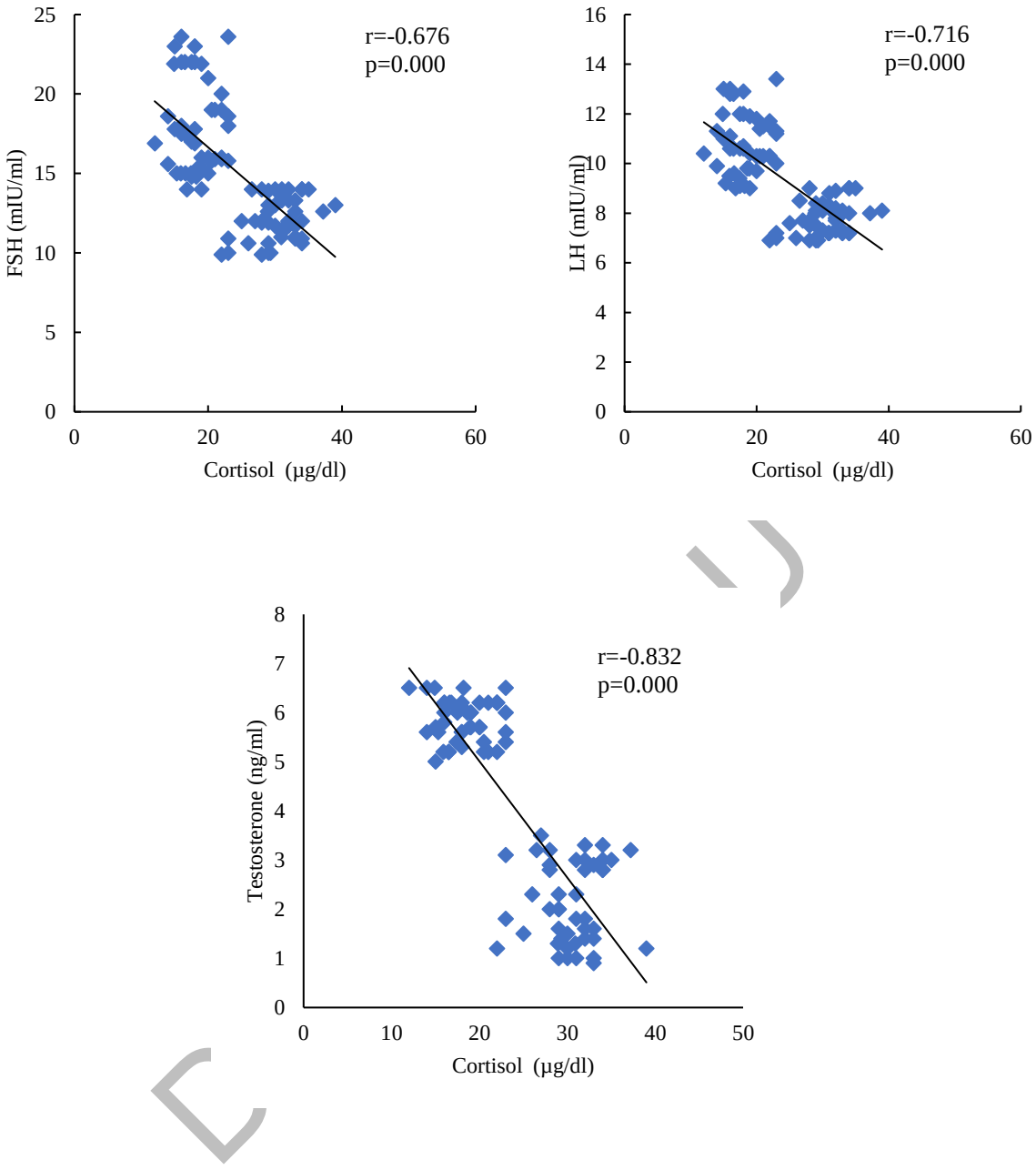


Figure 22. Correlation of plasma FSH (mIU/ml), LH (mIU/ml) and testosterone (ng/ml) with cortisol levels in male workers.

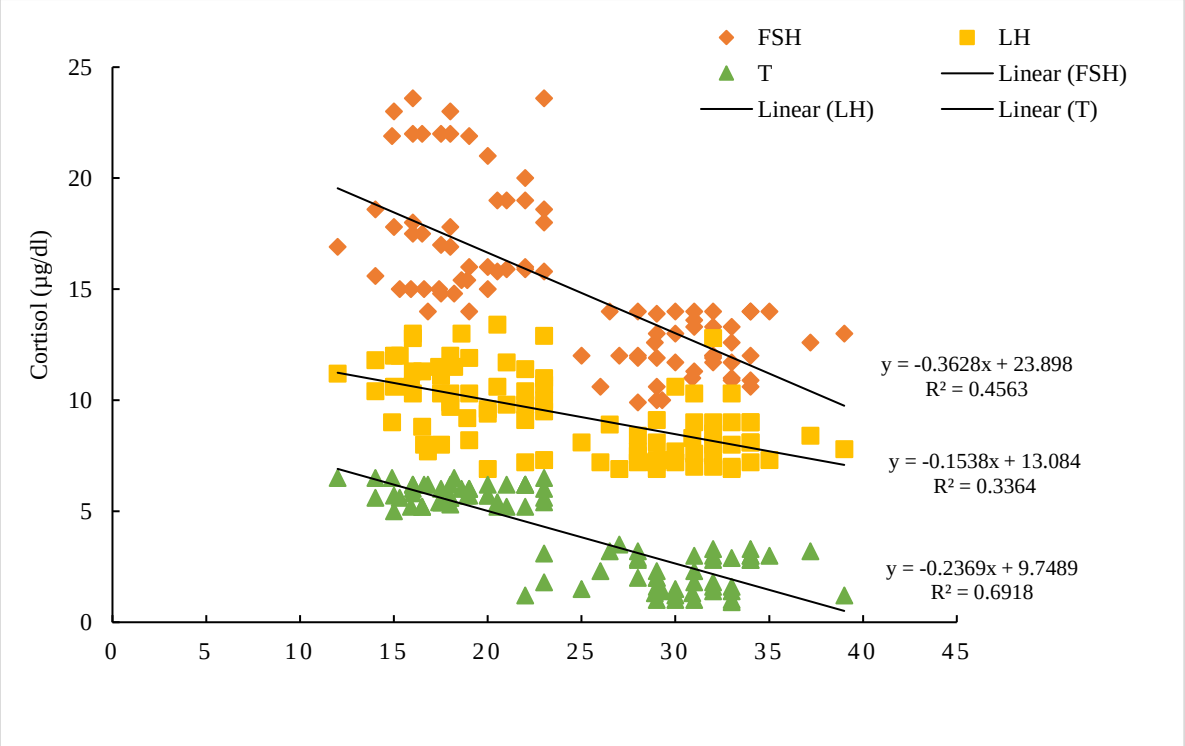


Figure 23. Summarized figure showing correlation of plasma LH, FSH, testosterone levels with cortisol in male workers.

DISCUSSION

The present study reviewed and reported the health status of male brick kiln workers living at Rawat, Punjab. In previous chapter, analysis of heavy metals in whole blood revealed a remarkable increase in heavy metal burden in blood among workers exposed to brick kiln pollutants. These heavy metals collectively induce multiple metabolic as well as reproductive disorders. Thus, it is suggested that heavy metal burden in circulation is transported to all the organs of the body and may affect the normal physiology and functions as monitored by biochemical and hormonal analysis in present study.

The findings of blood parameters showed decrease in RBC and HGB levels that might be linked with heavy metals emitted from kilns. As RBC are produced from the hematopoietic tissues of kidney and spleen, decrease in RBC number could result from the internal bleeding by damaged kidneys (Kori *et al*., 2006). Our findings are further supported by previous work of Fazio *et al*. (2014) where lowered RBC and WBC levels were evident among fish exposed to metal pollution. The molecular mechanism by which chromium (VI) induces cellular toxicity in human blood lymphocyte is through the formation of ROS with subsequent induce cellular damage (Seydi *et al*., 2020). Another study showed that in vitro incubation of erythrocytes and lymphocytes with different concentrations of K_2Cr_2O7 resulted in a dose-dependent increase in ROS number with reduction in antioxidant capacity of the cells (Husain & Mahmood, 2017). In our studies, decrease in percent hematocrit, MCV, MCH and MCHC was noted among brick kiln workers that might be due to toxic effects of heavy metals. Kamal *et al*. (2014) have suggested that variety of brick kiln emitted particles may induce cellular toxicity in actively dividing cells such as bone marrow cells, further supporting our results (Kamal *et al*., 2014).

Wąsowicz *et al*. (2001) found that work-related exposure to heavy metals e.g., cadmium or lead disturbs the normal homeostasis of the body by upsetting antioxidant capacity of the body. This, in turns, alters the levels and activity of trace elements and other enzymes (Wąsowicz *et al*., 2001). Antioxidant enzymes such as CAT and POD are known to play an important role in reducing the threatening effects of heavy metals. Turkez *et al*. (2012) showed that exposure to heavy metals increases oxidants levels and reduces antioxidants concentrations (Turkez *et al*., 2012). In present study, significant decrease in SOD and POD level was observed in the workers group. Our results are being supported by previous findings of Jahan *et al*. (2016) where brick kiln workers, exposed to emitted heavy metals exhibited reduced levels of antioxidant enzyme (Jahan *et al*., 2016).

Studies suggest that transition metals such as Cd, Ni and Cr act as catalysts in the oxidative reactions of biological macromolecules (Ercal *et al*., 2001). Due to their high degree of toxicity, some of them for instance, As, Cd, Cr, Pb, and Hg are considered as systemic toxicants and are known to induce multiple organ damage, even at lower levels of exposure (Tchounwou *et al*., 2012). Redox-active metals, such as Cu and Cr act by redox cycling, whereas redox-inactive metals, such as Pb, Cd, Hg are involved in reducing antioxidants enzymes levels in the cell. Both of the mentioned redox reactions may induce an assembly of various reactive oxygen species (Ercal *et al*., 2001). Ni is known to generate reactive oxygen species as well (Lippmann *et al*., 2006). Our studies have also reported that metal exposed group has significant increased number of ROS and MDA produced as compared to control. However, further studies are required to determine the effect of antioxidant supplementation following heavy metal exposure.

Previous studies have reported that in male rodents, Cd changes the concentration of reproductive hormones such as FSH, LH and T and affects the steroidogenesis in the

Leydig cells (Jahan *et al*., 2016). The results of present findings reported an increase in FSH and LH levels among brick kiln workers as compared to control. The increase in FSH and LH levels might be linked to metal burden in blood that may have significant effects on the negative feedback of this hormone (Rami *et al*., 2011). This suggests that presence of heavy metals in blood may cause some primary damage to the seminiferous tubules in the testes that hinders LH and FSH to bind to their respective receptors and mediate their actions (McGregor & Mason, 1990). Our findings are in contrast with the previous work of Lafuente *et al*. (2001), where rats exposed to cadmium chloride in drinking water experienced decrease in LH and increase in FSH concentrations (Lafuente *et al*., 2001). As testosterone produced by the testes is the key regulator of reproductive axis that controls the negative feedback actions, maintaining a regulated HPG axis; any disturbance in its production at testicular site causes abnormal/reduced concentrations of testosterone in blood. We found decreased levels of testosterone in blood plasma of kiln workers as compared to control group that might be due to the presence of high concentration of heavy metals in blood that increased the oxidative stress. Lowered testosterone levels in blood indicate that occupational exposure to kiln pollutants might have deposited heavy metals in blood that can either inhibit steroidogenic activity at cellular level or interferes with gonadotropin binding on its receptors (Priya *et al*., 2004). Literature data also suggests that high metal concentration in blood may induce primary testicular defects, which reduced testosterone synthesis and hence, via lifting negative feedback, caused a rise in circulating gonadotropin levels.

As it has been reported in previous studies that many components of the hypothalamic pituitary gonadal (HPG) axis are downregulated by plasma glucocorticoids such as cortisol. These effects are mediated either at hypothalamus and pituitary level, or by actions on the responsiveness of target tissues to gonadal hormones (Rehman *et al*., 2019;

Thakore & Dinan, 1994). The findings of present study also reported negative correlation of cortisol concentrations with pituitary gonadotropins LH and FSH levels in blood plasma. Multiple other studies have also reported the negative correlation of cortisol hormone and sex steroids (Chen *et al*., 1997; Liening & Josephs, 2010; Tsigos & Chrousos, 2002; Viau, 2002). The negative correlation is explained due to reciprocal relationship between the hypothalamic-pituitary-adrenal (HPA) and HPG axes wherein the activation of one affects the function of the other and vice versa (Toufexis *et al*., 2014). Previous studies showed that male monkeys subjected to restraints had experienced elevated plasma ACTH and cortisol, measured as indexes of stress, within 15 min after initiation of restraint and remained elevated for most of the restraint period; whereas LH levels began to fall immediately after restraint and remained suppressed for several hours even after the removal of restraints (Norman & Smith, 1992). Similar results are obtained in our study, where negative correlation between cortisol concentration and testosterone level was observed. In humans, exposure to cortisol causes a significant decrease in testosterone production (Cumming *et al*., 1983). Another study suggested that animals subjected to stressors experience inhibited testosterone levels as compared to LH levels, a reflection of the fact that in some animals, testosterone remained low after the return of pulsatile LH secretion even after the removal of restraints (Norman & Smith, 1992). As the present study reported increase in concentration of cortisol among brick kiln workers, this increase in cortisol is thought to be responsible for decrease in sex steroid production from Leydig cells and therefore, can be considered accountable for reproductive function in men.

CONCLUSION

The present study concluded that;

- Heavy metal burden in blood of workers caused alteration in blood parameters, decrease in antioxidant enzyme levels and increased oxidant production.
- Further, due to heavy metal burden in blood, increased stress response was generated, that resulted in elevated production of cortisol, which ultimately affected hypothalamic pituitary gonadal axis by altered production of pituitary gonadotropins (LH, FSH).
- Prior to response to increased levels of LH and cortisol, while decrease in production of sex steroid, testosterone was evident.
- Therefore, it is determined that long-term compromised antioxidant enzyme levels in blood, increased production of ROS, higher oxidative stress and disturbed production of gonadotropins and sex steroids serve as contributing factors in disturbing the metabolic health of brick kiln men, putting them at the risk for the development of other health problems that ultimately affect their reproductive outcomes.

SUMMARY

BIOCHEMICAL ANALYSIS

HORMONAL ANALYSIS

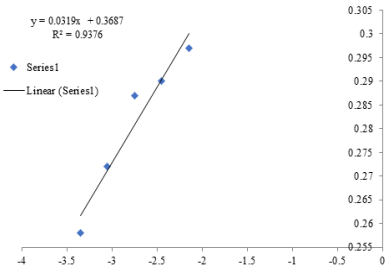
Antioxidants and Plasma Protein Content

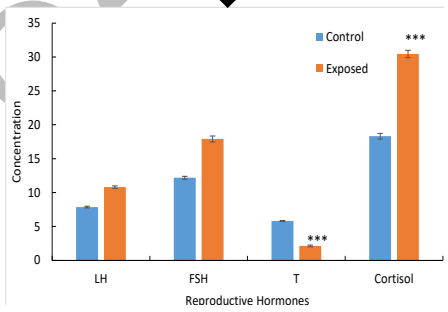
Biochemical parameters	Control	Exposed
SOD (U/min)	16.96±0.72	14.49±0.49**
POD (nmole)	21.72±2.22	19.23±1.44
ROS (µmol/min)	1.17±0.06	1.50±0.07***
MDA (nmol /ml)	4.69±0.36	5.28±0.40*
Protein estimation (g/dl)	11.65994	8.95808*

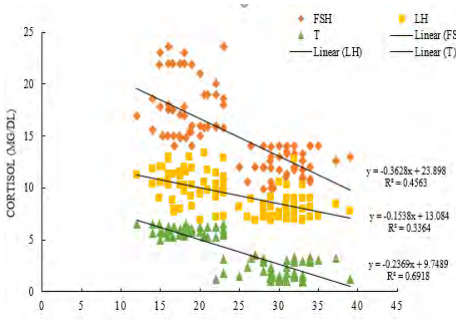
Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability

p<0.01 and p<0.001 compared to control







Reproductive Hormones and Correlation

HEAVY METAL BURDEN WITH COMPROMISED IMMUNE STATE IS A RISK FOR THE DEVELOPMENT OF PUBLIC AND REPRODUCTIVE HEALTH PROBLEMS IN MALE BRICK KILN WORKERS

Chapter 3

Determination of occupational exposure to brick kiln emissions on body mass index, lipid profile, and reproductive health of female kiln workers; A biochemical and hormonal study

ABSTRACT

The present study was planned to understand the toxic effects of kiln emitted metals on health profile of brick kiln female workers regarding BMI, reproductive health indicators, blood and lipid profile, antioxidant status, reproductive hormone concentrations and direct effect on hypothalamic pituitary adrenal axis. A total of 232 women were involved, of which 114 presented control subjects. Data regarding collection fertility indicators (such as family size and marital age) was also gathered. Plasma previously obtained and stored at −20°C was utilized to study biochemical variables (sodium dismutase, peroxidase, reactive oxygen species number, thiobarbituric acid reactive species, protein estimation), lipid profile, and hormonal analysis among the two groups. The findings showed a negligible change in mean family size and number of alive children among the two groups. The results also indicated increased platelet count; decreased sodium dismutase levels ($p=0.003$), peroxidases ($p=0.009$) and increased oxidants level; amplified total cholesterol ($p<0.001$), low-density lipoprotein ($p<0.001$) and triglyceride ($p<0.001$) levels; reduced total protein and high-density lipoprotein ($p=0.475$) concentrations; and increased cortisol levels in workers in contrast to control group. Significant decrease ($p<0.001$) in FSH, LH estradiol and progesterone concentration, while significant increase in prolactin levels were seen among workers groups as compared with control. The findings of studies further showed that plasma cortisol levels negatively correlated with FSH ($r=-0.872$), LH ($r=-0.856$), estradiol ($r=-0.923$) and progesterone ($r=-0.879$) concentrations with significance value of $p<0.001$, while it positively correlated with prolactin concentration ($r=0.874$). It is concluded that occupational workers had experienced alterations in hematology variables, decreased concentration of antioxidant enzyme, total protein and high-density lipoprotein, increased

oxidants level and total cholesterol, low-density lipoprotein and triglyceride levels, and decreased reproductive hormone (LH, FSH, E and P) concentrations, increased cortisol and prolactin levels. Additionally, negative correlation of cortisol with LH, FSH, E and P, and positive with prolactin was prominent. Thus, it is speculated that increased oxidative stress due to amplified oxidative enzyme concentrations, increased cortisol levels, early marital age, disturbed HPG axis, and reduction in antioxidant enzymes pose a risk to maternal health affecting family size as well as abortion index.

INTRODUCTION

Geographically, Pakistan is 3rd largest brick producing country in South Asian region with more than 45 billion bricks produced per year after China and India (Saeed, 2017). It is known that brick kiln sector contributes to 1.5% of its gross domestic product (GDP) in Pakistan. However, it is highly un-regulated and un-recognized area (CCAC secretariat, 2018). Pakistan has around 20,000 brick kilns in different cities across the country (CCAC secretariat, 2018). Approximately 1.8 million workers have been reported to work in the brick kilns of Pakistan (Kamal *et al*., 2014). The survey report by Labor and Human Resource Department, Government of the Punjab, presents that currently 10, 347 brick kilns have been identified in the Punjab province only, with 249, 682 brick kiln workers including men and women (http://dashboards.urbanunit.gov.pk/brick_kiln_dashboard/). The number of families who are serving at brick kilns in Punjab is 87,134. Due to a lack of proper allotment of space for the construction of brick kilns in Pakistan, these kilns are mostly developed on roadsides or in the vicinity of agricultural land and emissions from these kilns, poses health risks to the general population as well as brick kiln community (Ercelawn & Nauman, 2004).

Brick kiln emitted heavy metals induce reproductive disturbances among brick kiln workers (Jahan *et al*., 2016; Shahid *et al*., 2017). Occupational exposure to heavy metals serves as a risk factor for female fertility (Amadi *et al*., 2017). Studies suggest that some heavy metals (As, Cd, Pb, Hg) may act as endocrine disruptors and their exposure during pregnancy may adversely affect mother and the fetus, concerning low birth weight (Georgescu *et al*., 2011; A. Rahman *et al*., 2016). Further, recent studies report that female workers, occupationally exposed to Cr experience high threat for abortion and miscarriages (Amadi *et al*., 2017). Previous epidemiological studies have reported that

occupational exposure to Cr increases carcinogenic risk in the respiratory tract of individuals (Cohen *et al*., 1993). Additionally, occupational workers are also exposed to occupational factors, which play key role in shaping the employees' health as previous studies have suggested (Patil, 2017; Shaikh *et al*., 2012). Hence, it is endorsed that occupational risk factors affect the public health and reproductive outcomes of female workers by various mechanisms.

The determinants for maternal health are nutrition, age of marriage, rate of fertility, family size and contraception. In Pakistan, the problems associated with maternal health are prevalent on much larger scale; for example undernutrition in girls, early age marriage, and elevated fertility rates and unfulfilled needs for use of contraceptives are responsible for the ill maternal health (Khan *et al*., 2009). For a healthy mother and baby, maternal undernutrition is considered as crucial determinant of healthy pregnancy outcomes. According to the National Nutrition Survey 2001–2002, it has been reported that around 12.5% non-pregnant and 16.1% lactating mothers were malnourished in Pakistan (Khan *et al*., 2009). Its prevalence among pregnant and lactating women is also quite high (Khan *et al*., 2009). With malnutrition, other physiological conditions take place that may increase metal deposition and its uptake. For instance, in case of iron deficiency, upregulation in utilization of Fe occurs in the mucosa cell by duodenal metal transporter (DMT1), which has also attraction for Cd absorption (Amadi *et al*., 2017; Gunshin *et al*., 1997). This phenomenon has been further confirmed by other researchers in their respective studies as well (Akesson *et al*., 2002; Vahter *et al*., 2002). This suggests that during pregnancy, increase in Cd absorption is a result of iron deficiency and high body burden (Vaiserman, 2014). Complications that occur at the time of parturition are also the principal cause of death in women of all reproductive ages (Bhutta & Hafeez, 2015). It

has been reported previously that in Pakistan, approximately five million women become pregnant each year. Among these, 15% may experience obstetrical and medical complications (Khan *et al*., 2009). The maternal mortality ratio (MMR) is quite increased in rural areas in contrast to urban areas and is highest in Province Baluchistan (Bhutta & Hafeez, 2015). Thus, it is concluded that maternal health plays a key role in determination of child and mother health status.

Aims and Objectives:

In order to monitor maternal health and child mortality, huge challenges are being faced by Pakistan to meet its international responsibilities and fulfill Millennium Development Goal targets. Therefore, the current study was planned to measure the toxic effects of pollutants emitted from brick kilns on female workers regarding reproductive health indicators, blood and lipid profile, antioxidant status, reproductive hormone concentrations and direct effect on hypothalamic pituitary adrenal (HPA) axis. To address reproductive health in women of reproductive age working in brick kilns, the study aims to

- Find the association of fertility indicators and reproductive health risks on maternal health and pregnancy outcomes among workers
- Investigate the possible effects of heavy metal burden on blood parameters
- Assessment of effect of heavy metal burden on lipid profile
- Evaluation of oxidative stress markers among brick kiln workers
- Determine and compare the functionality of hypothalamic pituitary ovarian axis among brick kiln workers and control through hormonal bioassays
- Assessment of hypothalamic pituitary adrenal (HPA) axis via monitoring stress hormone concentrations in blood plasma
- Address the relationship of stress hormone on reproductive function of women

MATERIALS AND METHODS

Study population

Females employed for a varied number of years at the brick kiln sites were selected randomly who were willing to be included in this study, with everyone representing a population. The sample size for women subjects was calculated using same method mentioned in chapter 2 and calculated sample size for women was 322. However, the required number of samples were not achieved due to dominancy of male kilns and despite of large number of associated families with this occupation, number of female workers was comparatively less than men.

Subject selection

Different age groups (19– 45 years) of women working for varied years at brick kilns were considered, with a total number of 232 women, where 118 were kiln workers considered as Group I, and 114 were non-workers (control) considered as Group II.

Study of reproductive indicators

The questionnaires collected data regarding reproductive health indicators such as, age of menarche, period duration and cycle regularity, age at marriage, age at birth of first baby, number of children, number of dead and aborted children, number of miscarriages, and number of total pregnancies.

Blood profile on hematology analyzer

Hematology analysis was done using fresh blood as mentioned previously in chapter 2.

Serum Analysis for plasma enzymes

Blood plasma was examined for measuring levels of SOD, POD, ROS, TBARS, and total protein content following protocols of (Hayashi *et al*., 2007) and (Iqbal, *et al*.1996) as explained briefly in chapter 2. The total protein content was quantitatively calculated using commercially available kit by AMEDA Labordiagonstik GmbH Krenngasse, Graz/Austria (method given in chap 2).

Lipid Profile Analysis

Quantitative analysis of plasma levels of HDL and LDL cholesterol were performed on chemical analyzer with the help of AMEDA Labordiagonstik GmbH HDL and LDL cholesterol precipitation kits by following the given instructions.

High density lipoprotein determination (HDL)

Blank, Standard and Samples were prepared according to the method provided with the kit. Blank was prepared by mixing 1mL Reagent R (provided with the kit) with the 50µL distilled water. Standard and samples were prepared by mixing 1ml Reagent R with the 50µL Standard solution (provided with the kit) and 50µL plasma, respectively. Each sample was mixed gently and incubated for 10 minutes at 37℃. Absorbance of standard and samples were recorded against reagent blank on chemical analyzer. Chemical analyzer was set on 500nm wavelength and determination of HDL was done using following formula

$$\text{Cholesterol (mg/dL)} = A_{sample} / (A_{standard}) \times C_{standard}$$

Low density lipoprotein determination (LDL)

Blank, Standard and Samples were prepared according to the method provided with kit. Blank was prepared by mixing 220µL Reagent R1 (provided with the kit) with 3µL of distilled water. Standard and samples were prepared by mixing 220µL Reagent R1 with 3µL standard solution (also provided with the kit) 3µL plasma, respectively. Each sample was mixed gently and incubated for 5 minutes at 37˚C. Absorbance of standard and sample were recorded against reagent blank on chemical analyzer at 600 nm wavelength. This absorbance was considered as A1. After taking first absorbance, 55µL Reagent R2 (provided with the kit) was added to each sample and incubated for 5 minutes at 37˚C. Absorbance was recorded of each sample at 600 nm and considered as A2. The concentration of LDL cholesterol was calculated by using following formula

$$\text{Cholesterol (mg/dL)} = A_2 - A_1\,(_{\text{sample}}) \,/\, A_2 - A_1\,(_{\text{standard}}) \times C_{\text{Standard}}$$

Triglycerides

Triglycerides in blood plasma were determined by using Gesan Triglycerides mono reagent kit. Triglycerides are hydrolysed by lipoprotein lipase (LPL) to glycerol and fatty acids. Glycerol is then phosphorylated to glycerol-3-phosphate by ATP in a reaction catalyzed by glycerol kinase (GK) which is then further oxidized by glycerol phosphate oxidase producing dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen per oxide, in the presence of peroxidase (POD), catalyzes the oxidative coupling of 4-chlorophenol and 4-aminoantipyrine (4-AAP) to form a red-colored quinoneimine dye which can be spectrophotometrically measured at 500nm to determine triglycerides in the samples. The procedure provided with the kit was used for the determination of triglyceride levels in blood plasma.

Enzyme Linked Immunosorbent Assay

Hormonal concentrations for Luteinizing hormone (LH), Follicle stimulating hormone (FSH), Estradiol (E), Progesterone (P), Prolactin (PRL) and Cortisol were determined by different commercially available enzyme immune assay (EIA) tests kits.

Luteinizing hormone

The concentration of LH in plasma was measured quantitatively by Luteinizing hormone ELISA kit bought from Reddot biotech INC (method mentioned in chapter 2).

Follicle Stimulating Hormone

The concentration of FSH in plasma was measured quantitatively by using follicle stimulating hormone ELISA kit bought from Reddot biotech INC. The method provided with the kit was used (as mentioned in chapter 2).

Progesterone determination

Progesterone level in the plasma was determined with the help of ELISA kit provided by BioScience, INC., San Francisco by following the instruction provided with the kit.

Principle

There is a competition for binding between the progesterone of the test sample and HRP-labeled progesterone for the binding site of rabbit anti-progesterone enzyme. If the amount of progesterone in the test sample increases, HRP-labeled progesterone binding with the anti-progesterone enzymes decreases. Thus, value of absorbance is inversely related to the concentration of progesterone in the test sample.

Procedure

Standard, control and samples were taken (25 µL) and dispensed in the appropriate wells of microtiter plate coated with Goat anti-rabbit antibody. After that, 50 µL of rabbit anti-progesterone enzyme was added to each well. Then, 100µL HRP labeled progesterone was dispensed in each well, mixed thoroughly and incubated for 90 minutes at room temperature. After incubation, wells were washed with washing buffer of 1X for five times. After washing with buffer, 100 µL TMB substrate was added to each sample and incubated at room temperature for 20 minutes. In the end, 100 µL stop solution was put in each well and when blue color changed to yellow, absorbance was measured with the help of microplate reader at 450 nm wavelength.

Standard curve was constructed by plotting the absorbance and concentration of the standards on y-axis and x-axis respectively, and corresponding concentrations of the test sample were determined from standard curve by using their absorbance value.

Estradiol determination

Estradiol level in the plasma was determined with the help of ELISA kit provided by BioCheck, INC., USA by following the instruction provided with the kit.

Principle

Assay is based on the competition between estradiol in the test sample and HRP labeled estradiol for the rabbit anti-estradiol enzyme. HRP- labeled estradiol compete with the estradiol of the test sample and if the concentration of estradiol in the test sample is high, there is little binding of the HRP labeled estradiol with the rabbit anti-estradiol enzyme and absorbance will be low. Thus, value of absorbance is inversely related to the concentration of estradiol in the test sample.

Procedure

Standard, control and test samples were taken 25µL and dispensed in antibody coated wells of the microtiter plate. 100µL HRP labeled estradiol was dispensed in each well. After that 50µL rabbit anti-estradiol enzyme was put in each well, mixed through and incubated for 90 minutes at room temperature. After incubation, wells were washed 5 times with distill water and 100µL TMB was dispensed in them. After putting TMB, they were incubated for 20 minutes at room temperature and added 100µL stop solution. When color changed from blue to yellow, read the absorbance at 450 nm on microplate reader.

Prolactin

Prolactin level in the blood plasma was determined with the help of Human Prolactin/PRL ELISA Kit (ab226901) by following the instructions provided with the kit.

Principle

The sandwich ELISA was used for the determination of PRL whose detailed procedure has been diagrammatically shown in following figure (figure 24).

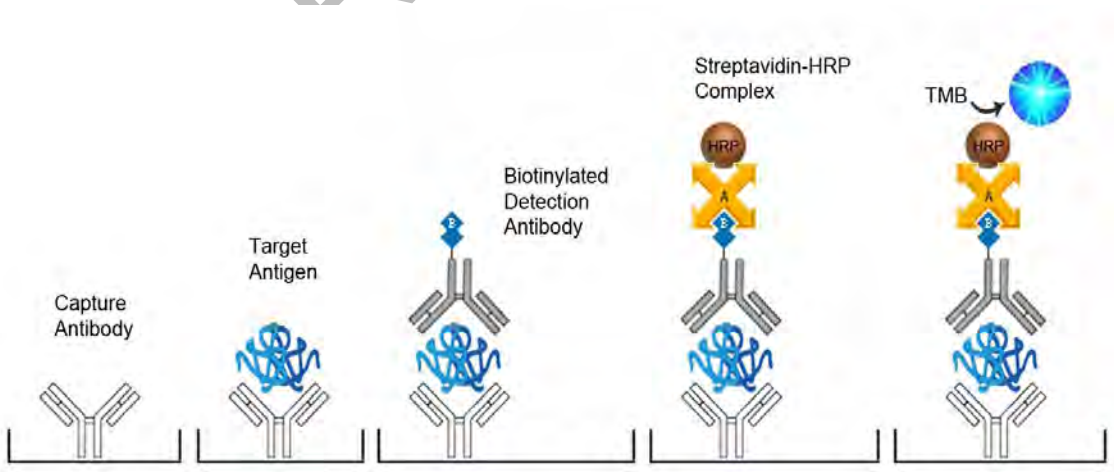


Figure 24. Diagrammatic representation of prolactin (PRL) ELISA Principle

Procedure

50 µL of all samples /standard were dispensed followed by addition of 50 µL of the Antibody cocktail. Plates were sealed and incubated for 1 hour at room temperature on a plate shaker set to 400 rpm. Later, plates were washed three times using 350 µL 1X Wash Buffer, followed by inversion and gentle tap against clean paper for removal of excess liquid. Afterwards, 100 µL of TMB Development Solution was added to each well and incubated for 10 minutes in the dark on a plate shaker set to 400 rpm. 100 µL of Stop Solution was added and plate was kept on plate shaker for 1 minute for mixing and OD was taken at 450 nm.

Determination of serum Cortisol hormone

Using Cortisol hormone ELISA test kit (The Calbiotech, Inc. USA), its concentrations in blood serum were measured. The assay protocol and minimum detection limit is mentioned in Chapter 2.

Principle of the test:

The Calbiotech, Inc. Cortisol test kit used works on the principle of solid phase competitive ELISA. The detailed procedure has been shown in following figure (fig 25).

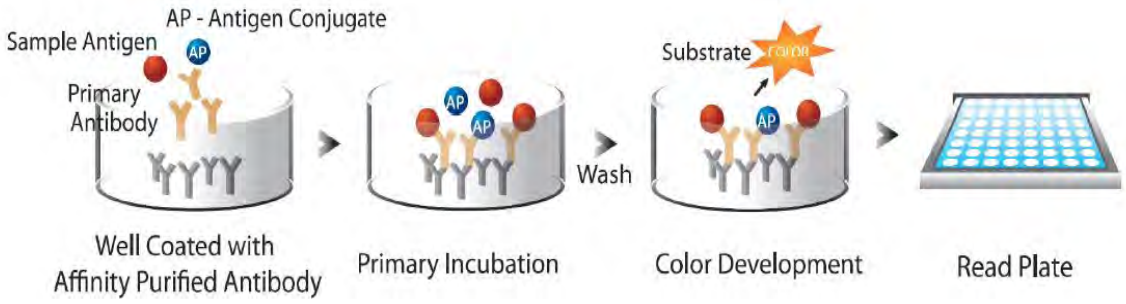


Figure 25. Diagrammatic representation of solid phase competitive ELISA Principle for cortisol.

Statistical analysis

The data was denoted as mean and standard error of mean (Mean±SEM). With the help of independent sample t-test, carried by computer software SPSS 17, statistical analysis for blood parameters, antioxidant enzymes profile and hormonal concentrations was carried out to determine the significance of our results. Correlation was determined among hormones in pairwise fashion using Pearson's correlation (r), and correlation (r) and significance (p) values were measured.

RESULTS

Reproductive health indicators

The outcomes of present study disclosed notable changes in reproductive indicators of brick kiln workers and non-workers. Table 12 shows the average age of menstrual onset among studied subjects. The mean age of menarche among brick workers subjects was 11years as compared to control that were experiencing menarche at the mean age of 14years. No significant change in duration of menstruation (in term of days) was distinguished among the two groups with an average of 5 days/menstrual cycle (figure 26). The mean age of marriage among brick workers was 23 years and the mean age of birth of first baby child was 24 years of age. Most of these mothers were lean and malnutritional. One female brick worker in our study was encountered that had experienced 19 abortions till her marriage and was found to possess weakened endometrium. There was no significance change in mean family size and number of alive children among the two groups. However, the number of dead children in control group was negligible as compared to workers group where a ratio of 0:1 was seen. The results of mean of total number of pregnancies among both groups revealed that the number was much higher in workers groups as compared to control, however due to increased number of miscarriages, abortions and child death, it did not affect family size.

Table 12. Comparison of indicators of fertility among control and brick kiln workers.

Females' Reproductive indicators		
Parameters	**Control (average)**	**Workers (average)**
Menarche age (years)	14	11
Menstrual Duration (days)	5	5
Age at marriage (years)	23	23
Age at 1st baby birth (years)	24	23
Family size (n)	6	6
No. of alive children (n)	3	4
No. of dead children (n)	0	1
No. of aborted children (n)	0	1
Average of Total pregnancy numbers (no of times-1X)	1*	6
*Most of the females in control group were unmarried		

(n=number)

Figure 26. The figure presenting periods regularity among (A) women working at brick sites and (B) control. (C) The figure shows the marital status of brick kiln and control subjects.

Haematological parameters

Drop in number of white blood cells occurred from 10.19±0.37 to 8.84±1.61, however, the change was not significant. Similarly, no significant change in RBC count (1×10^6), total haematocrit (%) and haemoglobin (g/dL) was evident in female workers and control ones. The MCV, MCH and MCHC decreased in female workers as compared to control. The RDW-CV and RDW-SD, somehow increased in workers. The increased platelet (PLT) count was seen in female workers in comparison with control, though, the mean platelet volume was lowered in worker group. No significant variance was observed in PDW among female workers as compared to control subjects.

Table 13 shows the effects of kiln emissions on various blood parameters as well normal range of blood variables. It was noticed that all the measured values lied within the normal range for adults.

Table 13. Effects of heavy metal burden on various blood parameters of female workers and control.

Blood parameters	Control	Workers	Normal range	P value statistics
WBC (1×10^3)	10.19±0.37	8.84±1.61	4-11	p=0.312
HGB (g/dL)	10.92±0.20	7.40±0.91	11.5-16.5	p=0.125
RBC (1×10^6)	4.14±0.07	3.62±0.12	3.8-5.5	p=0.151
HCT (%)	33.79±0.60	33.27±6.05	37-47	p=0.064
MCV (fL)	80.37±1.19	77.45±14.08	79-96	p=0.138
MCH (pg)	26.55±0.36	22.1±0.33	27-32	p=0.247
MCHC (g/dL)	32.32±0.19	30.71±0.33	30-36	p=0.976
RDW-CV (%)	15.02±0.27	14.96±0.22	11.8-14.5	p=0.118
RWD-SD (fL)	42.48±0.35	40.23±0.35	40.0 - 55.0	p=0.111
PLT (1×10^3)	266.78±7.78	327±13.49	150-450	p=0.955
MPV (fL)	10.92±1.11	9.00±1.64	7-12	p=0.301
PDW (%)	15.39±0.03	15.67±2.85	10.0-17.9	p=0.194

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control

Biochemical parameters

A significant decrease in SOD (p=0.003) and POD (p=0.009) levels were seen in blood plasma of female workers in contrast to control group, while decrease in protein estimation was also recorded in female workers exposed to brick kiln emissions, however, this change was not significant (p=0.274) (figure 27-29). A significant upsurge (p=0.059) in number of ROS was observed among brick worker's plasma as shown in table 14. No significant change in levels of thiobarbituric acid reactive species (TBARs) was seen among the two groups with p value of 0.802.

Table 14. The concentrations of oxidants/antioxidants among control and female workers.

Oxidative stress markers	Experimental groups		
	Control	Workers	P value statistics (p)
Sodium dismutase (U/min)	7.31±0.16	6.74±0.09**	P=0.003
Peroxidases (nmole)	22.54±0.73	19.17±1.11**	P=0.009
Reactive oxygen species (µm/min)	2.24±0.22	3.41±0.22*	P=0.059
TBARs (nmole/ml)	2.78±0.40	2.85±0.20	P=0.802
Protein Estimation	8.06±0.30	7.64±0.13	P=0.274

*, **, *** indicates significant difference at probability p<0.05, p<0.01 and p<0.001 compared to control

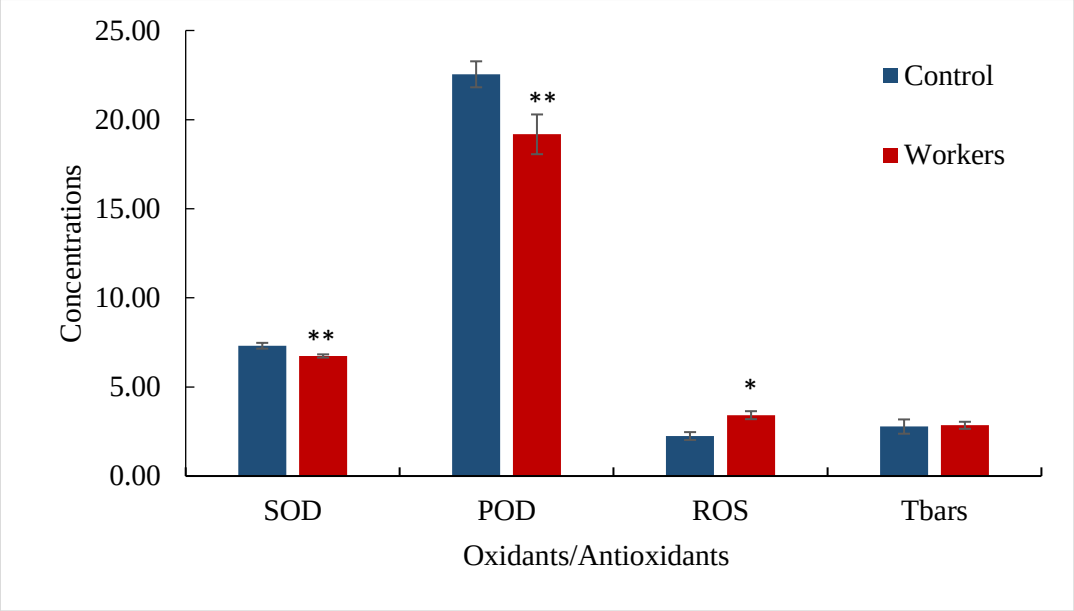


Figure 27. Effect of heavy metal burden on activity of sodium dismutase (U/min), peroxidases (nmole), reactive oxygen species (µm/min), and Thiobarbaturic acid reactive species (nmole/ml) in blood plasma among female workers and control.

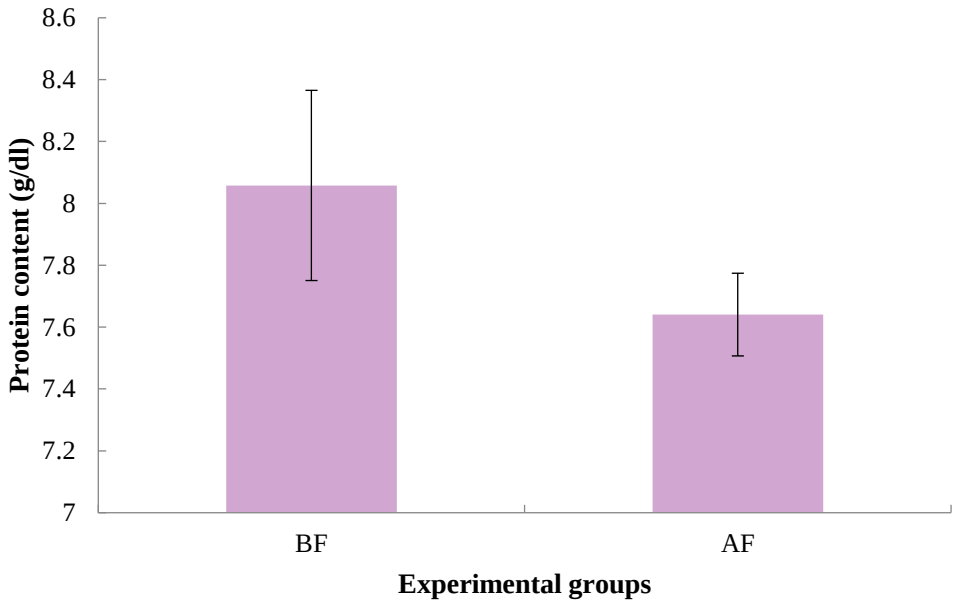


Figure 28. Effect of heavy metal burden on the total protein content (g/dl) in blood plasma among female workers and control.

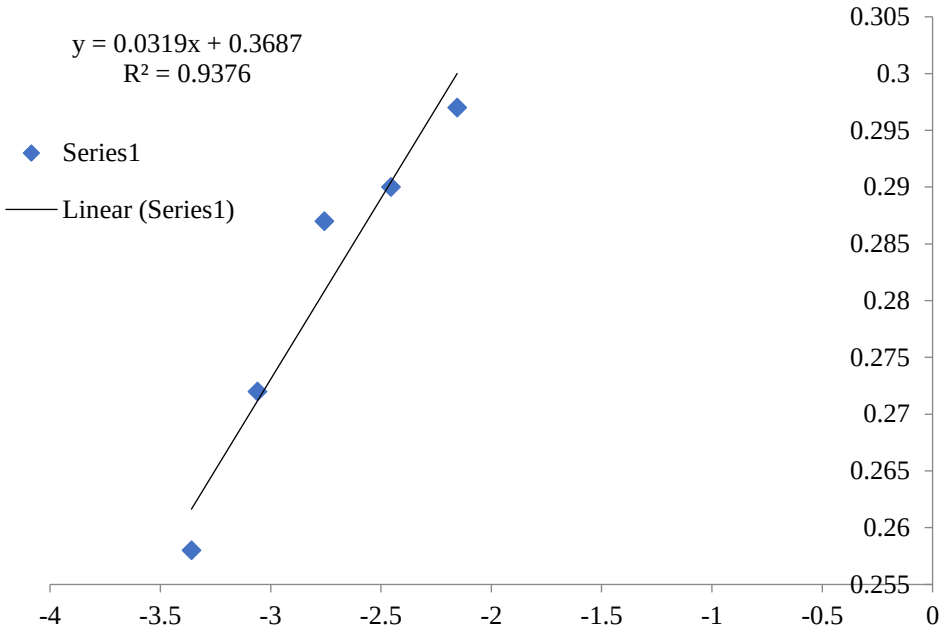


Figure 29. Linear graph showing protein estimation.

Lipid profile

The LDL level observed in control subject was 23.95±2.22, while among brick kiln workers, it was 117.68±1.45 (figure 30). The triglyceride levels in brick kiln workers and control females measured were 142.37±7.37 and 335.86±5.94, respectively. Total cholesterol level was increased ($p<0.001$) in workers compared with control group (Table 15).

Table 15. The effect of heavy metal burden on lipid profile of brick kiln workers and control.

Lipid profile	Control	Workers	Reference range[a]	P value statistics
HDL (mg/dL)	161.10±8.87	171.70±11.05	54.5 ± 15.9	p=0.475
LDL (mmol/L)	23.95±2.22	117.68±1.45***	165 ± 39	p<0.001
Triglyceride (mmol/L)	142.37±7.37	335.86±5.94***	138 ± 79	p<0.001
Total cholesterol (mmol/L)	213.52±8.36	356.56±12.99***	218.9 ± 37.9	p<0.001

[a] The plasma concentrations of lipid profile in healthy American women are given (Vogel *et al*., 1997).

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control

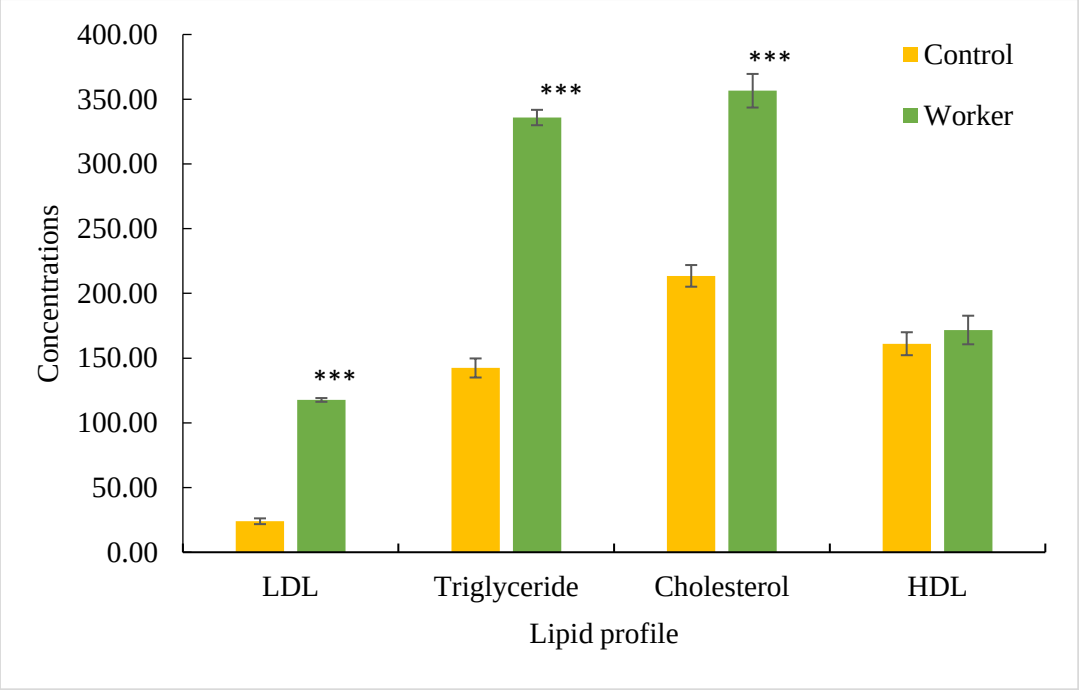


Figure 30. Comparison of low-density lipoproteins (mmole/L), total cholesterol (mmole/L), triglyceride (mmole/L), and high-density lipoproteins (mg/dL) concentration in blood plasma of control and brick kiln female workers.

Hormone assay

A highly significant increase ($p<0.001$) in blood plasma level of cortisol was seen among control (0.54±0.03ng/ml) and female workers (0.73±0.02ng/ml). A drop in LH concentration was evident among control group from 20.96±0.42IU/L to 18.02±0.10IU/ in workers group with significance value of $p<0.001$ (figure 31). Significant decrease ($p<0.001$) in FSH concentration was seen among workers groups from 10.50±0.25IU/L in control group to 8.09±0.07 IU/L. The estradiol (154.58±0.78pg/ml) and progesterone levels (10.73±0.20ng/ml) were significantly decreased ($p<0.001$) in blood plasma of brick kiln female workers as opposed to control group (E=160.47±0.72pg/ml) (p= 12.36±0.17ng/ml), respectively. A significant increase in level of prolactin hormone in blood plasma of worker subject was observed (303.78±0.71mIU/L) as compared to control (280.95±0.54mIU/L) with significance value of $p<0.001$ (table 16).

Table 17 shows the concentrations of hormone concentration during different phases of menstrual cycle and during different ages in workers samples. The reference ranges for FSH, LH and estradiol during different menstrual phases as suggested by Centre for disease control (CDC) have been mentioned as well (Release, Set, This, Lbxfsh, *et al*., 2002; Release, Set, This, Lbxlh, *et al*., 2002; Unicel, 2012).

Table 16. Effects of heavy metal burden on reproductive hormone profile of adult female workers and control.

Hormones	Control	Workers	P value statistics
Cortisol (ng/ml)	0.54±0.03	0.73±0.02***	p<0.001
LH (IU/L)	20.96±0.42	18.02±0.10***	p<0.001
FSH (IU/L)	10.50±0.25	8.09±0.07***	p<0.001
Estradiol (pg/ml)	160.47±0.72	154.58±0.78***	p<0.001
Progesterone (ng/ml)	12.36±0.17	10.73±0.20***	p<0.001
Prolactin (mIU/L)	280.95±0.54	303.78±0.71***	p<0.001

*, **, *** indicates significant difference at probability p<0.05, p<0.01 and p<0.001 compared to control

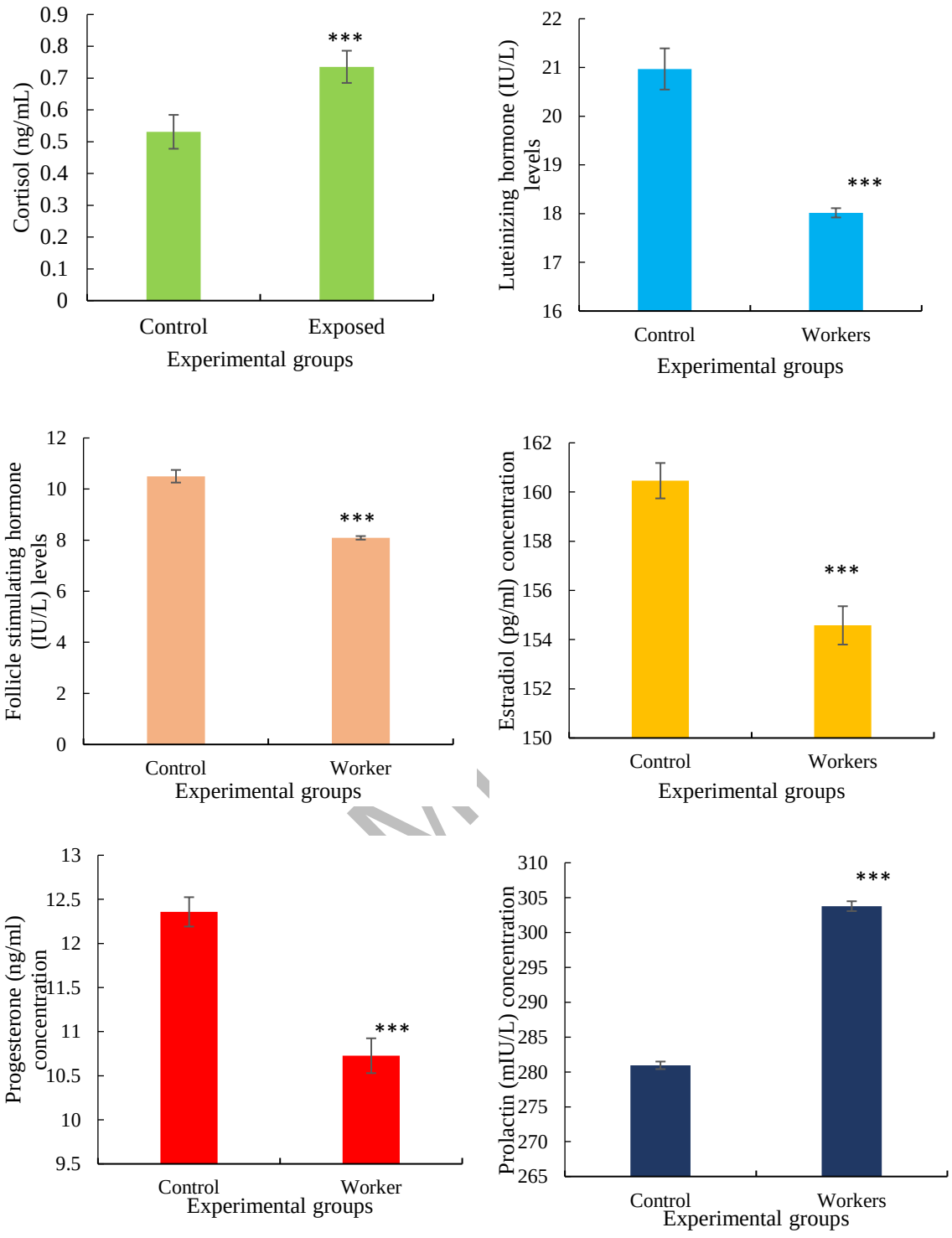


Figure 31. Comparison of cortisol (ng/ml), Luteinizing hormone (IU/L), Follicle stimulating hormone (IU/L), Estradiol (pg/ml), Progesterone (ng/ml), and Prolactin (mIU/L) concentration in blood plasma among female workers and control.

Table 17. Table showing hormone concentration during different phases of menstrual cycle.

Age groups	**Menstrual cycle phases**					
18-28 (n=46)	**Follicular Phase**	**Normal range**	**Luteal phase**	**Normal range**	**Secretory phase**	**Normal range**
FSH (IU/L)	5.50±0.15	2.53-8[a]	6.50±0.05	1.50-8.77[a]	10.50±0.25	4.7-21.5
LH (IU/L)	3.02±0.02	2-15.0[b]	12.06±0.34	0.6-19[b]	2.06±0.04	-
Estradiol (pg/ml)	114.58±0.16	20-350[c]	154.08±0.78	30-450[c]	224.58±0.28	-
Progesterone (ng/ml)	0.23±0.10	0.1-0.7[d]	10.73±0.20	2-25.0[d]	0.23±0.10	-
Prolactin (mIU/L)	137.18±0.71	-	139.78±0.01		230.78±0.31	-
29-38 (n=30)						
FSH (IU/L)	6.30±0.11	2.53-8[a]	5.80±1.25	1.50-8.77 [a]	12.58±0.21	4.7-21.5
LH (IU/L)	3.68±0.61	2-15.0[b]	11.96±0.66	0.6-19 [b]	3.55±0.42	-
Estradiol (pg/ml)	112.58±0.11	20-350[c]	152.08±0.18	30-450 [c]	234.58±0.18	-
Progesterone (ng/ml)	0.19±0.08	0.1-0.7[d]	11.73±0.20	2-25.0[d]	0.28±0.11	-
Prolactin (mIU/L)	185.91±0.45		160.56±0.11		280.81±0.52	-

39-45 (n=18)						
FSH (IU/L)	5.23±0.10	2.53-8[a]	6.11±0.34	1.50-8.77[a]	8.25±0.42	4.7-21.5
LH (IU/L)	3.81±0.05	2-15.0[b]	13.05±0.78	0.6-19[b]	4.06±0.14	-
Estradiol (pg/ml)	116.58±0.16	20-350[c]	156.08±0.31	30-450[c]	228.58±0.72	-
Progesterone (ng/ml)	0.21±0.09	0.1-0.7[d]	13.36±0.23	2-25.0[d]	0.22±0.12	-
Prolactin (mIU/L)	280.18±0.71	-	139.78±0.01	-	230.78±0.31	-
45+ (n=24)	**Postmenopausal**	**Normal range**				
FSH (IU/L)	100.95±0.10	18-153 [a]				
LH (IU/L)	35.67±0.21	16-64 [b]				
Estradiol (pg/ml)	10.35±0.18	<20 [c]				
Progesterone (ng/ml)	0.95±0.09	<1 [d]				
Prolactin (mIU/L)	20.20±0.11	<30				

[a](Release, Set, This, Lbxfsh, *et al*., 2002), [b](Release, Set, This, Lbxlh, *et al*., 2002), [c](Unicel, 2012)**,** [d](*Progesterone - Health Encyclopedia - University of Rochester Medical Center*, n.d.)

Correlation analysis

The relationship between plasma concentrations of cortisol and reproductive hormones including FSH, LH, estradiol, progesterone, and prolactin was determined through a Pearson's correlation in a pairwise fashion as shown in table 18 and figures 32 and 33. The findings of studies showed that blood plasma cortisol levels negatively correlate with FSH (r=-0.872), LH (r=-0.856), estradiol (r=-0.923) and progesterone (r=-0.879) concentrations with significance value of $p<0.001$. The blood plasma cortisol level positively correlated with prolactin concentration (r=0.874). Further correlation analysis revealed positive correlation of FSH with LH (r=0.989), progesterone (r=0.987) and estradiol (r=0.943, $p<0.001$) with significance value of $p<0.001$, while negative correlation of FSH was seen with prolactin (r=-0.974). Moreover, a positive correlation was evident between plasma concentrations of LH with estradiol (r=0.924) and progesterone (r=0.984), and negative correlation among LH and prolactin (r=-0.965) was noted. Negative correlation between prolactin and estradiol (r=-0.961) was present. The negative relationship between prolactin and progesterone (r=-0.991, $p<0.001$) concentration was found. A positive correlation among estradiol and progesterone level (r=0.958) was observed with $p<0.001$.

Table 18. A precise table showing Pearson's correlations among plasma LH, FSH, estrogen, progesterone, prolactin and cortisol in female workers and control.

Correlations						
Hormones	**Cortisol**	**LH**	**FSH**	**PRL**	**P**	**E**
Cortisol (ng/ml)	r=1					
	r=-0.856**					
LH (IU/L)		r=1				
	p<0.001					
	r=-0.872**	r=0.989**				
FSH (IU/L)			r=1			
	p<0.001	p<0.001				
	r=0.874**	r=-0.965**	r=-0.974**			
PRL (mIU/L)				r=1		
	p<0.001	p<0.001	p<0.001			
	r=-0.879**	r=0.984**	r=0.987**	r=-0.991**		
P (ng/ml)					r=1	
	p<0.001	p<0.001	p<0.001	p<0.001		
	r=-0.923**	r=0.924**	r=0.943**	r=-0.961**	r=0.958**	
E (pg/ml)						r=1
	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	

** Correlation (r) is significant at the 0.01 level (2-tailed).

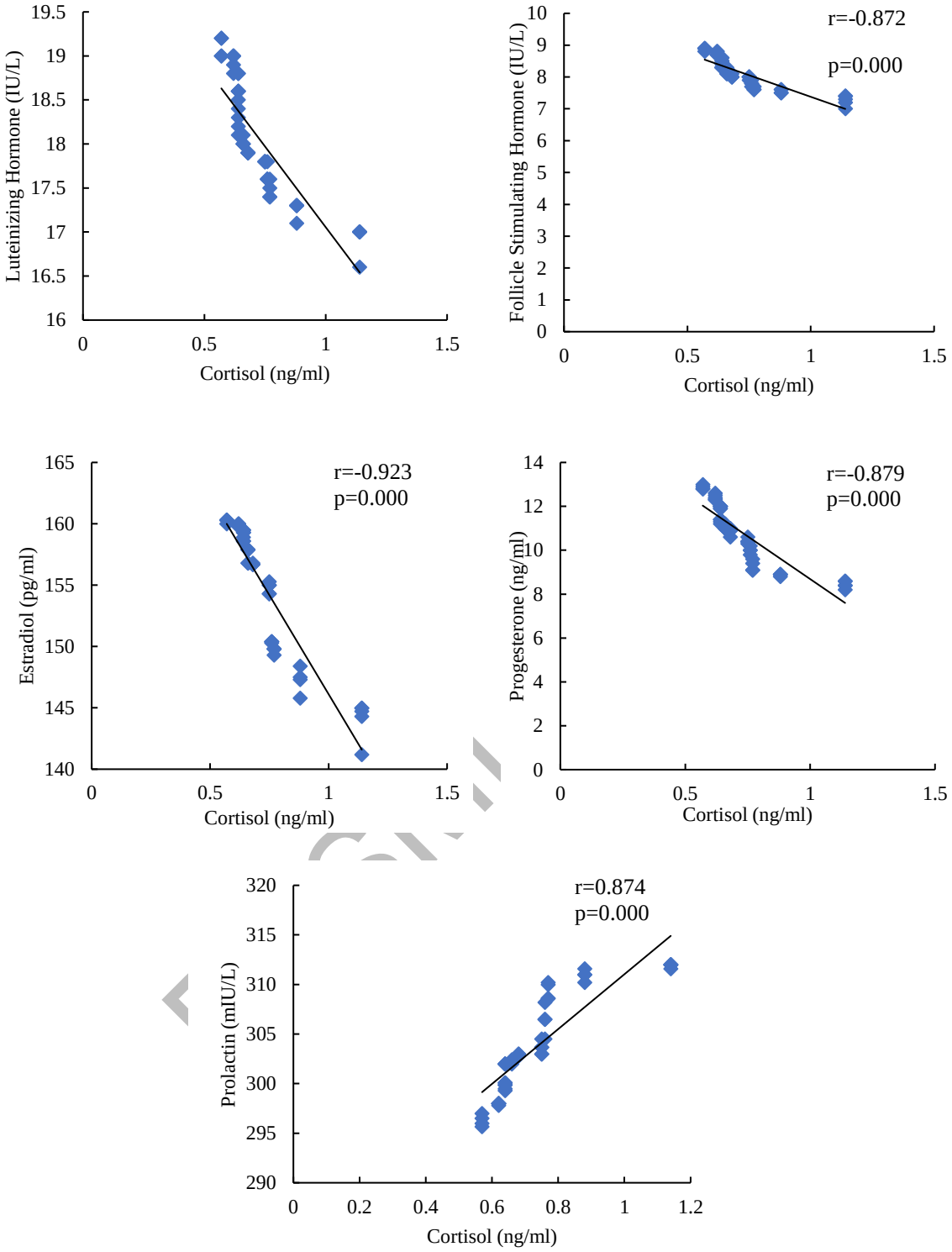


Figure 32. Correlation of plasma LH (IU/L), FSH (IU/L), estradiol (pg/ml), progesterone (ng/ml), prolactin (mIU/L) and cortisol levels in female workers.

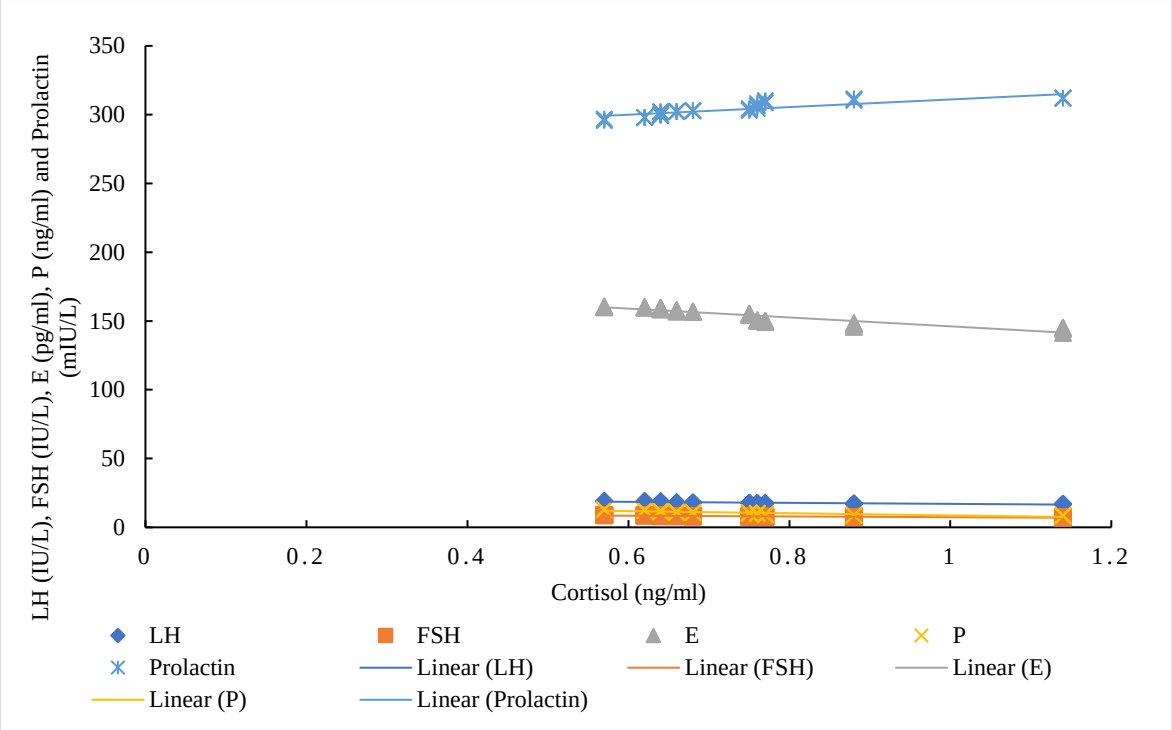


Figure 33. Figure showing summarized correlation of plasma LH, FSH, Estradiol, progesterone, prolactin with cortisol levels in female workers.

DISCUSSION

In present study, we reviewed and reported the health status of brick kiln female workers living at Rawat, Punjab. Considering brick kiln industry, brick manufacturing is the fastest-growing industrial sector in many countries including Pakistan (Bhat *et al*., 2014). Punjab province of Pakistan has the largest number (10,000) of active brick kilns according to the recent survey. With this mass number of brick kilns, the kilns smoke and emission is continuously polluting the environment, imparting hazardous effects on all life forms including plants, animals and humans (Khan & Vyas, 2008). Direct exposure to the toxic chemicals induce various types of musculoskeletal symptoms along with different types of cancers and therefore, they raise serious public health concerns among occupational workers (Rajesh & Niraj, 2010; Rzymski *et al*., 2014; Sanjel *et al*., 2016; Shaikh *et al*., 2012).

For a healthy mother and baby, maternal undernutrition and normal BMI are considered as crucial determinant of healthy pregnancy outcomes. Undernutrition can be defined as depleted circulating or stored levels of nutriments, that shows dietary scantiness (Wells *et al*., 2020). It is reported by Khan *et al*. (2009) that women of rural areas suffer more from undernutrition due to lack of several vitamins and essential nutrients in their diets (Khan *et al*., 2009). It is speculated that among Pakistani women of reproductive age (25-44years), the prevalence of malnutrition accounts for 30% in rural women (Khan *et al*., 2009). Our findings suggested that brick kiln workers women who participated in our study, were 18 to 45 years of age and most of them were malnourished. The maternal malnutrition is known to enhance the risk in developing offspring for metabolic and neurodevelopmental disorders, affecting immune responses and bringing neuroimmune consequences (Smith & Reyes, 2017). As malnutrition may bring hazardous and life

threatening risks for mothers and baby during childbirth, therefore, it is speculated that factor of malnutrition among brick workers may reduce reproductive potential and may increase the risk of blocked labor (Wells *et al*., 2020).

A regular menstrual cycle is an indication of healthy body with response to all the hormonal and physiological changes. However, various conditions may cause menstrual irregularities. Our findings suggested that menstrual irregularities were experienced among women working at kiln sites. During adolescence, variations in the menstrual cyclicity is a result of immature and disturbed hypothalamic-pituitary-ovarian (HPO) axis (Deligeoroglou & Creatsas, 2012). The menstrual irregularities may result into anemia, osteoporosis and ultimately, infertility (Sherly *et al*., 2017). The present study further reported that the trend of early age marriage and bonded labor was quite common among the brick kiln community. The rate of abortion was quite high among brick workers, as they had to do work, even during the conception that might be the major cause of simultaneous abortion and miscarriages. During conception, exposure to environmental pollution, occupational heavy metal and smoke may bring pathophysiological consequences and may accumulate in fetus by translocating through the placenta (Zhao *et al*., 2017). Cd inhaled via smoking integrates into the body tissues and is transported through the bloodstream to the ovaries, where it represses oocyte development, reduces steroid synthesis, and increases ovarian hemorrhage and necrosis (Thompson & Bannigan, 2008). Therefore, it is expected that menstrual irregularities, early age marriages, poor hygiene conditions and increased abortion rate among brick kiln workers impart a serious reproductive health concern on the maternal and child health of whole community and there is a need to address these issues at government and private level.

The findings of blood parameters showed a significant decrease in HGB and RBC number which is associated with toxic effects of heavy metals on blood cells. Our results

are strengthened by previous work of Fazio *et al*., (2014) where fish exposed to metal pollution experienced decreased RBC and WBC levels (Fazio *et al*., 2014). In another study by Kamal *et al*. (2014), it is suggested that brick kiln pollutants may cellular toxicity in actively dividing cells such as bone marrow cells. These cells, later differentiate and constitute blood components (Kamal *et al*., 2014a, 2014b). As hematopoietic tissues of kidney and spleen produce RBC, the decrease in RBC count might be contributed by an internal bleeding or kidney damage (Kori *et al*., 2006). We also reported reduction in WBC levels associated with metal toxicity, as it is known that the molecular mechanism by which Cr(VI) induces cellular toxicity in human blood lymphocyte is through the formation of ROS with subsequent cellular damage (Seydi *et al*., 2020). Increase in platelet number in whole blood of female workers was evidenced in our results. Comparable reports by Jahan *et al*. (2016) were followed, where male brick kiln workers exposed to heavy metals Cd, Cr and Ni displayed significant decrease in RBC , HCT and MCHC levels as compared to unexposed males (Jahan *et al*., 2016).

Turkez *et al*. (2012) showed that exposure to heavy metals increase oxidants levels and reduces antioxidants concentrations (Turkez *et al*., 2012). Wąsowicz *et al*. (2001) found that work-related exposure to heavy metals e.g., Cd or Pb disturb the normal homeostasis of the body by reducing antioxidant capacity of the body. This, in turns, alters the activity of many other enzymes (Wąsowicz *et al*., 2001). In present study, significant decrease in SOD and POD level was observed. Our results are being supported by previous results of Jahan *et al*. (2016) where brick kiln workers, exposed to emitted heavy metals presented reduced levels of peroxidase enzyme (Jahan *et al*., 2016). Ni is known to generate ROS (Lippmann *et al*., 2006). Our studies have also reported that brick kiln group has increased number of ROS as compared to control. As body antioxidants are the major

defensive mechanisms against ROS, and their reduced levels in exposed workers possibly make them prone to various health risks.

Different study groups have shown that decrease in levels of plasma total protein is considered as a conceded functioning of liver (Alam *et al*., 2017; Karakilcik *et al*., 2004). Liver damage may impart detrimental effects on cellular homeostasis through peroxidation of polyunsaturated fatty acids and with the formation of aldehydes, that may result in multiple pathological conditions (Yamaguchi *et al*., 2007). The present study showed decrease in plasma proteins among kiln workers when compared with control. This decrease in total protein in blood plasma suggests liver malfunctioning as experienced in our study subjects. Other liver enzymes and antioxidants concentrations have also been measured in present study and are discussed further.

Studies have also suggested that increase in production of fatty acids results in elevated cholesterol level and triglyceride level, that may result in liver dysfunction (Sayed, 2012). Cholesterol is an essential component of mammalian cell membranes, which plays major roles in membrane permeability and fluidity and also serves as a precursor of bile acids, steroid hormones and fat-soluble vitamins (Ihedioha *et al*., 2013). Thus, disturbed levels of cholesterol may interfere with other physiological functions such as metabolic and reproductive malfunctions. The current findings for lipid profile analysis showed an increase in plasma total cholesterol, LDL, and triglyceride (TG), and HDL levels in workers exposed to environmental pollutants; these findings are authenticated with our observed surge in BMI and abdominal fat. Our results are being supported by previous reports where heavy metal (nickel and chromium) induced rise in serum TC, LDL, and TG (Gupta *et al*., 2008). The ratio of TG to HDL is used clinically for the identification of metabolic disorders (Murguía *et al*., 2013). As LDL is considered as a bad cholesterol, therefore, elevated levels of plasma LDL in present findings could be due

to its receptors overactivation, as suggested before (Karacaoĝlu & Selmanoĝlu, 2010). Rehman *et al*., (2019) have proposed that rats exposed to food toxicant had increased LDL levels because their receptors permit cholesterol to enter hepatocytes, releasing its free cholesterol and triglycerides that in turn, inhibit cholesterol and formation of new LDL receptor, reducing LDL uptake, and promoting cholesterol storage (Rehman *et al*., 2019). The decrease in LDL uptake and loss of receptors function boost the cholesterol levels in serum (Adkison, 2012). The results of HDL cholesterol showed a comparable concentration among the control and workers groups; one of the possible explanations for these findings might be the active lifestyle of brick kiln women, who were engaged in prolonged strenuous activity that might have contributed in the production/accumulation of HDL, which is considered as a good cholesterol. Our results are being supported by previous studies which stated that HDL is inversely linked to BMI (Njelekela *et al*., 2002), this has been shown here that BMI (results from chapter 1) is near to normal, while little increase in HDL is found. Extensive literature review on public health and reproductive health concerning women subjects proposed that lipid profile analysis is important for determining the health status and reproductive studies of occupational women because components of lipid profile may vary during different phases of menstrual cycle and is an important indicator of reproductive health (Hatma, 2011; Knauff *et al*., 2008; Shohaimi *et al*., 2014; Wamala *et al.*, 1997). As we studied some of the reproductive parameters in women in this chapter, therefore lipid profile was performed for women samples but not in chapter 3 (men).

Previous studies have suggested that environmental exposure to even low levels of metals might be associated with variation in hormonal levels in women of reproductive age (Pollack *et al*., 2011). These variation in women are contributing risk factors for breast and ovarian cancers (Brinton *et al*., 1988; Kelsey *et al*., 1993). Literature data suggests

that heavy metals such as Cd is known to have inverse relation with FSH (Dutta *et al*., 2021). In present study, reduced LH and FSH levels were observed among occupational brick workers, that is consistent with earlier study of Lafuente *et al*. (2003), which concluded that Cd exerts dose dependent effects on the secretory patterns of the pituitary gonadotropins LH and FSH (Lafuente *et al*., 2003). Our findings are also supported by previous study of Pollack *et al*., (2011), where Cd exposure among healthy menstruating women resulted in reduced levels of FSH amplitude (Kawai *et al*., 2002; Pollack *et al*., 2011). This in turns, disrupts the overall ovarian function of hormone synthesis. The results of metal toxicity on LH and FSH concentrations here contrast with our previous results obtained for men (mentioned in chapter 2) where heavy metals induced elevated gonadotropin (LH, FSH) concentrations in blood, which might be due to lack of negative feedback mechanism controlling HPT axis or the presence of blood testes barrier (BTB) which prevented the direct interaction of testis with metals. Furthermore, previous reports also found that concentration and longevity of exposure of the rats under the influence of metals such as Pb, damage the signaling systems in the hypothalamus and pituitary gland, including their hyperfunction, leading to over-production of gonadotropin hormone GnRH and LH (Morphology, 2005). Whereas in case of females HPG axis is directly affected prior to metal toxicity, for example Cd is considered as a metalloestrogen and is capable to join the oestrogen receptors alpha and beta and stimulate it (Rzymski *et al*., 2015).

Metals are known to exert dose-dependent effects on both prolactin and ACTH secretion, with even exposure to low levels of Cd induce increase in plasma prolactin levels (Lafuente *et al*., 2003). Our findings presented that with circulation of heavy metals in blood, the occurrence of hyperprolactinemia among women working at brick kiln sites was also witnessed. The increase in prolactin levels might be due to induction by cortisol hormone (Bridges & Bridges, 2018). Our study has also reported increased cortisol levels

in blood of female workers that might be due to occupational exposure to environmental stress. It has been reported that exposure to physical or psychological stress results in activation of the hypothalamic pituitary system (HPA) system causing secretion of corticotropin releasing hormone (CRH) from the hypothalamus, which in turn stimulates adrenocorticotropin's to release adrenocorticotropic hormone (ACTH) and beta-endorphin from the hypothalamus, and eventually the release of corticosteroids from the adrenal cortex (Einarsson *et al*., 2008). We found decrease in estradiol and progesterone levels among kiln workers that might be due to increased cortisol in blood plasma, which usually decrease sex steroids production such as estradiol, by mediating its effects through malfunctioning of granulosa cell within the follicle, ultimately results in deterioration of oocyte quality (Baker *et al*., 2013; Prasad *et al*., 2016).

The correlation analysis showed that cortisol hormone which is produced because of activation of HPA axis negatively regulates the HPG axis, through mediating its effects on levels on pituitary gonadotropins LH, FSH, and ovarian steroids, estradiol and progesterone. Former study has also presented that increase in plasma cortisol level suppresses gonadotropin secretion from the pituitary and disrupts ovarian cyclicity (Oakley *et al*., 2009). Additionally, positive correlation was found among cortisol and prolactin levels as evidenced by previous studies in mice, where stress induced increase in prolactin levels through central and peripheral nervous system (Kirk *et al*., 2017). Correspondingly, evidence indicates that prolactin plays an important role in adrenal gland's response to stress in a way, that it not only stimulates the secretion of ACTH, but also increases the sensitivity of adrenal cortex, resulting in elevated corticosterone release (Levine & Delale, 2018). Further analysis showed that prolactin exhibits negative relationship with LH, FSH, estradiol and progesterone. Studies have suggested that prolactin mediates its direct effects on reproductive axis through suppression of kisspeptin

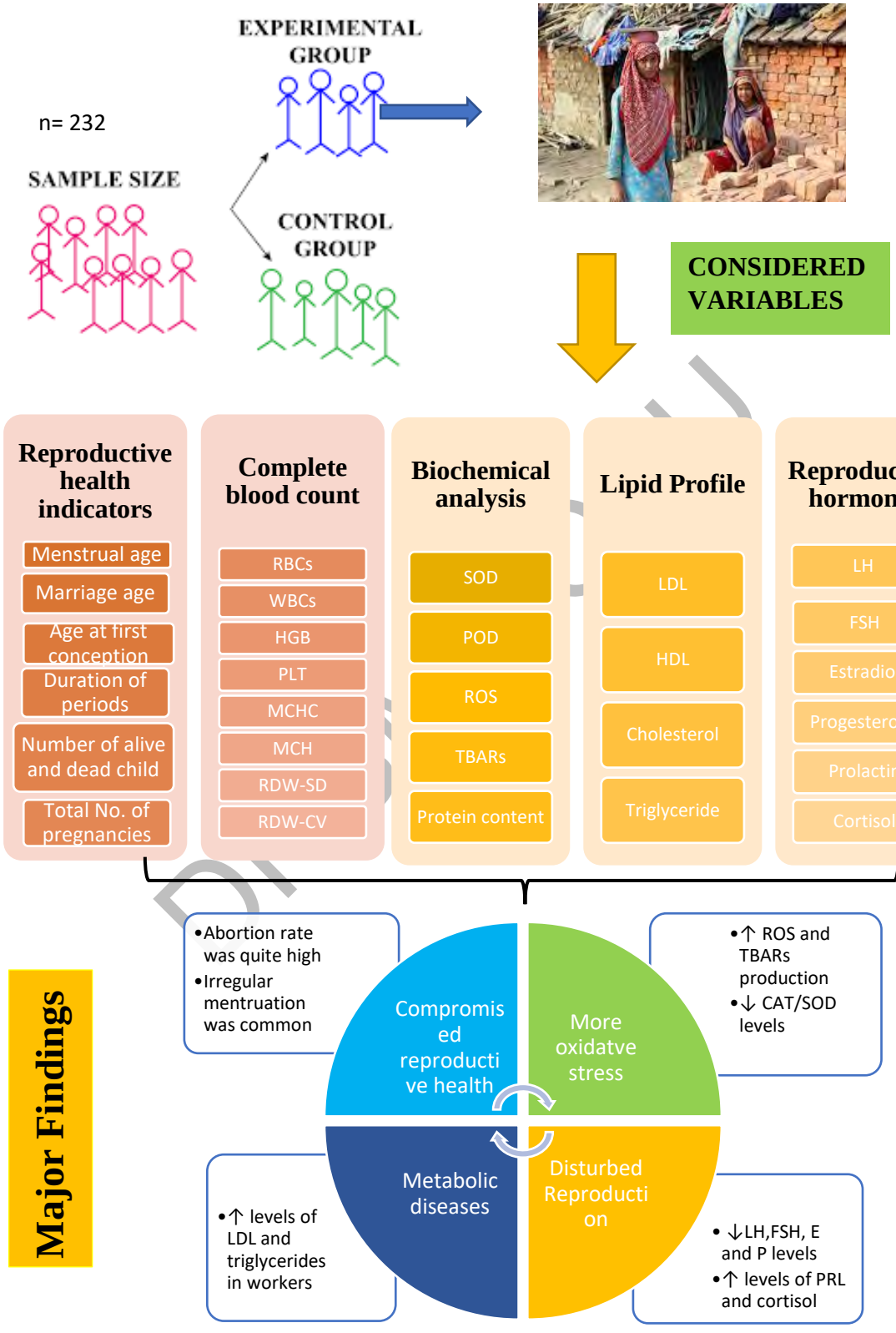
release at hypothalamic level, reducing activation of GnRH and lastly, decreasing gonadotropins secretion (Donato & Frazão, 2016). A positive relationship was found among LH, FSH and estradiol and progesterone justified by the fact that ovarian secretions are dependent on the pituitary gonadotropins. Estradiol and progesterone were found to have positive correlation between them.

CONCLUSION

- It is concluded that occupational exposure to environmental pollutants increases heavy metal burden in blood of labor women and may impart deleterious effects on maternal health as well as reproductive health.
- Observations of reproductive health and fertility indicators suggested that the female workers experienced issues of malnutrition, poor hygiene, irregular menstruation and missed abortions along with other pathological conditions and were at the verge of developing major reproductive disorders with complication in conception and pregnancy.
- The study concluded that occupational exposure to environmental pollutants also affects blood parameters, oxidant levels, antioxidant enzymes concentrations, total protein content, lipid profile, and plasma concentrations of reproductive hormones/stress hormones.
- The study also found negative correlation of cortisol with the reproductive hormones (LH, FSH, E, P), while positive correlation with prolactin was seen.

However, further such studies are needed to evaluate the molecular basis of the risks associated with heavy metals burden and other pollutants in maternal blood and their possible actions on women health.

SUMMARY


EXPERIMENTAL GROUP
n= 232
SAMPLE SIZE
CONTROL GROUP
CONSIDERED VARIABLES
Reproductive health indicators
Menstrual age
Marriage age
Age at first conception
Duration of periods
Number of alive and dead child
Total No. of pregnancies
Complete blood count
RBCs
WBCs
HGB
PLT
MCHC
MCH
RDW-SD
RDW-CV
Biochemical analysis
SOD
POD
ROS
TBARs
Protein content
Lipid Profile
LDL
HDL
Cholesterol
Triglyceride
Reproductive hormones
LH
FSH
Estradiol
Progesterone
Prolactin
Cortisol
Major Findings
•Abortion rate was quite high
•Irregular mentruation was common
Compromised reproductive health
More oxidatve stress
•↑ ROS and TBARs production
•↓ CAT/SOD levels
Metabolic diseases
Disturbed Reproduction
•↑ levels of LDL and triglycerides in workers
• ↓LH,FSH, E and P levels
•↑ levels of PRL and cortisol

Chapter 4

Mutational analysis of ABCG-2 gene in Pakistani brick kiln worker and control population; identification of multiple known and novel causative variants at the coding region of exon 3 and exon 5

ABSTRACT

Heavy metals are known to induce genetic changes by creating single nucleotide polymorphisms (SNPs) in important structural and functional genes. ABCG2 is a membrane transporter protein expressed in multiple organs including placenta, where it protects the developing fetus. The molecular alteration in the ABCG2 gene might interfere with the normal functioning of the protein; risking life of fetus by exposing it to multiple drug metabolites and xenobiotics compounds. The aim of present study was to identify genetic mutations in the ABCG2 gene in brick kiln worker and control subjects from Pakistani population. To understand the functional changes in the ABCG2 gene, two most mutating exons (exon 3 and 5) were selected from female participants. The previously collected blood samples were subjected to DNA extraction, gel electrophoresis and nanodrop, DNA amplification, gel electrophoresis, gene sequencing and post-sequencing analysis using BioEdit, Chromas and mutation taster. The findings showed that a total of twenty- eight genetic variations were found, including 25 novel ones: 24 of them were present in the coding exons, and 1 in the intron. Results revealed that mutations were found in both worker and control samples. In addition to three previously reported nonsynonymous single nucleotide polymorphisms, g.91361G>A (rs2231137), g.91461G>A (rs142634180) and g.91521C>T (no rsID), the novel variations found in workers samples were present at genomic position g.91474G>A, g.91482delinsAA, g.91410T>A, g.91414G>A, g.91416C>A (rs142634180), g.91419T>A, g.91427G>A, g.91436T>A, g.91450C>T, g.91454T>A, g.91456C>A, g.91459T>A, g.91461G>A, g.91463G>A, g.91416C>A, g.91427G>C, g.91430G>C, and g.91440_91441delinsAA in brick kiln workers while those found in control are g.91474G>A, g.91487C>A, g.91507G>A, g.91521C>T, g.91346G>C, g.91416C>A, g.91430G>A, g.80676T>A, and

g.91446A>T. The V12M variant (rs2231137) has been previously reported and is associated with multiple metabolic diseases. Therefore, it can be inferred from the present findings that occurrence of these polymorphisms might interfere with the normal function of ABCG2 protein expressed in placental tissue, bringing fetal exposure to multidrug metabolites and xenobiotics compounds and increasing chances of miscarriage.

INTRODUCTION

Heavy metals including Cd, Cr, and Ni are known to induce genotoxic effects in the human body. The presence of heavy metals even in lower concentrations in the living cells is toxic, due to their higher densities and metallic properties (Alaraidh *et al*., 2018). Cd and its compounds are known to exert mutagenic, genotoxic, and carcinogenic effects (Apykhtina *et al*., 2018). Tabrez *et al*. (2014) have mentioned in their respective study in detail, all the possible mechanisms through which heavy metals such as Cd, As, Co and Cr alter gene functions by exerting their toxic effects in cells. These include altered gene expression and DNA-protein cross-linking, interference in DNA repair mechanisms, increased cell cycle activity, production of chromium- DNA adducts, induction of redox stress, and alternation of signaling pathways such as MAPK/Src/Akt family kinases (Tabrez *et al*., 2014). Another study conducted by Li *et al*., (2019) indicated that *in vitro* exposure to heavy metals ions increases antibiotic resistance capacity against multiple drugs in *E. coli* through changes in the genes. The results of whole-genome analysis depicted that genetic changes were spotted in 17 genes (involved in translation/transcription/structure of cell wall/membrane transport) and 11 intergenic regions in the *E.coli* genome (Li *et al*., 2019). A study conducted by Alaraidh *et al*., (2018) reported that heavy metals alter the gene expression of antioxidant genes, and enhance the production and accumulation of reactive oxygen species and H_2O_2 in plants that toxify them (Alaraidh *et al*., 2018) Therefore, it is concluded that exposure to heavy metals present in the environment is responsible for inducing mutagenesis and genotoxicity in body systems via multiple mechanisms, affecting a wide range of gene families.

Previous studies have shown that heavy metals bring genetic variations widely through creating single nucleotide polymorphisms (SNPs) in important structural and

functional genes. Silver (Ag), Cu, and Zn exposure in *E.coli* is known to alter gene expression of cell wall-linked genes, antibiotic resistance genes (rpoB and rpoA gene), and genes of membrane transport (Li *et al*., 2019). Studies suggest that early-life exposure to Pb brings epigenetic changes in genes without any symptoms of disease (Modgil *et al*., 2014). A genome-wide association studies (GWAS) conducted by Ng *et al*., (2015) also showed that genetic variants in metal transporter genes was evident caused by toxic metals such as manganese (Mn), mercury (Hg) and Cd. These metals attach themselves to p and q arms of different chromosomes, create lead SNPs, resulting in formation of intronic and exonic variants (Ng *et al*., 2015). It has been found that there occurs a correlation among gene polymorphisms and heavy metals such as As, Pb and platinum (Hollman *et al*., 2016). Thus, it is confirmed that heavy metals induce genetic and epigenetic changes in the genome via displacing normal nucleotide sequence and creating variants of genes.

To understand the gene-environment interactions in present study, ATP-binding cassette (ABC) transporters (ABCG2), gene was selected. ABC transporters presents one of the most abundant active transport molecules families. The genes for ABC transporters encode for large group of functional membrane proteins which transport substrates across membranes using energy dependent pathways (Iida *et al*., 2002). These transporters are found in abundance in genomes of prokaryotes and eukaryotes. The function of eukaryotic transporters is to move materials to and from across the cytoplasm, plasma membrane and between organelles (Robey *et al*., 2009). In human genome, 48 genes of ABC transporters are found on different chromosomes. These transporter proteins have been grouped into subfamilies; seven of these transporter family groups (A–G) are present in the human genome (Robey *et al*., 2009). The seven distinct subfamilies of ABC genes are ABC1, multidrug resistance (MDR/TAP), multidrug resistance proteins (MRP),

Adrenoleukodystrophy (ALD), ATP-binding cassette, sub-family E (OABP), GCN20, and White family. The second member of G family ATP-binding cassette, sub-family G was selected, which is also denoted as junior blood group and abbreviated as ABCG2. Previously, it was also called as breast cancer resistant protein (BRCP), which functions as a xenobiotic transporter, thus, playing a major role in multi-drug resistance. It likely serves as a cellular defense mechanism in response to multiple drugs exposure.

The ABCG2 gene is found on chromosome 4 (66 kb) in human genome; contains 16 exons and 15 introns; the exons size ranges are between 60-532bp. The gene location on chromosome has been shown in figure 34 taken from GENELIB (https://www.genelibs.com).

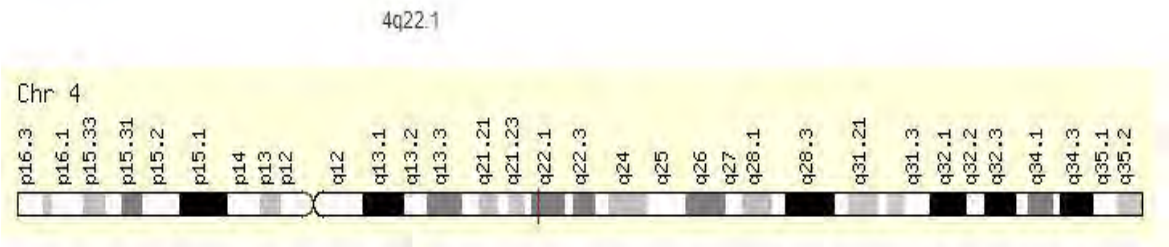


Figure 34. ABCG2 gene location on chromosome 21

It is a protein coding gene and its transcription results in the production of 75kD protein comprised of 665 amino acids (Zhang *et al*., 2018). The protein structure for this transporter has been given by Taylor *et al*. (2017) as mentioned in figure 35. Studies have indicated that the molecular mechanisms which regulate the ABCG2 expression are not well documented, however events such as gene amplification and translocations on chromosome 4 trigger the increased levels of ABCG2 expression in the cell lines (Bailey *et al*., 2001; Rao *et al*., 2005). Studies suggest that ABCG2 plays an important role in shielding body against xenobiotics and regulate oral bioavailability (Robey *et al*., 2009). As large number of substrates are being transported, the expression of ABC proteins in specialized cell types

varies with varied functions. ABCG2 or BRCP is expressed in multiple tissues such as, liver, kidneys, mammary glands, body barriers (blood brain barrier and blood testes barrier), and maternal-fetus barrier, where it performs various physiological roles (Taylor *et al*., 2017). Due to capability of multiple substrate specificity and widespread localization, ABCG2 is known to be involved in multidrug resistance. Therefore, the expression of ABCG2 in tissue barriers is justified.

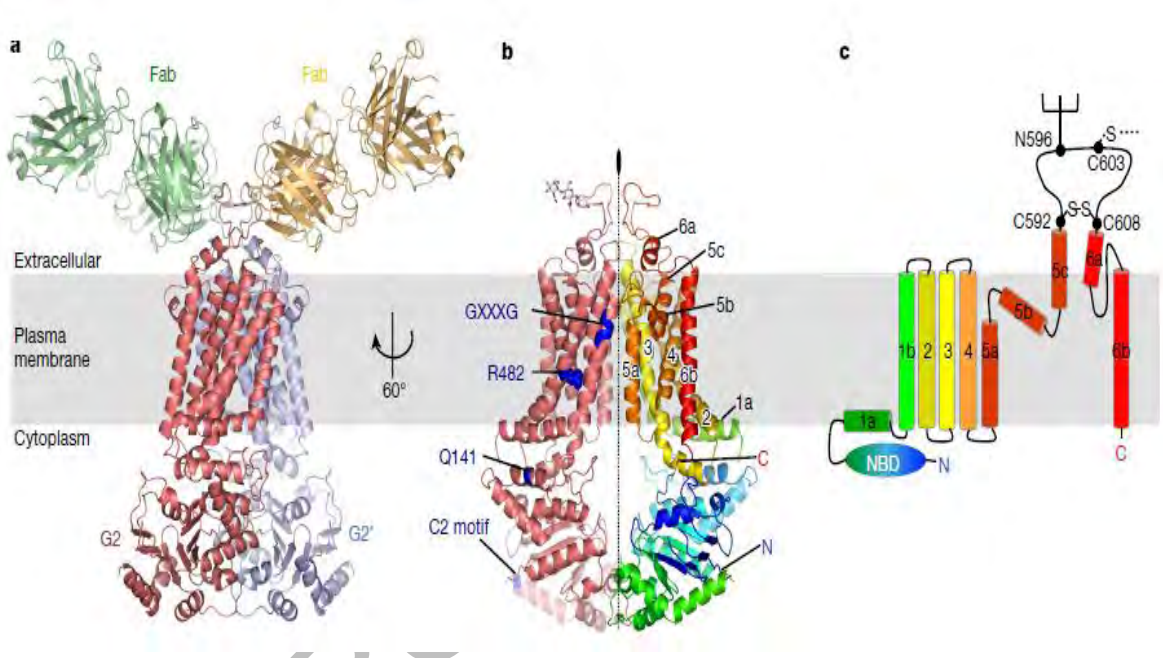


Figure 35. The detailed 3D structure of ABCG2 protein (Taylor *et al*., 2017)

Figure 36 shows in detail the gene expression of ABCG2 in cells. The ACG2 protein is translated from mRNA on ribosomes attached to endoplasmic reticulum, where dimerization and core glycosylation occurs. The nascent protein then moves towards Golgi complex, where its glycosylation is completed and from here, mature protein travels to the plasma membrane. The damaged/misfolded ABCG2 protein is degraded by several pathways within the cells, such as lysosomal or the ubiquitin-mediated proteasomal degradation, or by accumulation in aggresomes. (Mózner *et al*., 2019).

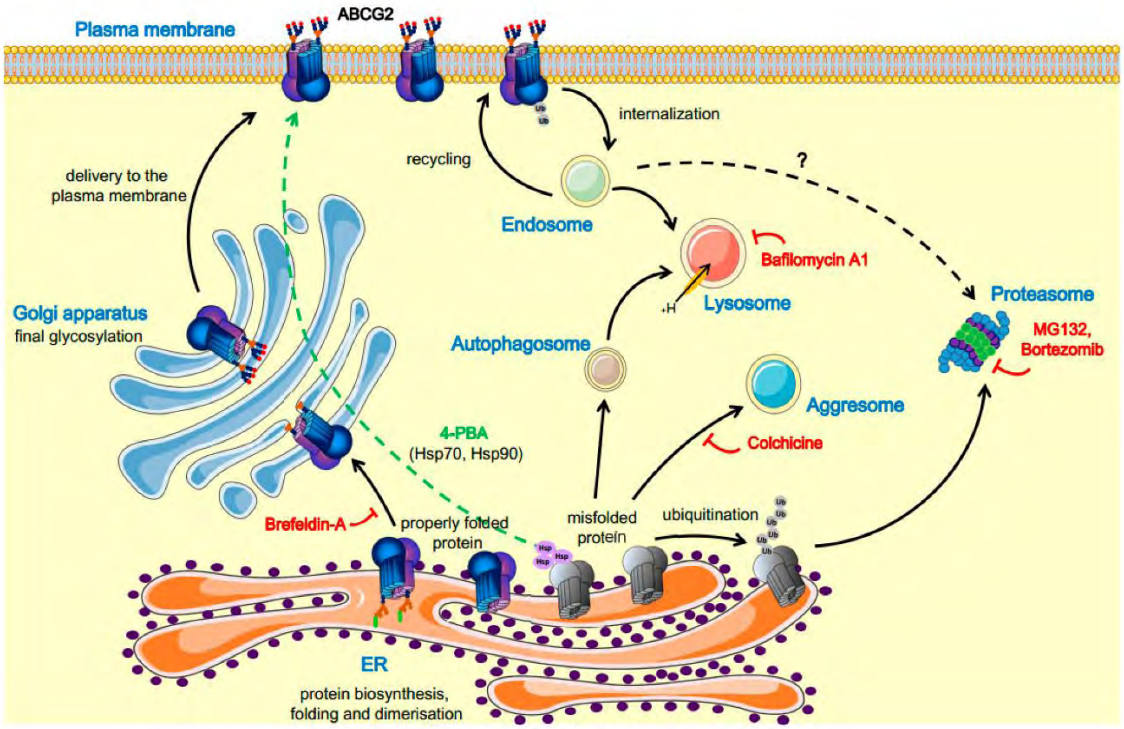


Figure 36. Figure shows the ABCG2 gene expression and trafficking pathways along with its modulators; The ABCG2 protein is synthesized on ER-bound ribosomes; followed by dimerization and core glycosylation, later it travels to the Golgi complex, where its glycosylation is completed; thereafter, the mature ABCG2 travels to the plasma membrane. In contrast, the misfolded ABCG2 protein can be degraded by several pathways, such as lysosomal or the ubiquitin-mediated proteasomal degradation, as well as by accumulation in aggresomes (Mózner *et al*., 2019).

Clinical trials have shown that among individuals, the drug response varies greatly due to alterations in multiple protein structures at the molecular level. These include drug transporters, receptors, targets, and enzymes metabolizing them (Kobayashi *et al*., 2005). The expression of ABCG2 gene in multiple vital and reproductive organs has been reported including ovaries, testes, prostate, uterus, placenta, brain, thymus, liver, kidney and others. Therefore, single nucleotide polymorphisms (SNPs) have been studied to explore the function or expression of these proteins. For ABCG2 gene, many study groups have identified naturally occurring SNPs in the gene (Iida *et al*., 2002; Pedersen *et al*., 2017;

Rachel *et al*., 2018; Robey *et al*., 2009). Following figure (figure 37) depicts the number of SNPs present in different coding and non-coding regions of ABCG2 (Bircsak *et al*., 2018).

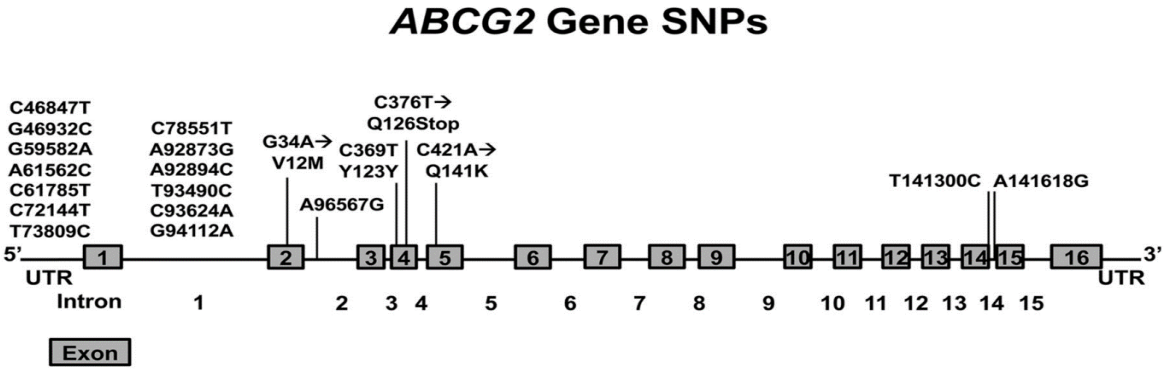


Figure 37. Map of SNPs in the noncoding and coding regions of the ABCG2 gene.

Similarly, figure 38 shows the effect of nucleotide polymorphisms on the amino acid (AA) sequence in ABCG2 transporter protein as reported previously (Tamura *et al*., 2006).

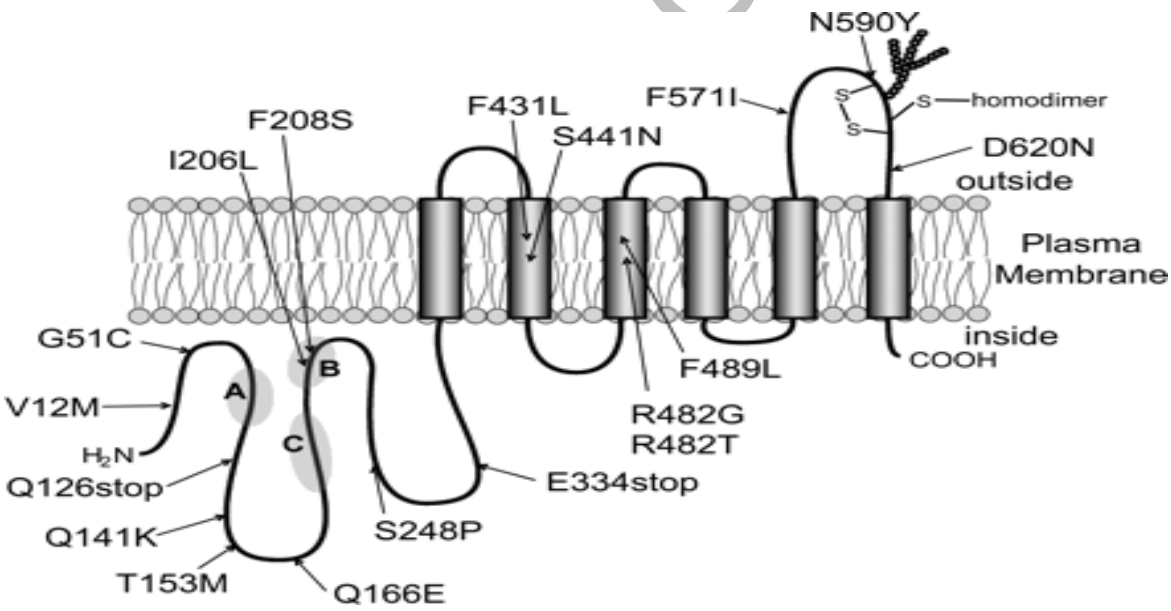


Figure 38. Schematic illustration of human ABCG2 and its nonsynonymous polymorphisms (Tamura *et al*., 2006)

As we have established from literature that high levels of ABCG2 gene are expressed in placenta, where it protects the developing fetus; it can be suggested that molecular

alteration in the ABCG2 gene might interfere with the normal functioning of the protein; risking life of fetus by exposing it to multiple drug metabolites and xenobiotics compounds (Mao, 2008). The results of fertility analysis (from chapter 3) in our studies showed that the rate of abortion among brick kiln workers was quite evident as compared to control females. Due to lack of proper medication and poor health conditions prevailing at brick kiln sites, the kiln workers were consuming multiple types of medicine without consultation of doctor. Therefore, the present study concluded that as results of health data indicated increased rate of miscarriage in kiln workers (chapter 3, table 12), due to lack of proper medical facilities and poor health conditions, this gene might act as one of the major factors responsible for weakening/disrupting feto-maternal placental barrier and allowing xenobiotics and kiln pollutants to cross placental barrier and move into the growing fetus and might be a cause of abortion. As it is established that alteration in gene expression of ABCG2 in placenta might interfere with normal transport of materials across placental membranes, therefore, we suggest that identification of known and novel disease-causing variants in ABCG2 protein might be the key players responsible for poor maternal health and increased rate of abortions. ABCG2 gene was selected for present study to identify the single nucleotide polymorphisms in selected exons of ABCG2 gene and thus, hindrance in the normal function of these proteins.

Aim and Objectives:

The current study aims to identify the known and novel SNPs in brick kiln workers and control samples to predict is normal function and find its associated linkage with metal toxicity.

The objectives are to

- Find the reported and novel disease-causing mutations in ABCG2 gene sequence using control and workers samples
- Analyze and predict the association of polymorphisms and variants with disease expectation

MATERIALS AND METHODS

Subject selection

Women of different age groups (19– 45 years) working for varied years at brick kilns were considered, with a total number of 232 women, where 118 were kiln workers considered as Group I and 114 were non-workers (control). Additionally, data gathered from fertility variables indicated that most of the brick kiln participants were married and rate of abortion in them was quite high as compared to control. Therefore, only women were selected for this study because the study gene is known to express in placenta and alteration in its transcription/translation are linked with fertility outcomes in females.

Samples used

Blood samples previously kept at 4°C for genetic analysis were processed by thawing and were subjected to DNA extraction.

DNA extraction

Chemicals used with concentration- Recipes of Solutions

1. Lysing solution A for 250 ml

Sucrose 27.36gm

Tris 0.303gm

Mgcl2 0.254gm

Then autoclaved the mixture. After autoclave 2.5 ml 1% triton X-100 was added.

2. Lysing solution B

Tris 0.182gm

Nacl 3.52mg

EDTA 0.11gm

3. Solution C of PCI (Phenol, Chloroform, Iso-amyl alcohol)

Phenol 25ml

Chloroform 24ml

Iso –amyl alcohol 1ml

PCI is always freshly prepared. PCI was prepared by mixing equal volume of solution C and phenol.

4. Solution D

Iso-amyl alcohol 2ml

Chloroform 48 ml

The two reagents mentioned above were taken in a 50 ml falcon tube and were mixed well with a ratio (chloroform and isoamyl alcohol 24:1).

20 % Sodium dodecyl sulphate (SDS)

12.5 gm of SDS were dissolve in 50 ml of autoclaved water and then chilled for 2 days.

Tris – EDTA buffer (TE Buffer)

Tris 109gm

Boric acid 55gm

EDTA 9.3 gm

All the ingredients mentioned above were taken in a reagents bottle and were dissolved in distilled autoclaved water. final volume of the solution was raised to 1 litter.

Procedure

Phenol chloroform (Organic) method was used for DNA Extraction .750ul volume of blood was used and dissolved in 750 µl of lysis solution A; followed by centrifugation for 1min at 13000rpm; the pellet was dissolved in 500µl lysis solution and was again subjected to centrifugation for 1min at 13000rpm at room temperature. Again, 500ul lysis solution B was added in pellet, and after gentle mixing, was incubated for 30 minutes at 60° Celsius. 15ul protein kinase (PK) and 20% SDS (Sodium Dodecyl sulphate) were added. Then samples were incubated overnight at 55° Celsius.

Next day, the incubated samples were subjected to cell digestion. Addition of 500µl of Solution C of PCI was done, followed by centrifugation for 10min. The supernatant was moved to another tube to which, 500µl of Solution D of solution was administered and centrifuged for 10 min. Again, the supernatant was transferred to another clean 1.5ml Eppendorf, to which sodium acetate (55µl) and chilled isopropanol (500µl) were supplemented; incubated at -20◦C for 45min; centrifuged for 10 min; and removal of aqueous layer. To eradicate all the impurities, the obtained pellet was augmented with 70% ethanol (500µl), and centrifuged for 5min at 7500rpm; again, aqueous layer was discarded, and the DNA pellet was saved and air dried. Later, DNA pellet was resuspended in TE (Tris EDTA) Buffer and stored at 4◦C.

Preparation and Running of Agarose Gel

To detect DNA, we performed agarose gel electrophoresis.

Solutions Required

10X Tris Boric EDTA buffer (TBE Buffer)

Tris 109 gm890Mm

Acetic acid 55 gm890mM

EDTA 9.3 gm25mM

All the ingredients mentioned above were taken in a reagent bottle and were dissolved in distilled autoclaved water. Final volume of the solution was raised to 1 litter.

Ethidium Bromide

For making ethidium bromide, 0.1gm of ethidium bromide was added in 10 ml of distilled autoclaved water and mixed well. It was then stored at 4°C.

Yield gel electrophoresis

1% agarose gel was used for DNA quantification. 1% gel was made by taking 1 gm agarose in a beaker with addition of 100ml of 1X TAE buffer and dissolving by heating the mixture for 2 minutes in the oven. After formation of clear solution, 7µl of ethidium bromide (EtBr) was added to the gel solution. Gel solution was poured in the gel plate having dual comb 16 teethed casters and was allowed to solidify at room temperature. When the gel was solidified, combs were carefully removed, and gel was moved to gel electrophoresis tank that was filled with 1X TAE buffer. Then, 5µl of diluted DNA was blended with 2µl loading dye (6X) and stacked in the wells. Control samples were also run in some wells. The gel was run at 70V and 500mA current for 60 minutes. UV trans-illuminator (Bio Rad, UK) was then used for visualizing gel. Following gel pictures are showing representative DNA bands with comparison to 1KB Ladder (figure 39).

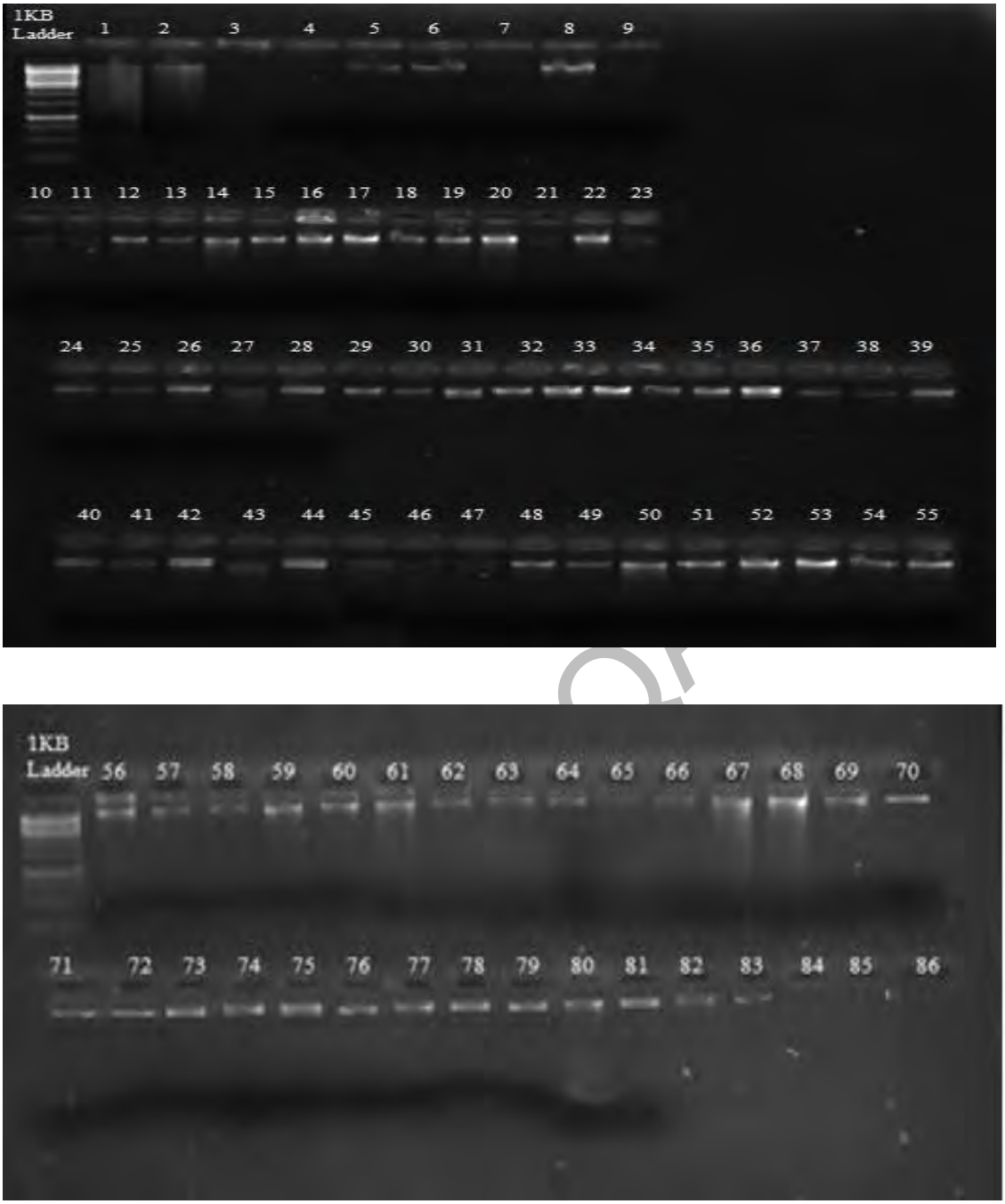


Figure 39. Gel electrophoresis images: 1KB ladder was loaded in first well with DNA samples in next wells. DNA is of highly intact and of more than 20kb size. (A) Sample 1-55 (B) Sample 56-83.

DNA Quantification

DNA quantity was measured using Thermo scientific Multi Skan Go Instrument as given in table 19.

Table 19. Table showing nucleic acid concentrations after DNA extraction

Sample No	Nucleic Acid 260/280	Nucleic Acid Conc. in (ng)
1	1.86	500
2	1.56	435
3	1.9	250
4	1.57	210
5	1.67	190
6	1.65	319
7	1.78	234
8	1.49	156
9	1.78	199
10	1.98	497
11	1.56	320
12	1.54	675
13	1.78	600
14	1.45	321
15	1.765	545
16	1.81	566
17	1.543	320
18	1.65	345

19	1.67	545
20	1.76	235
21	1.56	245
22	1.89	344
23	1.76	874
24	1.56	678
25	1.76	433
26	1.87	456
27	1.45	432
28	1.54	543
29	1.90	345
30	1.89	321
31	1.45	456
32	1.63	214
33	1.78	123
34	1.34	765
35	1.56	321
36	1.54	344
37	1.56	654
38	1.76	543
39	1.87	432
40	1.96	343
41	1.59	436
42	1.54	621

43	1.78	234
44	1.76	345
45	1.56	234
46	1.76	654
47	1.68	321
48	1.91	432
49	1.65	432
50	1.87	432
51	1.65	321
52	1.87	432
53	1.89	567
54	1.67	234
55	1.89	532
56	1.54	432
57	1.67	765
58	1.89	353
59	1.76	543
60	1.98	543
61	1.56	432
62	1.89	543
63	1.98	654
64	1.76	345
65	1.56	432
66	1.76	654

67	1.78	432
68	1.98	432
69	1.67	345
70	1.89	234
71	1.89	543
72	1.80	676
73	1.65	345
74	1.56	129
75	1.12	543
76	1.32	192
77	1.56	145
78	1.76	169
79	1.43	279
80	1.56	396
81	1.78	506
82	1.80	701
83	1.54	756

Primer selection and design

Primers were designed for two exons (Exon3. Exon 5) with maximum single nucleotide polymorphism (SNPs) reported for ABCG2 gene located on chromosome 4. Both forward and reverse primers were considered using online tool, Primer3web version 4.1.0. Later, these primer pairs were checked with various other tools such as *ENSEMBL* genome browser 96, Sequence Manipulation Suite-PCR primer stat, UCSC-InSilico PCR, BLAT search genome. Primers were prepared by Humanizing Genomics Macrogen, Rawalpindi. The primer sequences for forward and reverse primer are given (table 20).

Table 20. Detail of ABCG-2 primers.

No.	Primer Name	5'-3' Sequence	bps	tm	gc%	Product size
1	Exon 3-3FP	CAAGTTGTGCCTGTCTTCCC	20	60.7	55	653
2	Exon 3-3RP	GGAAATAGCCAAAACCTGTGAG	22	60	45.45	
3	Exon 5-5FP	AGCCAATGGTGTCTTGCTTT	20	59.7	45	490
4	Exon 5-5RP	GCTGCTGTAAAGAACGTCAGT	21	57.3	50	

DNA amplification by PCR

The PCR intensification was performed using reagents mentioned in table 21.

Table 21. PCR reagents, their concentrations and volume.

Reagents Used	**Stock Conc.**	**Working Conc.**	**Vol/Rec**
Extracted DNA (template)	-	-	1 µL
$Primer_{forward}$	10 µM	0.2 µM	0.4 µL
$Primer_{reverse}$	10 µM	0.2 µM	0.4 µL
DNTPs	10 mM	0.2 Mm	0.4 µL
$MgCl_2$	25 mM	2.5 Mm	2 µL
Buffer	10X	1X	2 µL
taqPolymerase	5U/ µL	1.5 U	0.3 µL
PCR Water		13.5 µL	
Final Volume			**20** µL

PCR was completed using Galaxy XP Thermal Cycler (BIOER, PRC).

Forward & reverse Primer (BGI Company), Taq polymerse enzyme 5U/ µL (Solis BioDyne FIREPol DNA polymerase, 01-01-00500), PCR buffer (Solis BioDyne FIREPol DNA polymerase, 01-01-00500), $MgCl_2$ (Solis BioDyne FIREPol DNA polymerase, 01-01-00500), dNTPs (Solis BioDyne, dNTPs Set, 02-21-00400), PCR water (Invitrogen RT PCR grade water, AM9935)

Optimization of PCR products

Primer for exon 3 and exon 5 of ABCG-2 gene was optimized at 58˚C with touch down temperature range of 56-66 ˚C. PCR profile after optimization consists of an initial melting step at 96˚C for 10minutes, 96˚C for 60sec, annealing temperature of 66˚C for about 60sec

with 2-steps for 10cycles at -1˚C, 96˚C for 60sec, 56˚C for 60sec, 72˚C for 60sec through 4 steps of 28 cycles and lastly, a final extension step at 72˚C for 1min and then at 4˚C for infinity. Optimized PCR conditions are shown in table 22.

Table 22. Optimized PCR conditions

Steps	Sub-cycles	Conditions	PCR cycles
Initial Denaturation		95 °C, 10min	1
PCR Cycles	Denaturation	95 °C, 1min	40
	Primer annealing	56/58 °C, 1min	
	Primer extension	72 °C, 1min	
Final extension		72 °C, 10min	1
Hold		04 °C, ∞	1

DNA determination by Gel Electrophoresis

Amplified products of DNA were run on 2 % agarose gel. 2% gel was made by taking 2 gm agarose in a beaker with addition of 100ml of 1X TAE buffer and dissolving by heating the mixture for 2 minutes in the oven. After loading and completing run, the gel was pictured under UV.

DNA Sequencing

83 PCR Amplified product samples with Exon 3 primer were sent for sanger sequencing. Out of 83, 18 samples passed. 19 amplified products with Exon 5 primer were sent for sanger sequencing out of which 20 samples passed.

Statistical analysis

The sequenced data was analyzed to reference DNA sequence from Ensemble genome browser by using BioEdit Sequence Alignment Editor version 7.2.5 and Chromas. Mutation taster was used for the identification of variants and mutations present in two exons.

RESULTS

Genotypic analysis

The results of DNA sequencing for studied exons (Exon 3 and exon 5) showed that no changes or variations were evident in sequence of exon 5 among all the worker and control samples. However, using mutation taster, Bio-Edit and Chromas, multiple novel disease-causing, and single nucleotide polymorphisms were seen in DNA sequence of exon 3 of ABCG2 gene as shown in tables 23 and 24.

- ***Genetic variations in sample AF2***

In brick kiln worker sample AF2, a first single nucleotide **polymorphism** was found at genomic position g.91361G>A where a single substitution of G with A was present, that resulted in replacement of valine (V) to methionine (M) at position 12 in protein structure of ABCG2 protein. This observed change has been previously reported as well and assigned rs2231137. According to dbSNP, the genomic location for present variation is mapped on NCBI website as shown in figure 40.

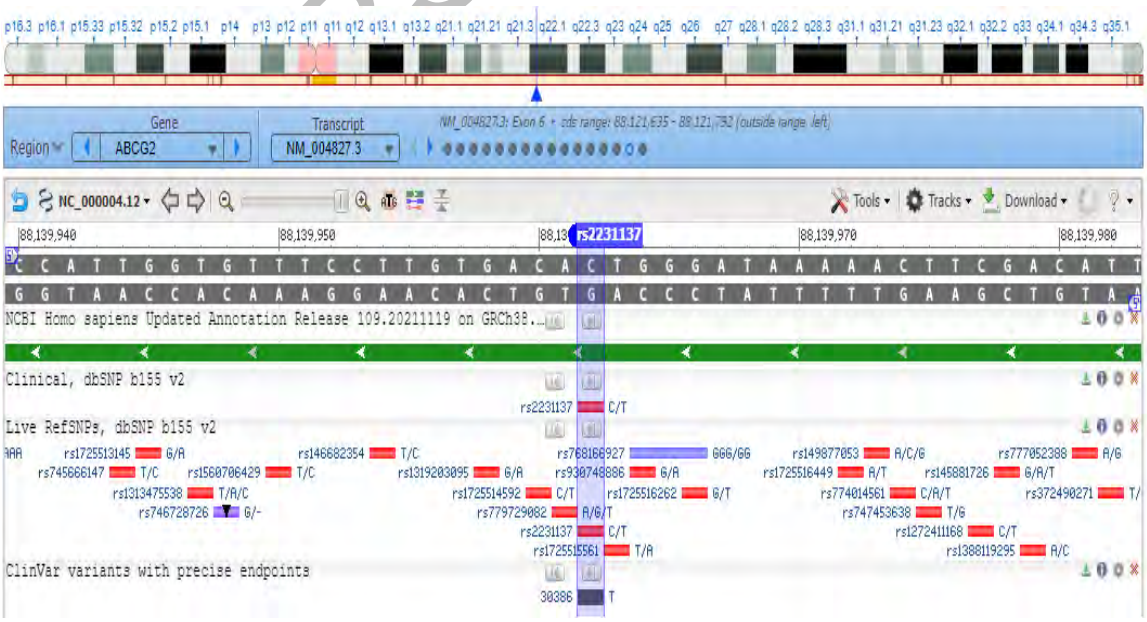


Figure 40. The genomic location of rs2231137 mapped on NCBI website (VarView)

Another novel **disease-causing mutation** was evident at position g.91474G>A that did not affect the amino acid sequence, leaving normal protein structure.

There was also found a novel **frameshift mutation** at genomic position g.91482delinsAA where events of deletion followed by insertion were evident. The amino acid chain was shifted with substitution of phenylalanine codon to stop codon at position 52 in protein structure. This frameshift mutation was predicted as novel disease-causing mutation as it has been not reported previously and may hinder with the normal function of ABCG2 protein.

- ***Genetic variations in sample AF58***

Following analysis of worker sample AF58, multiple polymorphisms and disease-causing mutation were observed at genomic positions g.91410T>A, g.91414G>A, g.91416C>A, g.91419T>A, g.91427G>A, g.91436T>A, g.91450C>T, g.91454T>A, g.91456C>A, g.91459T>A, g.91461G>A (rs142634180), and g.91463G>A.

The first novel **polymorphism** was seen at position g.91410T>A which brought AA structural changes from leucine to glutamine at AA28.

Some other novel polymorphisms were detected at positions; g.91416C>A that converted alanine to glutamic acid at 30th AA position; g.91419T>A that converted phenylalanine to tyrosine at AA31; g.91427G>A where protein structure of ABCG2 was altered at position 34 from glycine to arginine; g.91436T>A that changed amino acid sequence of ABCG2 protein at position 37 from leucine to isoleucine; and g.91454T>A where AA change at position 43 was observed with substitution of cysteine with serine.

All the above-mentioned polymorphisms were novel except g.91461G>A (rs142634180) that has been reported before and has assigned rsID, it presents a single

nucleotide substitution which brings protein change from arginine to glutamine at position 45 in protein structure (R45Q). According to dbSNP, the genomic location for present variation is mapped on NCBI website as shown in figure 41.

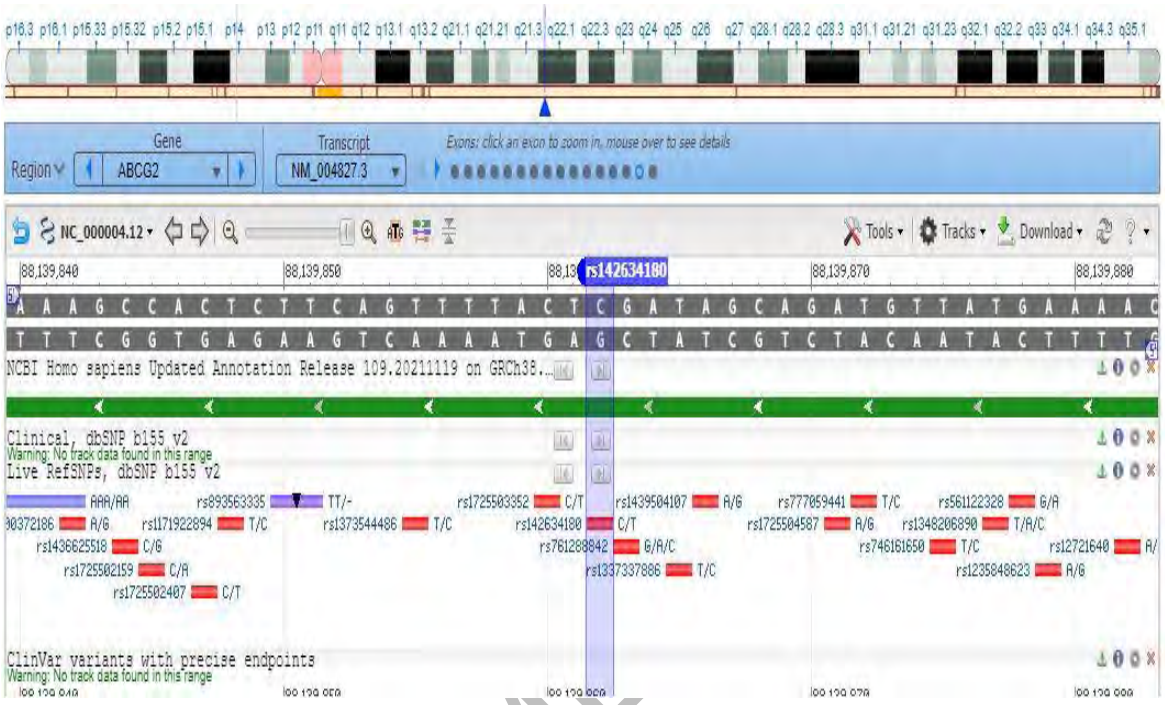


Figure 41. The genomic location of rs142634180 mapped on NCBI website (VarView)

Novel **disease-causing mutations** in sample AF58 were also found at many positions, these are g.91414G>A and g.91450C>T, where single substitution of G>A and C>T occurred respectively, however, no change in AA sequence was present and normal protein was produced.

Similarly, other disease-causing mutations that altered AA structure of ABCG2 protein were g.91456C>A that replaced cysteine at position 43 with stop codon, and g.91459T>A that also shifted AA sequence from tyrosine to stop codon at AA position 44.

Another disease-causing change that occurred at genomic position g.91463G>A was a single nucleotide substitution that affected AA sequence in ABCG2 protein replacing valine to isoleucine at position 46.

- ***Genetic variants in sample AF59***

Three novel **polymorphisms** were observed in worker sample AF59, present at genomic positions g.91416C>A, g.91427G>C, and g.91430G>C where single substitution of nucleotide in coding sequence of gene were identified. At g.91416C>A, AA sequence at position 30 was shifted from alanine to glutamic acid, while at g.91427G>C and g.91430G>C, amino acids in protein structure were replaced at position 34 from glycine to arginine and at position 35, from alanine to proline.

A novel **disease-causing mutation** was also observed at position g.91440_91441delinsAA where deletion and insertion in nucleotide sequence shifted AA sequence as well from serine to lysine at AA position 38.

- ***Genetic variations in sample BF16***

Single nucleotide polymorphisms and disease-causing mutations were also found in control samples as shown in table 23 and 25.

The sequence analysis of sample BF16 showed presence of two novel **disease-causing mutations**; the first one was present at genomic position g.91474G>A, while the other was present at g.91507G>A, where a single substitution at both spots did not bring protein structural change through AA substitution.

Two novel **polymorphisms** were also seen at position g.91487C>A, where proline was replaced with threonine at position 54, and g.91521C>T, where serine was substituted

with leucine at position 65, this change has been reported before but no rsID has been assigned yet.

- ***Genetic variations in sample BF50***

Analysis of another control sample BF50 revealed presence of four novel single nucleotide **polymorphism** at genomic positions g.91346G>C, g.91416C>A, g.91430G>A, and g.80676T>A.

The first three polymorphisms g.91346G>C, g.91416C>A, g.91430G>A, brought changes in AA sequence as glutamic acid was replaced with glutamine at AA7, alanine with glutamic acid at AA30 and alanine with threonine at AA35.

Another novel polymorphism was seen at genomic position g.80676T>A that was present in intronic region, however whether this change brings any AA structural change is not known.

A **disease-causing mutation** was seen at position g.91446A>T where a single base exchange caused replacement of histidine with leucine at position 40.

Following figure (42) can be memorized to identify the amino acid and protein structural changes in DNA sequences of control and worker samples.

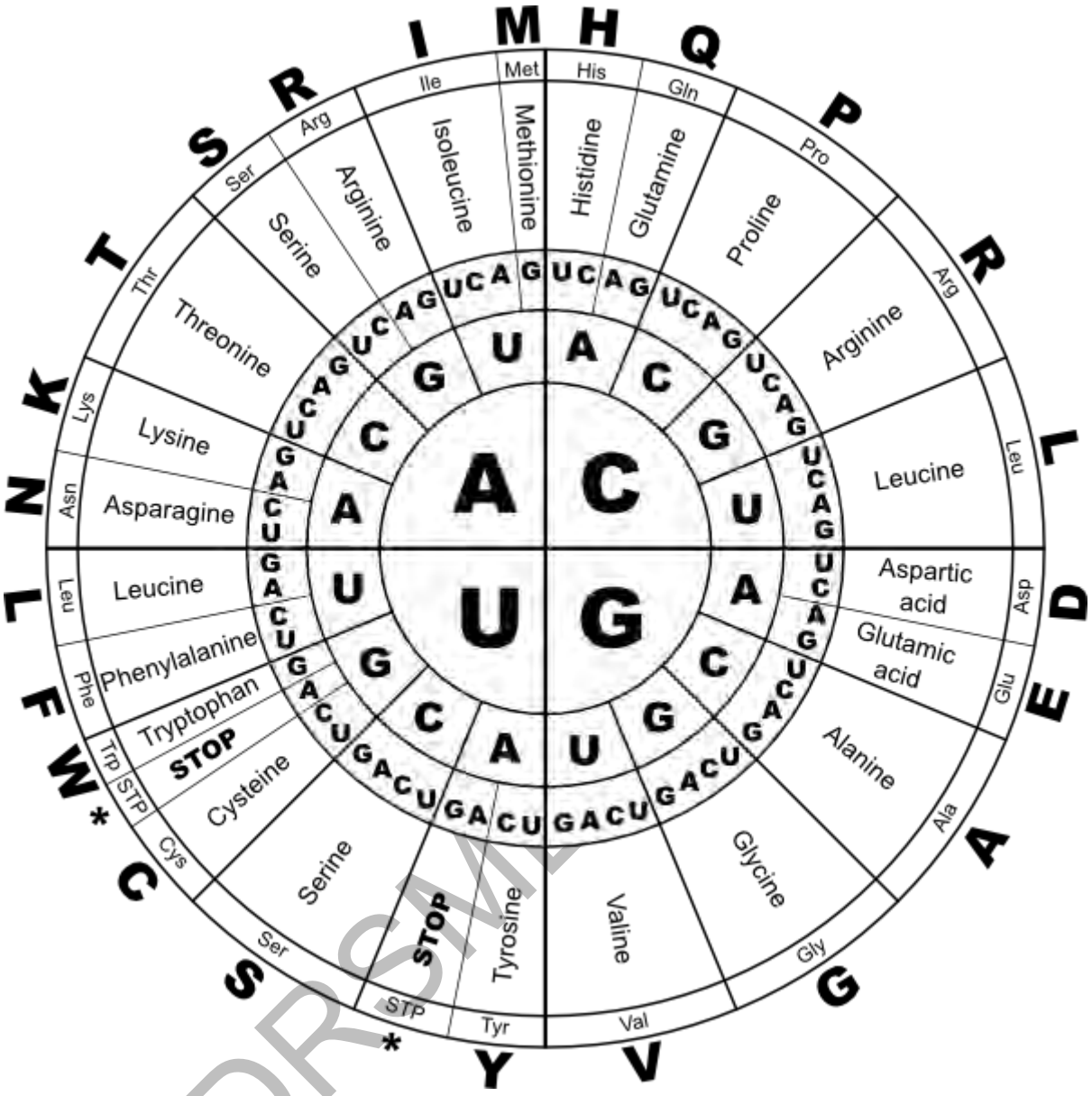


Figure 42. Figure showing abbreviations for amino acids used to identify protein changes.

Table 23. Table showing genomic DNA changes, physical location, alteration area and type, and mutation prediction observed in DNA sequence of ABCG2 gene in workers samples.

Sample ID	rsID	Change	Physical location	cDNA change	g. DNA	Alteration type	Protein structure change	Alteration area	Alteration name	Mutation prediction
AF2	rs2231137	G>A	Chr4:89061114C>T	c.34G>A	g.91361G>A	Single base pair change	V12M	CDS SSC	Substitution	Polymorphism
	Novel	G>A	Chr4:89061001C>T	c.147G>A	g.91474G>A	Single base pair change	No AA change	CDS SSC	Substitution	Disease Causing
	Novel	T>A ->A Insertion	chr4:89060993delinsTT	c.155delinsAA	g.91482delinsAA	Deletion and insertion	F52*	CDS SSC	Frameshift mutation	Disease Causing

AF58	Novel	T>A	Chr:89061065A>T	c.83T>A	g.91410T>A	Single base pair change	L28Q	CDS SSC	Substitution	Polymorphism
	Novel	G>A	chr4:89061061C>T	c.87G>A	g.91414G>A	single base exchange	No AA changed	CDS SSC	Substitution	Disease Causing
	Novel	C>A	chr4:89061059G>T	c.89C>A	g.91416C>A	single base exchange	A30E	CDS SSC	Substitution	Polymorphism
	Novel	T>A	chr4:89061056A>T	c.92T>A	g.91419T>A	single base exchange	F31Y	CDS SSC	Substitution	Polymorphism
	Novel	G>A	chr4:89061048C>T	c.100G>A	g.91427G>A	single base exchange	G34R	CDS SSC	Substitution	Polymorphism

	Novel	T>A	chr4:8906 1039A>T	c.109T> A	g.91436T >A	single base exchange	L37I	CDS SSC	Substitution	Polymorp hism
	Novel	C>T	chr4:8906 1025G>A	c.123C> T	g.91450C >T	single base exchange	No AA change	CDS SSC	Substitution	Disease Causing
	Novel	T>A	chr4:8906 1021A>T	c.127T> A	g.91454T >A	single base exchange	C43S	CDS SSC	Substitution	Polymorp hism
	Novel	C>A	chr4:8906 1019G>T	c.129C> A	g.91456C >A	single base exchange	C43*	CDS SSC	Substitution	Disease Causing

	Novel	T>A	chr4:8906 1016A>T	c.132T> A	g.91459T >A	single base exchange	Y44*	CDS SSC	Substitution	Disease Causing
	rs142 63418 0	G>A	chr4:8906 1014C>T	c.134G> A	g.91461G >A	single base exchange	R45Q	CDS SSC	Substitution	Polymorp hism
	Novel	G>A	chr4:8906 1012C>T	c.136G> A	g.91463G >A	single base exchange	V46I	CDS SSC	Substitution	Disease Causing
AF59	Novel	C>A	chr4:8906 1059G>T	c.89C>A	g.91416C >A	single base exchange	A30E	CDS SSC	Substitution	Polymorp hism
	Novel	G>C	chr4:8906 1048C>G	c.100G> C	g.91427G >C	single base exchange	G34R	CDS SSC	Substitution	Polymorp hism

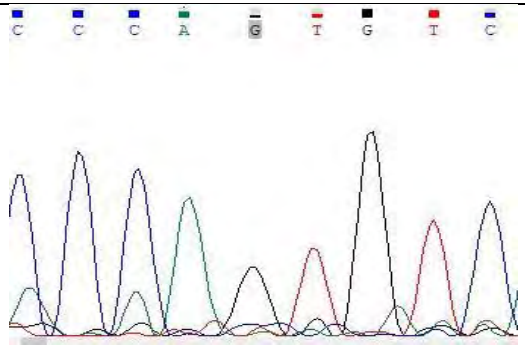
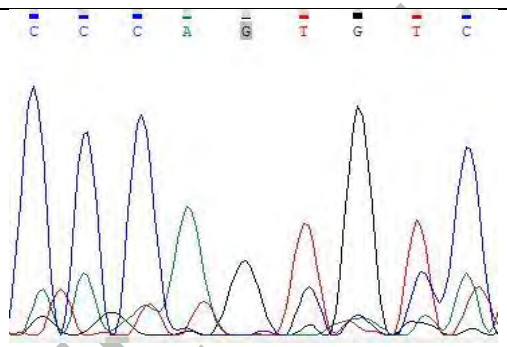
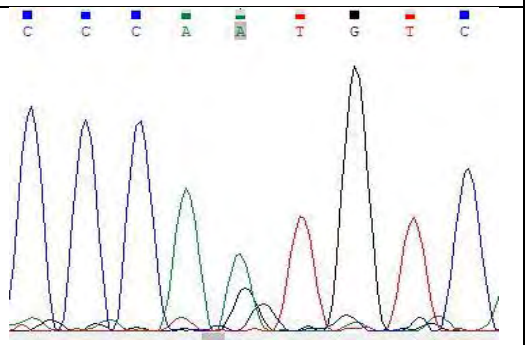
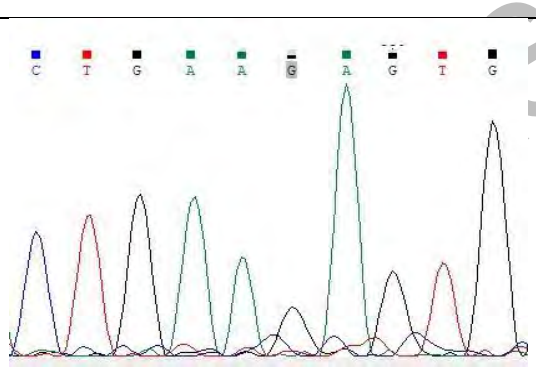
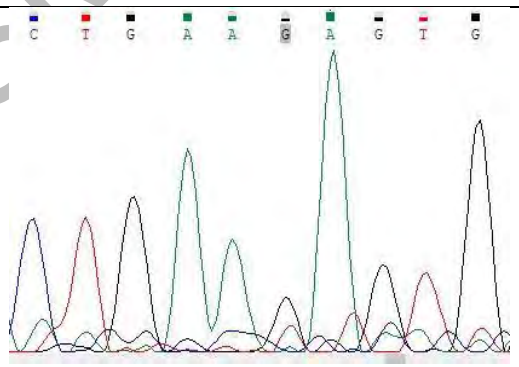
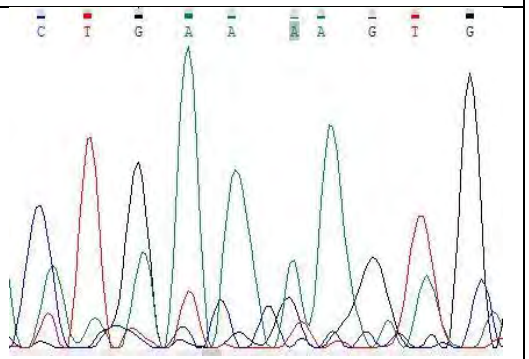
	Novel	G>C	chr4:8906 1045C>G	c.103G> C	g.91430G >C	single base exchange	A35P	CDS SSC	Substitution	Polymorp hism
	Novel	G>A T>A	chr4:8906 1034_890 61035deli nsTT	c.113_11 4delinsA A	g.91440_ 91441deli nsAA	Deletion and insertion	S38K	CDS SSC	Substitution	Disease Causing
BF 16	Novel	G>A	chr4:8906 1001C>T	c.147G> A	g.91474G >A	single base exchange	No AA change	CDS SSC	Substitution	Disease Causing
	Novel	C>A	chr4:8906 0988G>T	c.160C> A	g.91487C >A	single base exchange	P54T	CDS SSC	Substitution	Polymorp hism

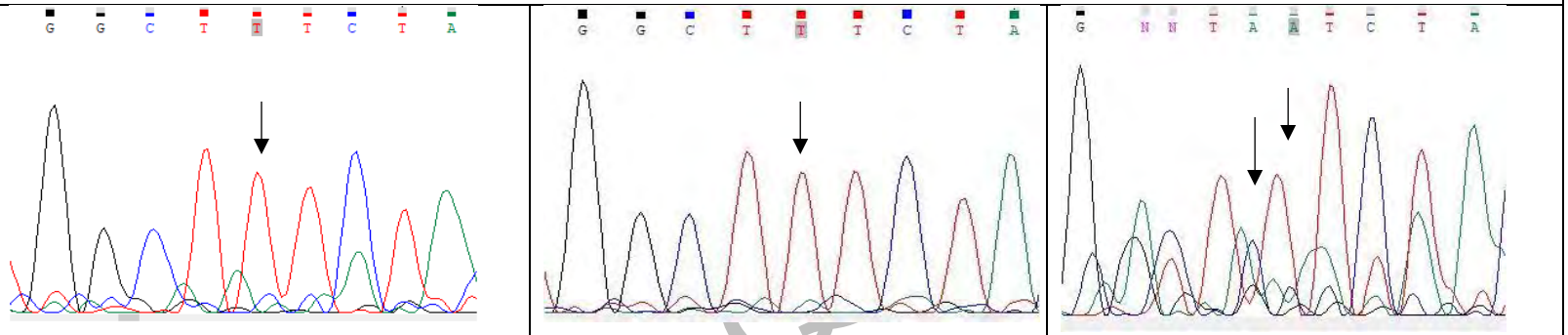
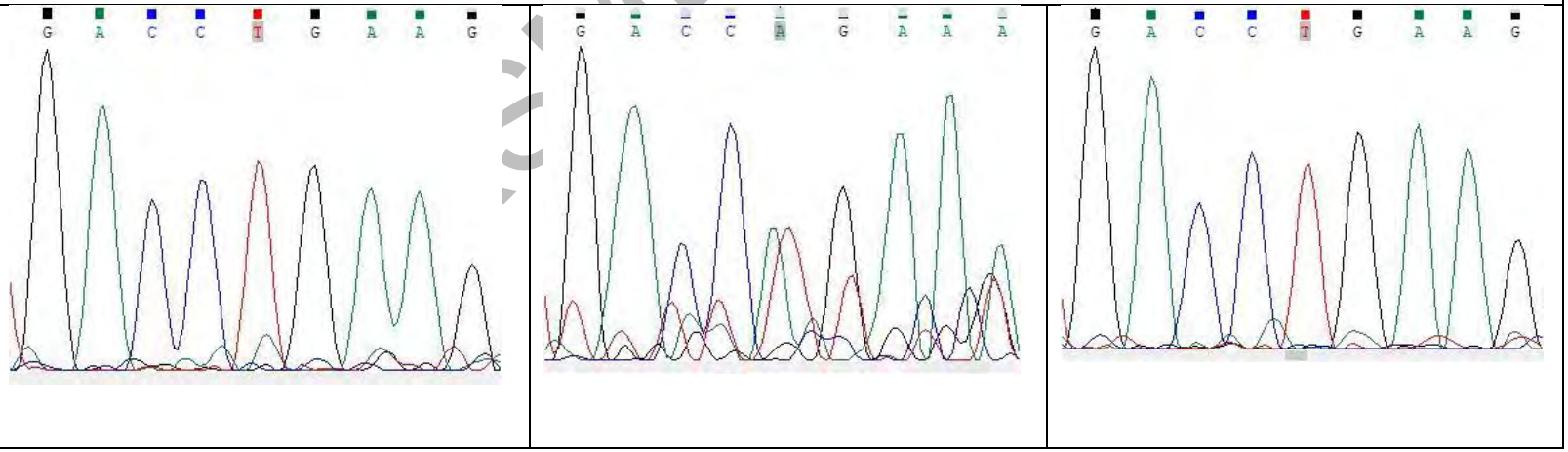
	Novel	G>A	chr4:8906 0968C>T	c.180G> A	g.91507G >A	single base exchange	No AA change	CDS SSC	Substitution	Disease Causing
	Repor ted but no rsID	C>T	chr4:8906 0954G>A	c.194C> T	g.91521C >T	single base exchange	S65L	CDS SSC	Substitution	Polymorp hism
BF 50	Novel	G>C	chr4:8906 1129C>G	c.19G>C	g.91346G >C	single base exchange	E7Q	CDS SSC	Substitution	Polymorp hism
	Novel	C>A	chr4:8906 1059G>T	c.89C>A	g.91416C >A	single base exchange	A30E	CDSSS C	Substitution	Polymorp hism

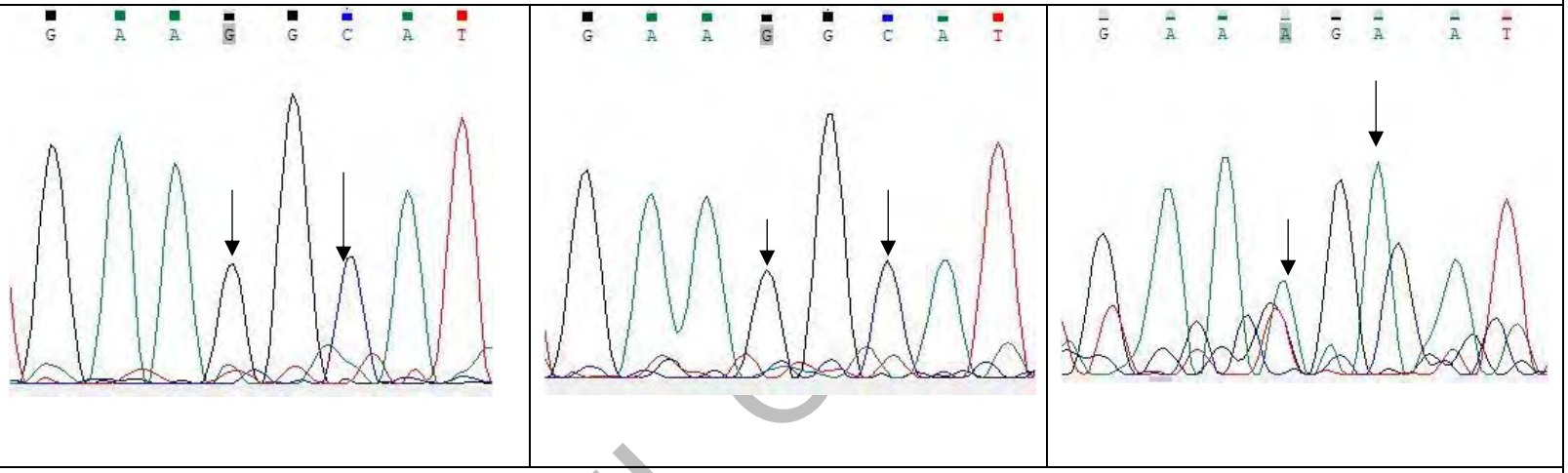
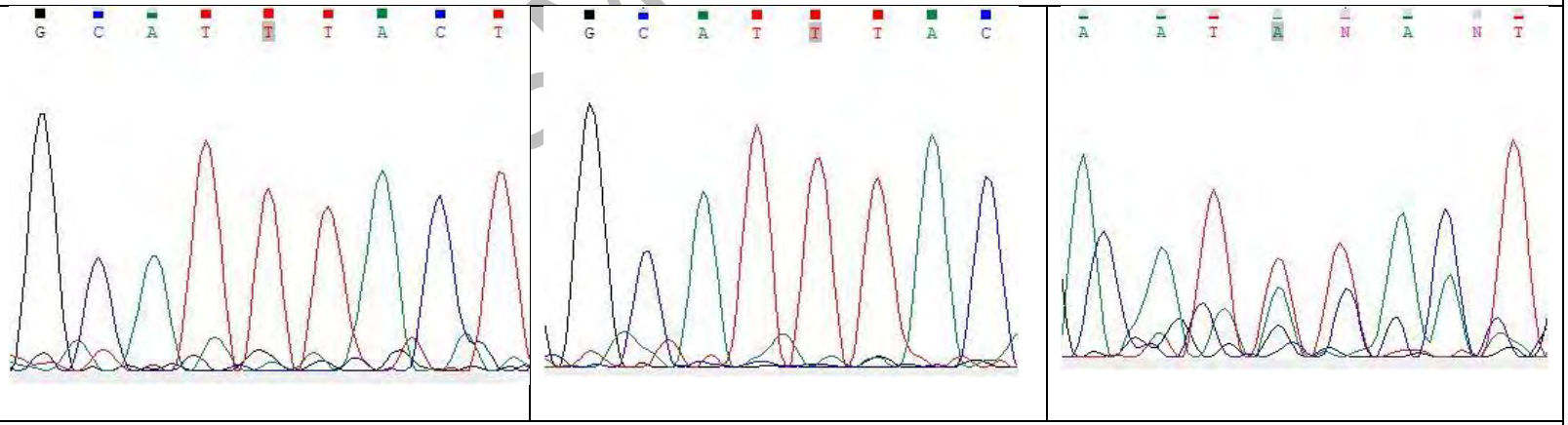
	Novel	G>A	chr4:8906 1045C>T	c.103G> A	g.91430G >A	single base exchange	A35T	CDS SSC	Substitution	Polymorp hism
	Novel	T>A	chr4:8907 1799A>T	N.A	g.80676T >A	single base exchange	N.A	Intron	Substitution	Polymorp hism
	Novel	T>A	chr4:8906 1029T>A	c.119A> T	g.91446A >T	single base exchange	H40L	CDS SSC	Substitution	Disease Causing

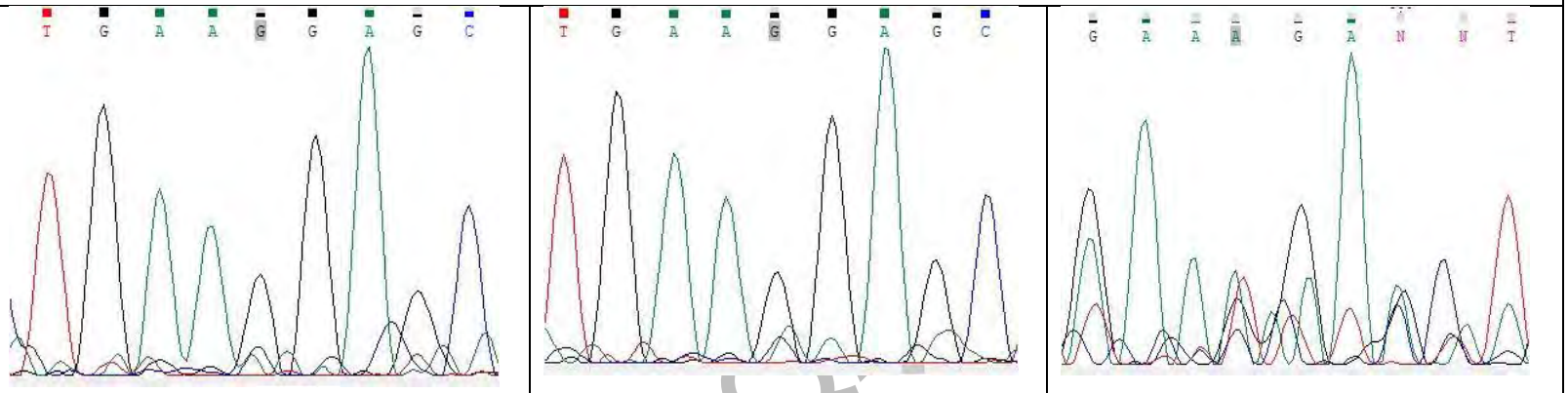
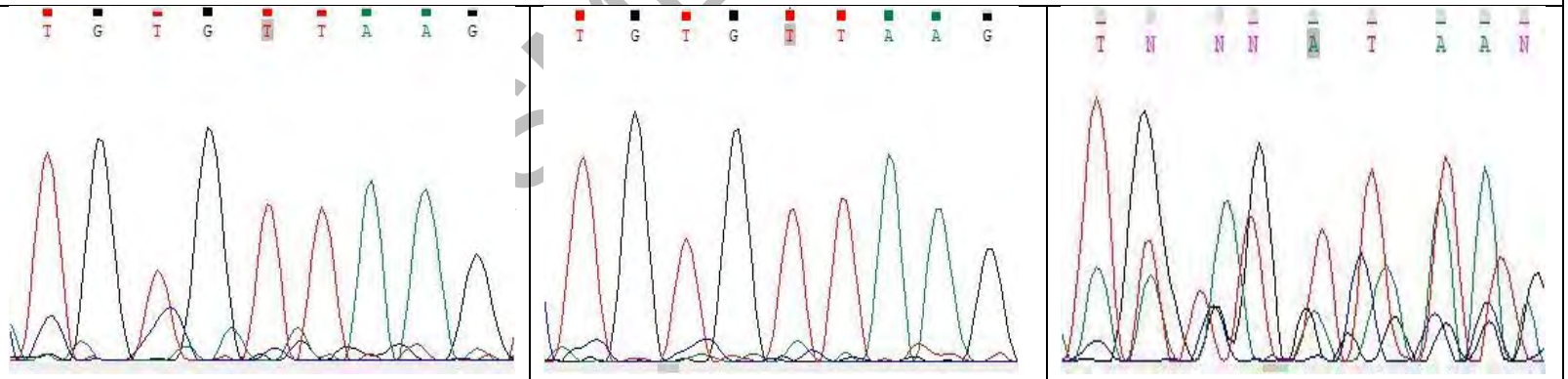
CDS-coding sequence; N.A- not available; SSC- Splice site change

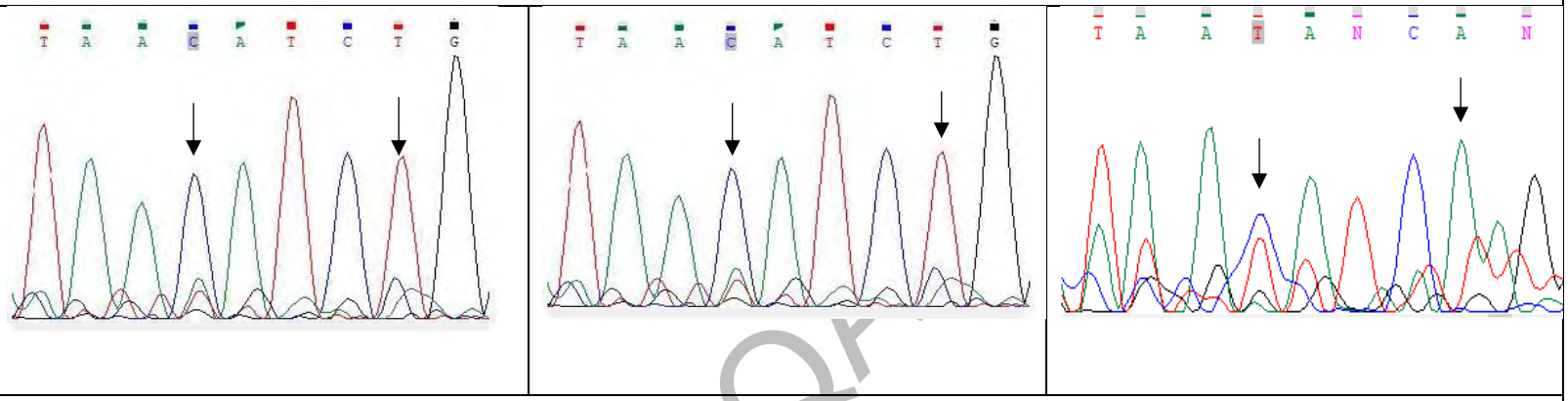
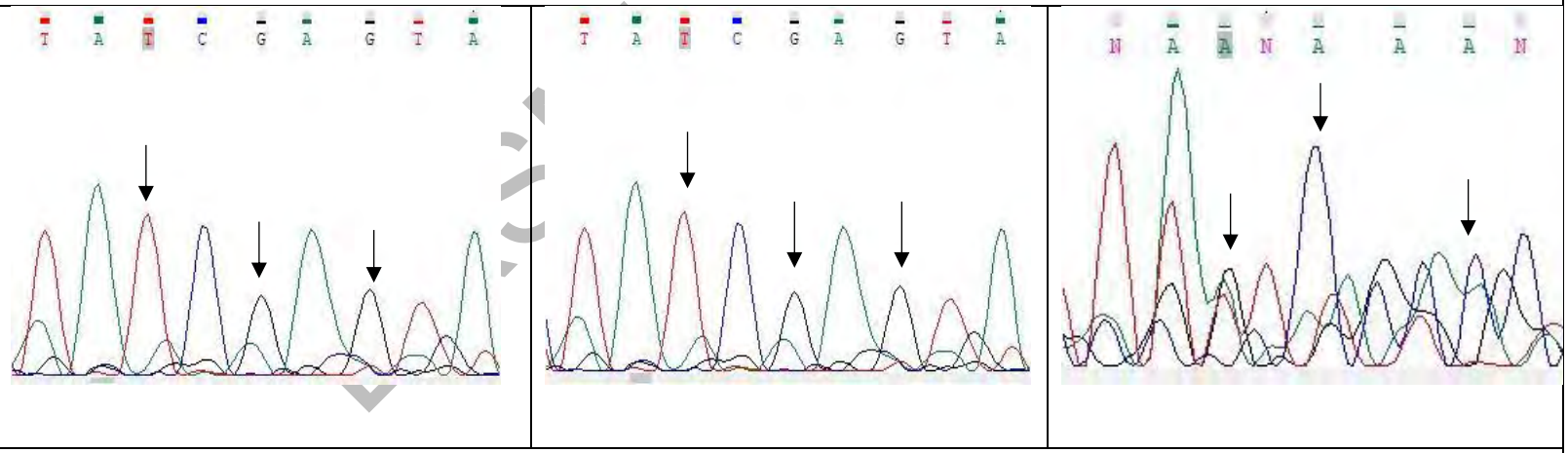
Table 24. Table showing single nucleotide polymorphisms (SNPs) and their genomic locations in worker samples.

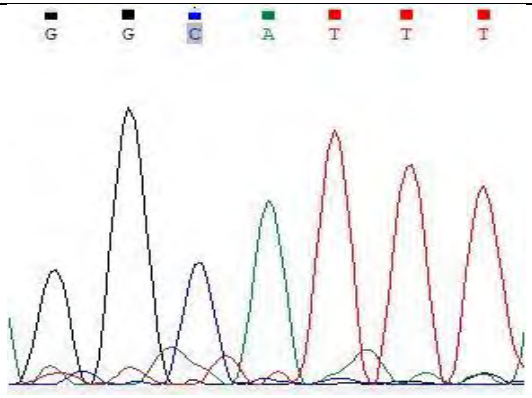
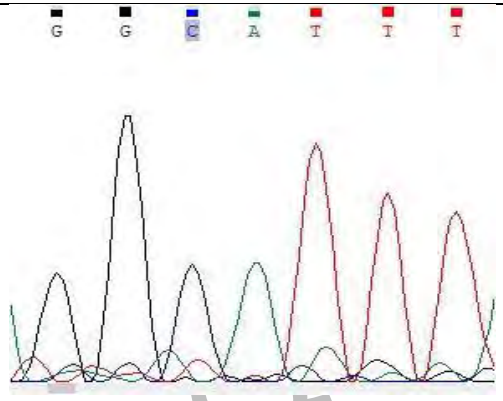
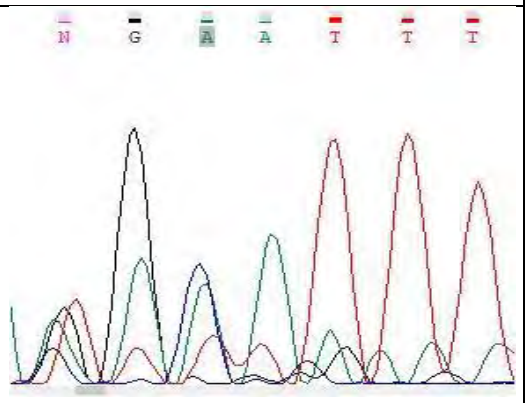
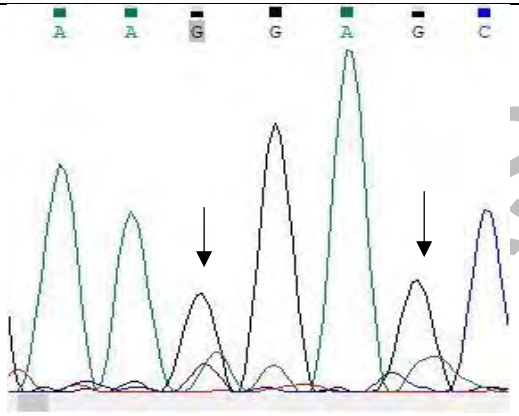
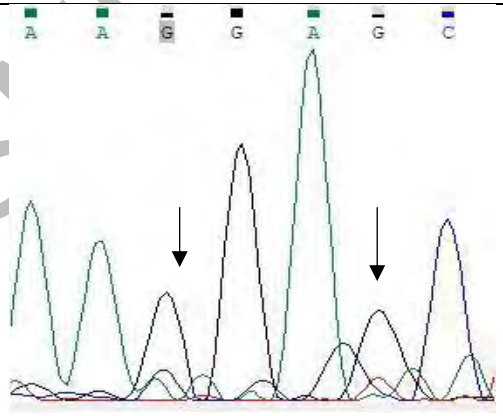
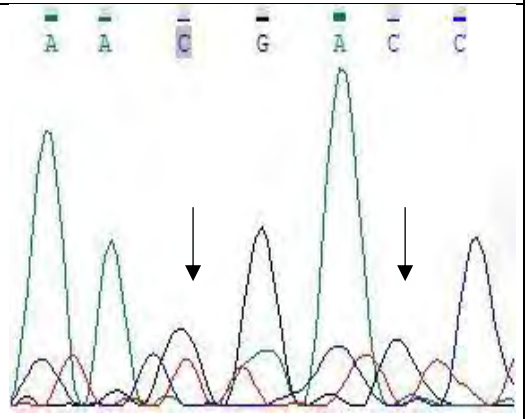
Sample ID	Wild type	Control	Worker
AF2	g.91361G>A		
			
	g.91474G>A		
			

	g.91482delinsAA
	
AF58	**g.91410T>A**
	

	g.91414G>A, g.91416C>A
	
	g.91419T>A
	

	g.91427G>A
	
	g.91436T>A
	

	g.91450C>T, g.91454T>A
	
	g.91459T>A, g.91461G>A, g.91463G>A
	

AF59	**g.91416C>A**		
			
	g.91427G>C, g.91430G>C		
			

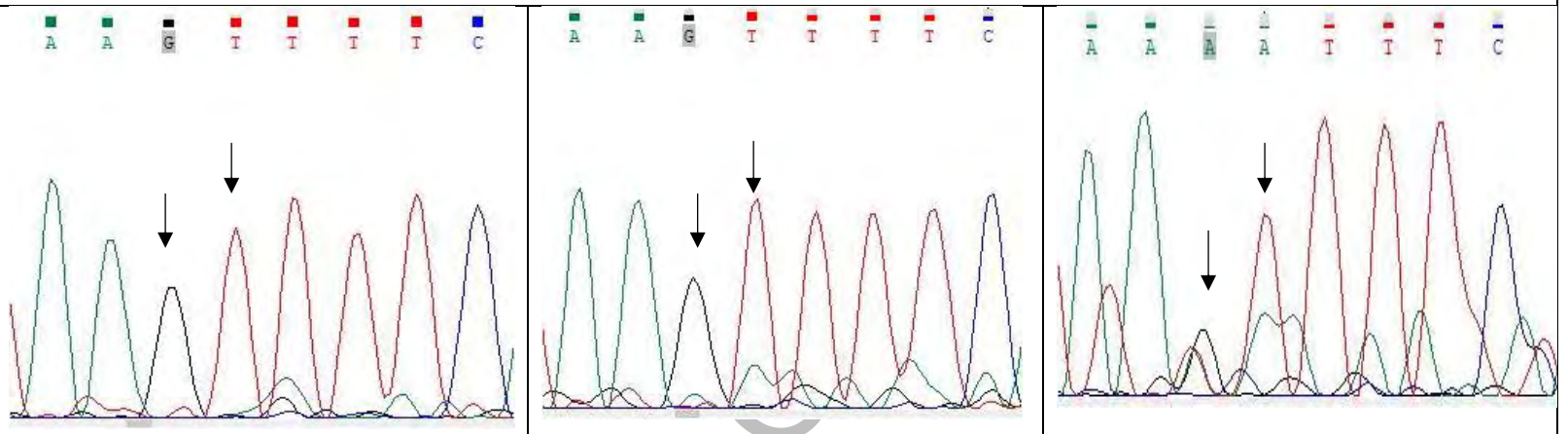
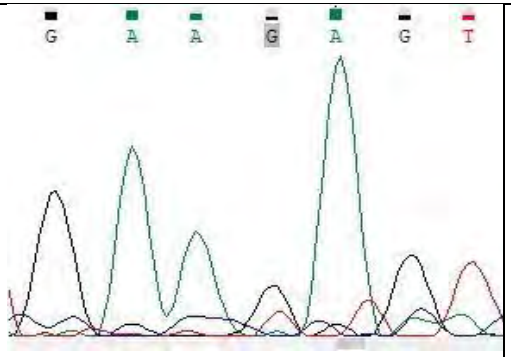
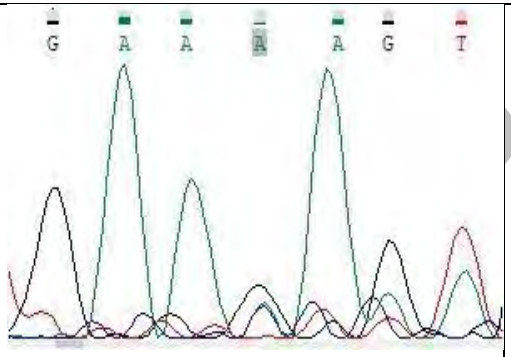
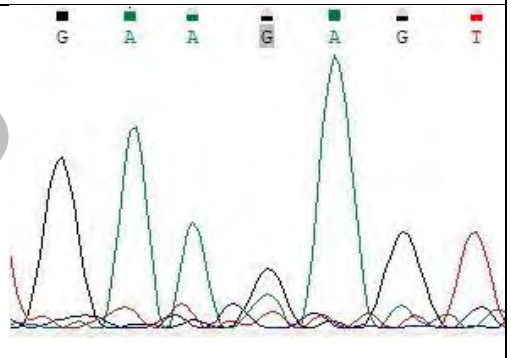
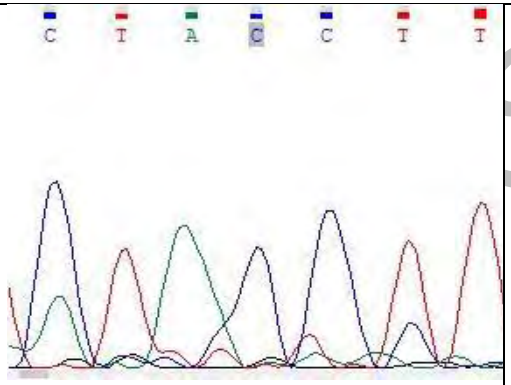
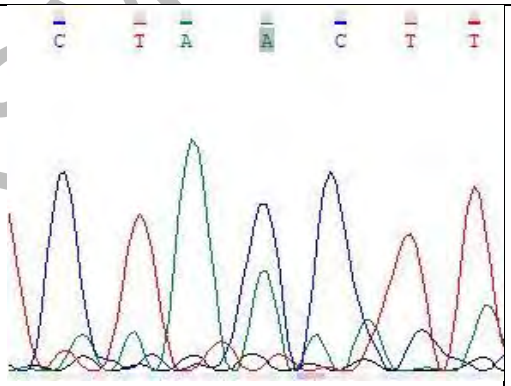
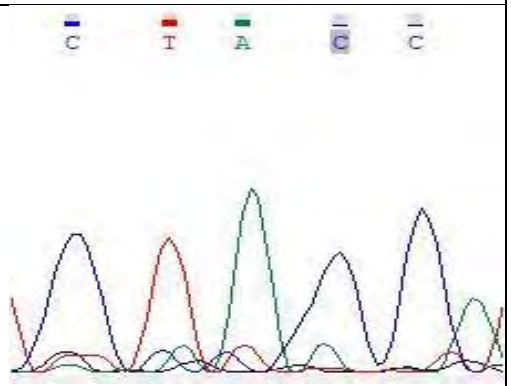
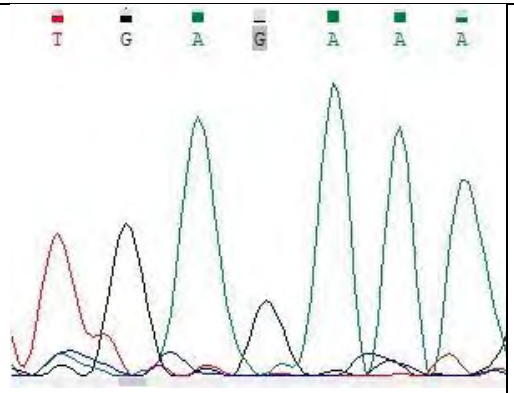
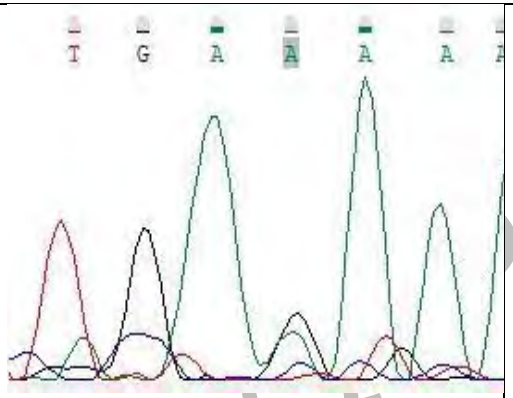
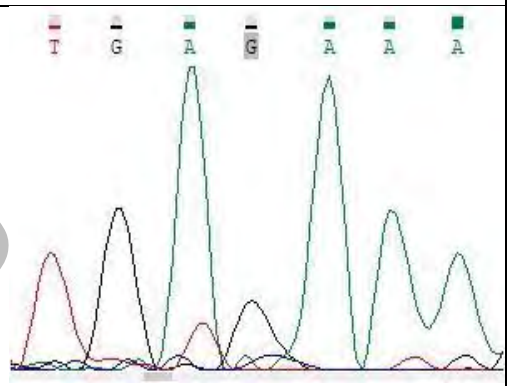
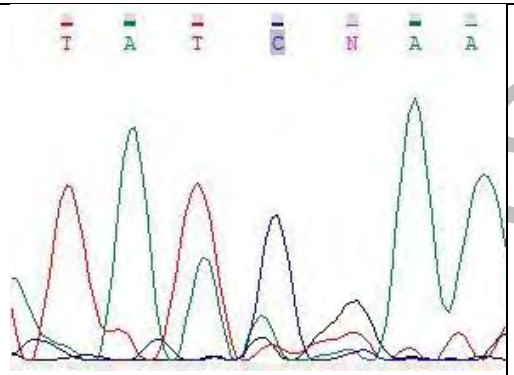
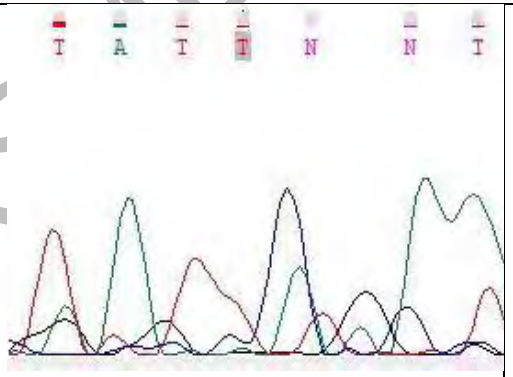
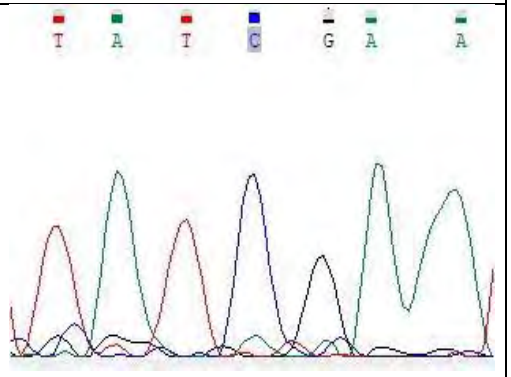
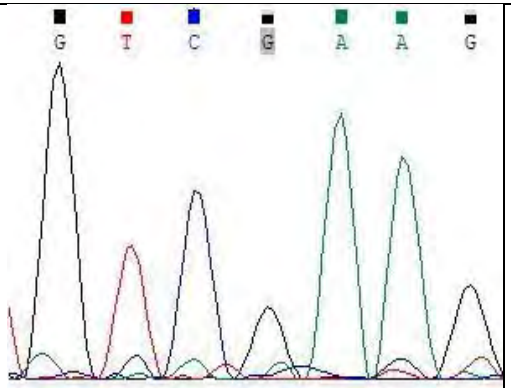
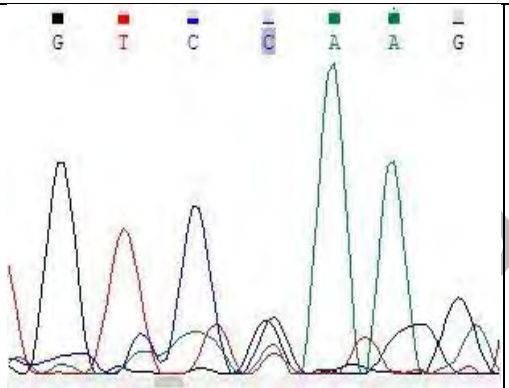
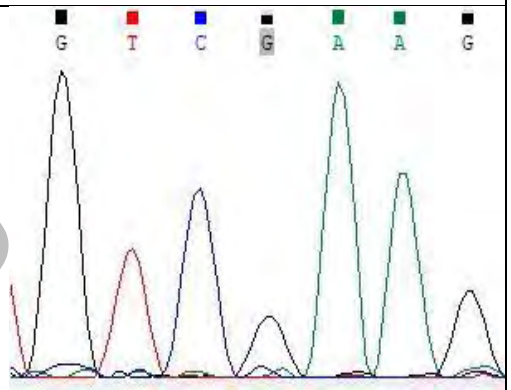
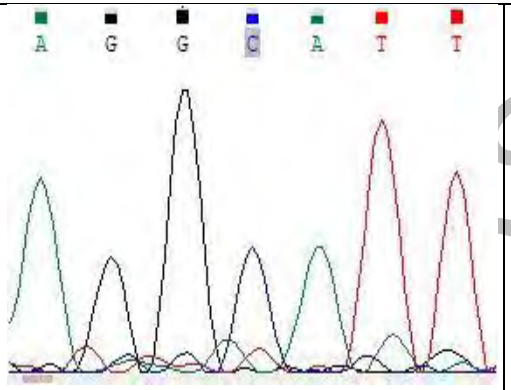
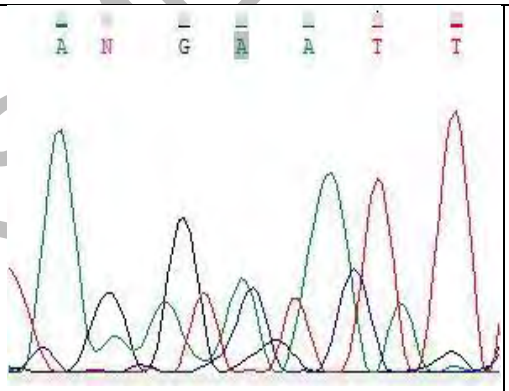
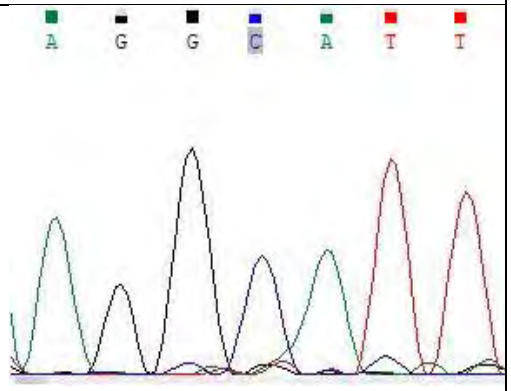
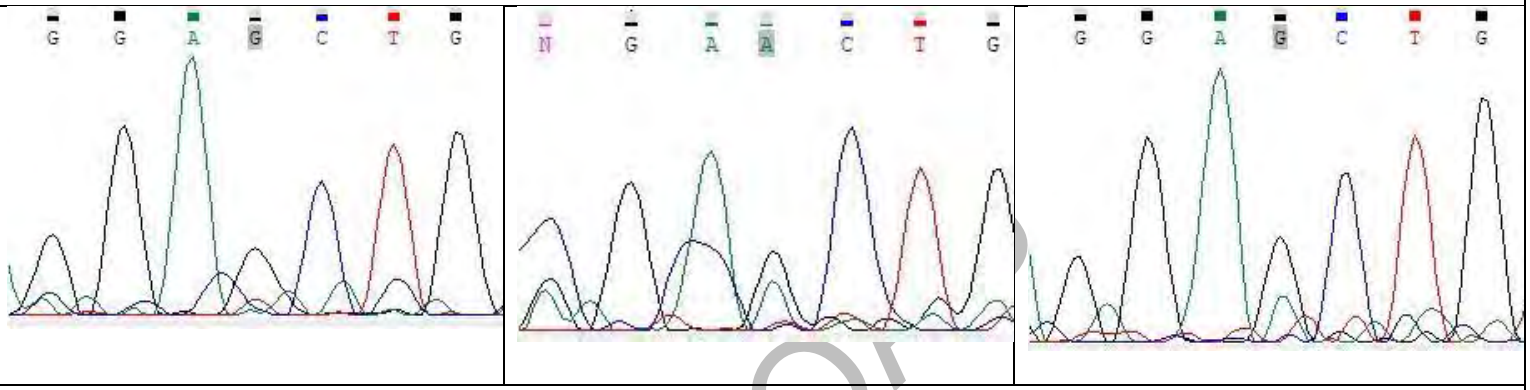
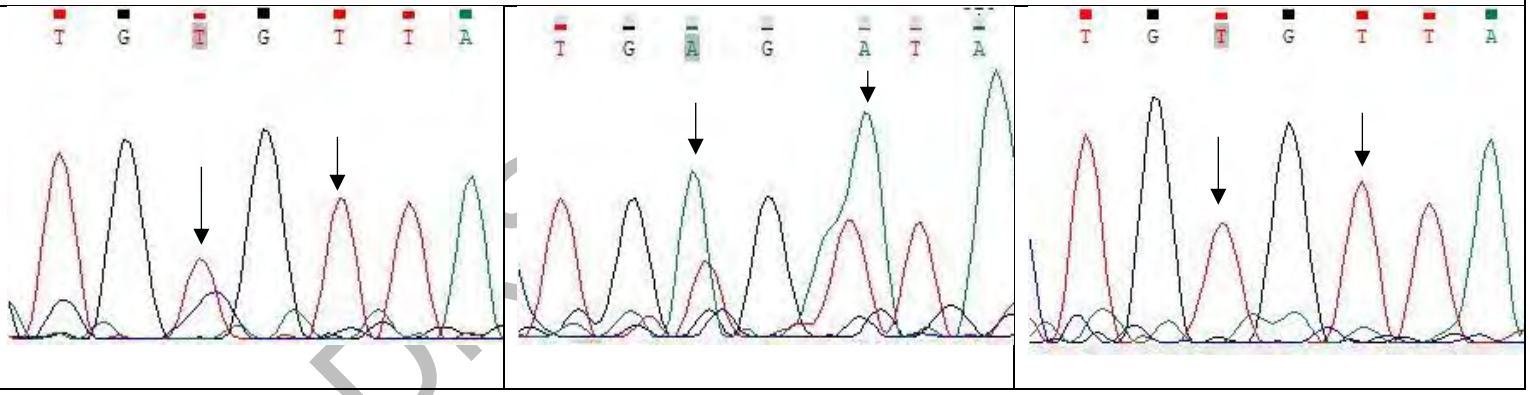
	g.91440_91441delinsAA
	

Table 25. Table showing single nucleotide polymorphisms (SNPs) and their genomic locations in control samples.

Sample ID	Wild type	Control	Worker Sample
BF16	**g.91474G>A**		
			
	g.91487C>A		
			

	g.91507G>A		
			
	g.91521C>T		
			

BF50	**g.91346G>C**		
			
	g.91416C>A		
			

	g.91430G>A
	
	g.80676T>A, g.91446A>T
	

DISCUSSION

Brick kilns release enormous amounts of metals into the atmosphere. Some of these metals are deposited in the human body and bring genotoxic reactions in cells, affecting gene function. In the present study, we found multiple disease-causing mutations and polymorphisms in two selected exons of the ABCG2 gene, which is a protein-coding gene. The novel variations in the current study were present in the coding and intronic region of the gene signifying their importance in determining the functional potency of ABCG2 protein. The membrane-associated protein encoded by this gene is included in the superfamily of ATP-binding cassette (ABC) transporters that transport various molecules across extra- and intra-cellular membranes. This protein functions as a xenobiotic transporter which may play a major role in multi-drug resistance (Imai *et al*., 2002). It is called a half transporter with a molecular weight of 72kDa. It contains six transmembrane domains and only one N-terminal binding domain (Alsanosi *et al*., 2014). It likely serves as a cellular defense mechanism in response to mitoxantrone and anthracycline exposure.

An important function of the ABCG2 transporter is the secretion of urate in the intestines, which is a byproduct of biochemical reactions that usually occur in the body (Matsuo *et al*., 2014). It functions as an antioxidant against free radicals in the bloodstream. However, genetic polymorphisms in ABCG2 expression disrupt their normal function, resulting in pathological conditions. Previous studies have found that an epistatic interaction pair of polycystin-2 (PKD2) and ABCG2 gene (rs2728121: rs2231137) is found among Chinese individuals; that is associated with increased urate levels since it acts as a urate transporter. This SNP pair is identified to influence the development of gout from both hyperuricemia and healthy individuals males (Dong *et al*., 2020). Another study showed that ABCG2 exhibit protective role against oxidative stress in colorectal cancer. ABCG2

mediates its actions by inhibition of expression of inflammatory genes and reducing oxidative stress (Nie *et al*., 2018). Former studies also reported that toxicity via chemotherapy induces genetic polymorphisms in cells, bringing adverse actions. The found SNP rs2231137 is also associated with ischemic stroke, as testified in Chinese population (Liu *et al*., 2018). Further, literature review suggests that polymorphisms in different regions of the ABCG2 gene are associated with ischemic strokes, hyperuricemia, and gout (Dong *et al*., 2020; Liu *et al*., 2018; Yang *et al*., 2021).

Although this gene is expressed in BBB, liver, and intestine as well, however, significant expression of this protein has been observed in the placenta, which may suggest a potential role for this molecule in placenta tissue (Mao, 2008; To *et al*., 2020). It has been reported that ABCG2 protein expresses on the outer membrane of syncytiotrophoblast facing maternal blood, therefore it acts as an efflux transporter. It functions to move hazardous compounds away from the fetal compartment to the maternal circulation (Karttunen *et al*., 2017). Multiple variants encoding different isoforms have been found for this gene in the intestine and placenta (Kobayashi *et al*., 2005; Zamber *et al*., 2003). The polymorphisms in ABCG2 gene expression that result in inhibition of these transporters might bring feto-toxicity in human placenta (Karttunen *et al*., 2017).

The first single nucleotide variation was found in the brick kiln worker sample at genomic position g.91361G>A where a single substitution of G with A was present, which resulted in a replacement of valine (V) with methionine (M) shift at position 12 in the protein structure of ABCG2 protein. This observed change presents a missense variation that brings alleles to change from C>T and is a naturally occurring nonsynonymous SNP (Itoda *et al*., 2003). This variation has been reported in humans 43 times and assigned reference ID as rs2231137. This variant (V12M) has been studied worldwide and its high prevalence has

been reported in South and East Asia, America, Europe, Japan and Africa (Bäckström *et al*., 2003; Honjo *et al*., 2002; Iida *et al*., 2002; Zamber *et al*., 2003). As ABCG2 acts as a G protein coupled receptor, analysis of protein structure shows that Val12Met (G>A) is present near amino terminal of ABCG2 receptor protein within the cell. Previous literature suggested that the novel ABCG2 mutation (ABCG2-M71V) is also involved in the removal of uric acid, causing build-up in serum uric acid levels resulting in development of gout. Studies have indicated that polymorphism that result in variant rs2231137 is linked with decrease in protein expression of ABCG2 in vitro as well as altered substrate specificity (Maekawa *et al*., 2006). The rs2231137 variant (G>A) has been recently reported in patients with esophageal squamous cell carcinoma, exposed to chemotherapy radiation (Yang *et al*., 2021). The study depicted that chemotherapy toxicity brings genetic polymorphisms in cells, bringing adverse actions. The mentioned SNP rs2231137 is also associated with ischemic stroke, seen in Chinese population (Liu *et al*., 2018). Other studies found that an epistatic interaction pair of polycystin-2 (PKD2) and ABCG2 gene (rs2728121: rs2231137) is found among Chinese individuals; that is associated with urate levels, because ABCG2 gene acts as a urate transporter. This SNP pair is identified to influence the development of gout from both hyperuricemia and healthy individuals males (Dong *et al*., 2020).

Two more novel variations were found in sample AF2, these included disease-causing mutation at position g.91474G>A that did not affected the amino acid sequence, leaving normal protein structure; and a novel frameshift mutation (F52*) at genomic position g.91482delinsAA, where events of deletion followed by insertion were evident. The amino acid chain was shifted with the substitution of phenylalanine codon to stop codon at position 52 in protein structure, that may hinder with the normal function of ABCG2 protein.

Analysis of worker sample AF58 depicted 11 novel polymorphisms at multiple genomic positions. One of the above-mentioned polymorphisms has been reported earlier at g.91461G>A and has assigned rsID as rs142634180, it presents a single nucleotide substitution which brings protein change from arginine to glutamine at position 45 in protein structure (R45Q). This variant (rs142634180) of ABCG2 is a missense variant and has been cited nowhere so far. Novel disease-causing mutations were also found at g.91414G>A and g.91450C>T, where single substitution of G>A and C>T occurred respectively, however, no change in AA sequence was present and normal protein was produced.

Three novel polymorphisms were seen in worker sample AF59, present at genomic positions g.91416C>A, g.91427G>C, and g.91430G>C where single substitution of nucleotide in coding sequence of gene were identified. A novel disease-causing mutation was also seen at position g.91440_91441delinsAA where deletion and insertion in nucleotide sequence shifted AA sequence as well from serine to lysine at AA position 38.

The sequence analysis of sample BF16 showed presence of two novel disease-causing mutations; at genomic position g.91474G>A and g.91507G>A, where a single substitution at both spots did not bring protein structural change through AA substitution. Two novel polymorphisms were also seen at position g.91487C>A and g.91521C>T. the variant g.91521C>T has been reported before but no rsID has been assigned yet.

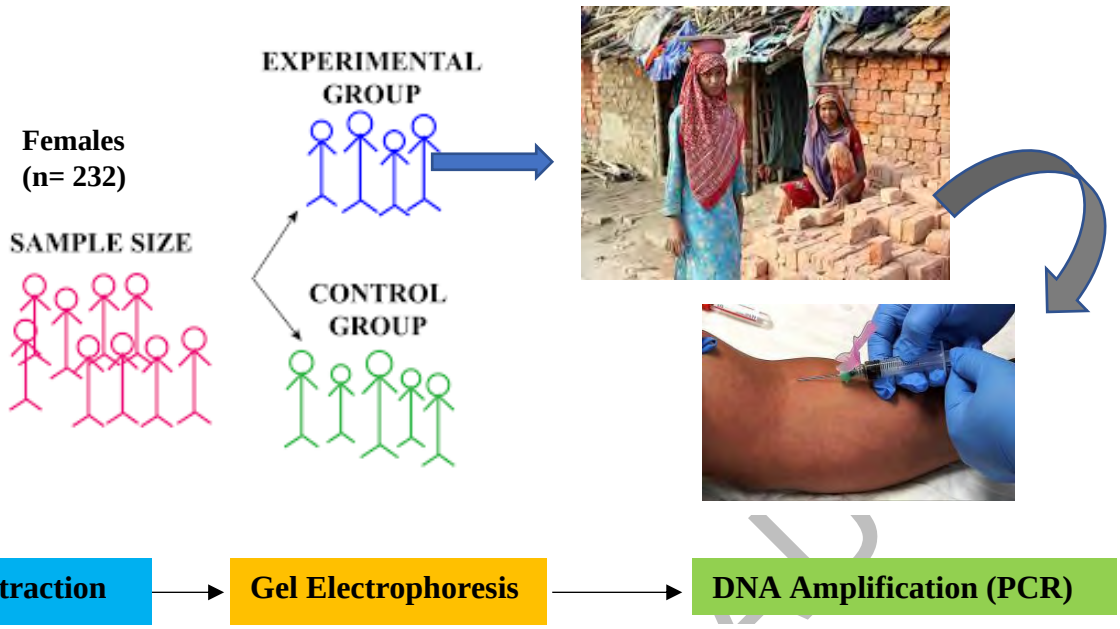
Analysis of another control sample AF50 revealed presence of four novel variants at g.91346G>C, g.91416C>A, g.91430G>A, and g.80676T>A. Another novel polymorphism was seen at genomic position g.80676T>A that was present in intronic region, however, whether this change brings any AA structural change is not known. A disease-causing mutation was seen at position g.91446A>T where a single base exchange caused replacement of histidine with leucine at position 40.

The study summed up that ABCG2 plays an important role in transport of multiple drugs, chemicals, and materials across the tissues especially placenta. The presence of polymorphisms in ABCG2 gene brings AA changes and may hinder the normal function of ABCG2 protein. Previous studies also reported ABCG2 polymorphisms, linked with development of risk for metabolic diseases such as gout, hyperuricemia, and other ischemic strokes. Our results found the presence of multiple single nucleotide polymorphisms and disease-causing mutations, that alter the function of ABCG2 gene and may affect the normal healthy pregnancy.

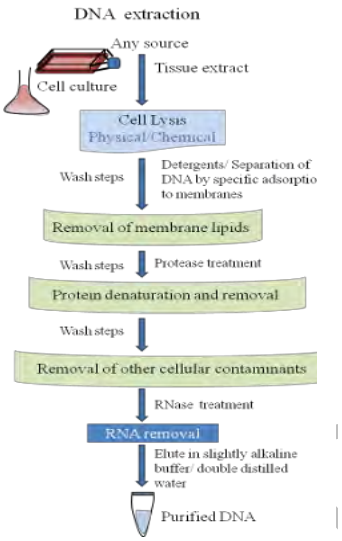
CONCLUSION

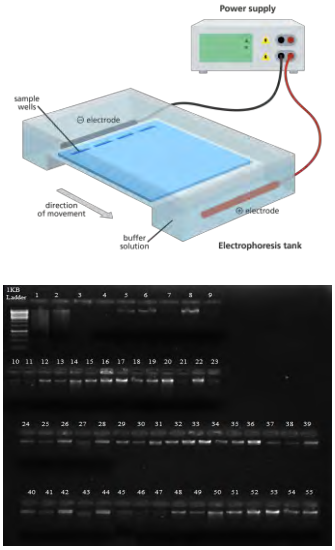
- The present study concluded that heavy metal burden in blood serves as one of the major factors in inducing genetic changes by creating SNPs in important structural and functional genes such as ABCG2 gene (which functions to protect developing fetus) in women, as depicted earlier in fertility analysis, where rate of miscarriage in brick kiln workers was greater than control.
- We found the presence of 28 novel SNPs in two studied exons of ABCG2 gene from brick kiln and control population, where most of the mutations were disease causing and others were polymorphisms.
- Therefore, it can be inferred from the present findings that occurrence of these polymorphisms might interfere with the normal function of ABCG2 protein expressed in placental tissue, bringing fetal exposure to multidrug metabolites and xenobiotics compounds and increasing chances of miscarriage.

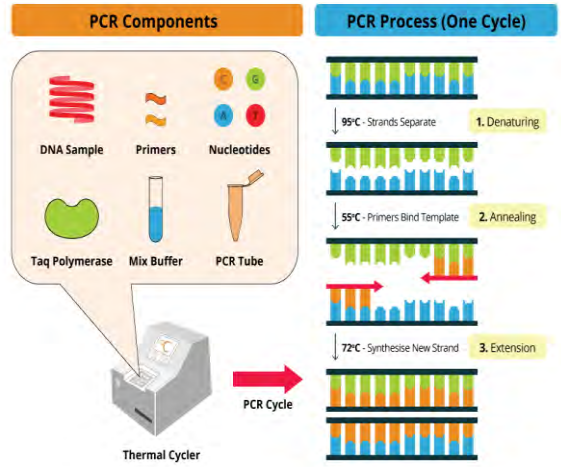
SUMMARY



DNA Extraction → **Gel Electrophoresis** → **DNA Amplification (PCR)**

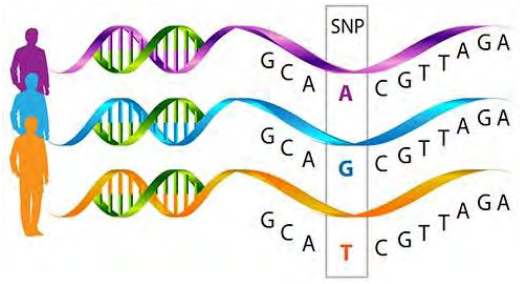


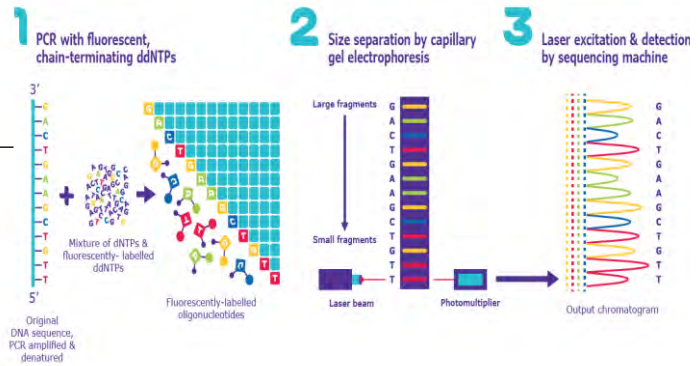




Identification of single nucleotide polymorphisms (SNPs) and disease-causing mutations

DNA sequencing through Sanger sequencing





We found 28 variants with 3 already reported and 25 novel single nucleotide polymorphisms (SNPs) and disease-causing mutations in workers and control samples

Chapter 5

A biochemical and hormonal approach to evaluate the heavy metal burden in blood of brick kiln children and monitor its possible effects on metabolism and puberty

ABSTRACT

In Pakistan, the number of children associated with brick kiln industry is quite high, that raises public health concerns. In present study, aim was set to monitor the socio-demographic determinants, blood profile, antioxidant status, oxidative stress response, content of DNA damage and growth outline of children working at brick kiln sites. A total of 275 children (n=175 brick kiln, n=100 control) including boys (control n=50, brick kiln workers n=85) and girls (control n=50, brick kiln workers n= 90) aged 4-18 years were included, from whom demographic data along with blood samples were gathered. Blood was subjected to hematological and genotoxic analysis while, blood plasma was used for the determination of biochemical and hormone assays. The results showed that all the brick kiln children were illiterate, weak and malnourished, and 72% of boys, while 71% of girls had underweight BMI. The findings presented a significant decrease in the RBC count ($p<0.05$) and percent hematocrit, significant increase in white blood cells ($p<0.05$), reactive oxygen species ($p<0.001$), and decrease in catalase ($p<0.001$), peroxidase ($p<0.001$) and sodium dismutase ($p<0.01$). There was observed a drop in GH levels from 1.66±0.08 ng/mL in control to 0.87±0.13ng/mL in brick kiln children. Similarly, increase in cortisol concentration from 0.83±0.14 ng/mL in control to 1.81 ± 0.05 ng/mL in brick kiln children was evident. The findings of comet assay showed a decrease in percent DNA in head region ($p<0.001$), and increase ($p<0.001$) in comet tail of brick kiln children than control. Conclusively, it is stated that children subjected to brick kiln emissions experience heavy metal burden in blood; altered blood profile; decreased antioxidant levels, and increased oxidant species production; augmented oxidative stress and reduced production of GH. Thus, it can be proposed that children residing at brick kiln sites experience poor hygiene; poor health conditions and growth spurt; and their exposure to industrial emissions may cause malfunctioning of reproductive axis in girls and boys.

INTRODUCTION

The production of bricks by human beings is traced back to 10,000 BCE (Zhang *et al*., 2018). Each year, the global production rate of bricks is 1500 billion; while continent Asia alone contributes the production of almost 1300 billion bricks with China, producing 1000 billion bricks that constitutes 66.67 percent of the world's brick production (Asia, 2017). Second, comes the India and then Pakistan. The production of bricks is crowded in less developed countries mostly. Different methods have been introduced for brick making. These include Along with other industries, such as vehicle exhaust, brick kiln industry contributes to environmental pollution for all life forms.

Brick kiln industry is the fastest growing industry in South Asia with increased number of bonded labor (Guttikunda *et al*., 2013). After China and India, Pakistan is the third largest brick producing country with more than 45 billion bricks produced per year (Saeed, 2017). Pakistan has around 20,000 brick kilns in different cities while, Punjab alone has 10,000 active brick kilns. Most of these kilns are of Fixed Chimney Bull's Trench Kiln type and 25 percent of Pakistani brick kilns operate throughout the year. In Pakistan, brick kiln industry depicts the increased prevalence of bonded labor (Ercelawn & Nauman, 2004). According to Labour and Human Resource Department, Government of the Punjab, approximately 87,134 families are associated with this profession (http://dashboards.urbanunit.gov.pk/brick_kiln_dashboard/). Child labor is a social issue around the globe and particularly, in Pakistan. An article reported that prevalence of child and bonded labor is quite common in Pakistan, despite being illegal. Whereas, according to Federal Bureau of Statistics survey conducted in 2004, it was found that around 90 percent of brick kiln workers in Punjab were bonded (Saeed, 2017). In the brick kiln industry, different roles are assigned based on the sex and age of the workers, for instance, immature/pubertal boys and girls usually mold and plough the kiln soil, and carry/

transport bricks, therefore, are exposed directly to polluted soil. In Punjab the total number of children aged 4-14 years, related to brick kiln is 126,779, of which 94,052 are school going while, 32,727 children do not attend the schools. Further, survey has shown that the total number of girls and boys labor at brick kiln is 57,679 and 69,100 (http://dashboards.urbanunit.gov.pk/brick_kiln_dashboard/). Due to such increased number of child labor working/living at brick kiln industry, the concerns for public and reproductive health risks are much increased among them.

A number of studies have reported that the brick kiln emissions are associated with multiple ill-health effects in humans (Jahan *et al*., 2016; Kamal *et al*., 2014b; Kaushik *et al*., 2012; Raza *et al*., 2014; Sett & Sahu, 2014; Tomei *et al*., 2009). A bulk of gaseous toxic substances and by-products are formed and released during the baking of bricks at kilns (Ishaq *et al*., 2010a). These hazardous gases as well as heavy metals are released through natural means or by human activities and become part of the environment, thus, affecting all life forms including humans (Ismail *et al*., 2012). Emissions from kiln include P.M, metals, and gaseous pollutants (sulfur and CO_2, NO_2, CO, hydrogen fluoride, and VOCs) (Madden & Fowler, 2000). Animals, plants and human are directly/indirectly exposed to these emissions (Madden & Fowler, 2000). Some heavy metals play crucial role in regulating physiological processes of animals (Ismail *et al*., 2012). They also have adverse effects on human health (Hu, 2002; Mehra & Thakur, 2010). Heavy metals induce toxicity by getting themselves attached with the protein sites that are not compatible for them. They disrupt the original metal from their natural binding position, thereby destroying the cells (Flora *et al*., 2008; Satoh *et al*., 2008). However, due to their oxidation-reduction and chemical coordination potential, they bypass the control mechanisms as well, such as nutrient transport, homeostasis compartmentalization and binding ability cells (Hu, 2002; Mehra & Thakur, 2010). Cr, Zn, Ni, and Cd are known to

impart adverse actions among humans (de-Angelis *et al*., 2017; Jahan *et al*., 2016; Wicke *et al*., 2012). Among brick kiln workers, children living/working at brick kiln sites are at increased risks of exposure to environmental pollutants (Madden & Fowler, 2000). Different cities of Punjab province have many brick kiln operations, especially District Rawalpindi, where an estimated 450 brick kilns are located. In Rawat, these brick kilns and road transport smoke pose a huge burden of air pollution on human health and atmosphere (Ismail *et al*., 2012).

The direct exposure to brick kiln pollutants is known to induce various public health concerns and reproductive problems in occupational workers including women and children (Joshi & Dudani, 2008; Malik & Kayani, 2014). Different studies have shown that children and youth working at brick kiln sites experience respiratory diseases in South Asia due to association of brick kiln air pollutants and premature deaths. A study from Bangladesh, reported that yearly, 750 premature deaths occur due to brick kiln industry. Not only public health concerns are important to consider, but also, maternal, newborn and child health issue raise a serious question among people residing at brick kiln sites and occupationally exposed to brick kiln emitted pollutants. The social determinants play key part in determining the maternal and child health (Bhutta & Hafeez, 2015). Previous studies found that around 800 pregnant women decease each day due to pregnancy related complications such as unsafe abortions, infections, severe bleeding and hypertensive disorders, worldwide and pregnancy related death is principal cause of death among women of reproductive age after HIV (Malik & Kayani, 2014b). As maternal health is essential to ensure the health of children and the whole family, therefore, child mortality rate also depends on mother's health. Researchers have reported that on the globe, Pakistan ranks 26th for under-5 child mortality rate and approximately 202,000 neonates die each year during the first month of life (Bhutta & Hafeez, 2015). Others leading causes of death

responsible for under-5 children reported by Pakistan Demographic and Health Survey 2006–2007 are diarrhea (27%), pneumonia (26%), and malaria and are more common among children with low birth weight or malnutrition (Liu *et al*., 2015; National Institute of Population Studies, 2008). Another study suggested that Pakistan has the third and second highest rates of newborn mortality and stillbirths (Cousens *et al*., 2011; Rajaratnam *et al*., 2010). Approximately, 21% of Pakistani population is living below the poverty level and brick kilns are mostly developed outside the cities, in the urban or per-urban areas (Bhutta & Hafeez, 2015). Therefore, it is concluded that a number of socio-economic issues such as poverty, undernutrition, poor hygiene, illiteracy and absence of health facilities along with these diseases, further strengthen the risk factors (Bhutta *et al*., 2013).

Aims and objectives:

Studies show that heavy metals affect children growth by effecting hypothalamic pituitary somatotropic axis (Le-Gac *et al*., 1993). Jahan *et al*. (2016) reported that exposure to some heavy metals (Cd, Cr, and Ni) causes homeostatic imbalances among male brick kiln workers via decreasing antioxidant concentration and reproductive enzymes levels (testosterone) (Jahan *et al*., 2016), to explore the effects of heavy metal burden in blood and children's health has not been assessed so far. Therefore, the study was designed to investigate the toxic effects of kiln emitted heavy metals on child health by monitoring their blood parameters, antioxidant enzyme status of the body, reproductive hormones, induced DNA damage, somatotropic and stress hormones concentrations, that eventually may affect reproductive health. To address public health and growth concerns among boys and girls of different age groups working at brick kilns, the study aims to

- Find the association of social factors with children health and GH
- Understand the growth and developmental health risks among children of pubertal age
- Assessment of effect of brick kiln emitted heavy metal burden on body mass index and body weight of brick kiln girls and boys
- Investigate the possible effect of heavy metal burden on blood parameters of children of pubertal age
- Evaluation of oxidative stress markers among brick kiln girls and boys
- To assess the effect of heavy metals on the hypothalamic pituitary somatotropic axis
- Address the relationship of physiological response of stress on onset of puberty in girls and boys
- Assessment of hypothalamic pituitary adrenal (HPA) axis via monitoring stress hormone concentrations in blood

MATERIALS AND METHODS

Subject selection

Due to dominancy of male workers kilns and despite of large number of associated families with this occupation, the number of children was negligible. Children of different age groups working at brick kilns were considered as they are more exposed to brick kilns emissions. The number of brick kiln children (n=175) exposed to brick kiln pollutants and non-workers living in same District (n=100) aged 4 to 17 years, including boys (control n=50, brick kiln workers n= 85) and girls (control n=50, brick kiln workers n= 90) were considered. The sample size for children subjects was calculated using same method mentioned in chapter 2 and calculated sample size for girls was 171 and 288 for boys, however the desired number of samples was not achieved due to their reservedness and unwillingness of their parents.

Body mass index

BMI for children was calculated by the online BMI calculator centered by National Health Centre for Statistics (2000). The online calculator considers parameters age, sex, height in meter, weight in kilogram (Kg). BMI percentile was categories as

1. **Underweight** - < 5th percentile
2. **Healthy Weight** - 5th percentile up to the 85th percentile
3. **At Risk of Overweight** - 85th to < 95th percentile
4. **Overweight** - >/= 95th percentile

Following formula was used for determination of BMI:

$$\text{BMI} = \frac{Weight\ (Kg)}{Height\ (m2)}$$

Assessment of reproductive indicators and pubertal onset

The questionnaires collected data regarding reproductive variables, indicators and stage of development. It also that gathered information related to pubertal onset and physical changes observed in children were noticed. The knowledge about reproductive diseases, reproductive history, fertility issues, alive and dead sibling number and information of other transmissible diseases was also collected.

Sample collection

The blood was withdrawn through venipuncture using 5 ml sterile syringe. Blood was transferred to gel vacutainers. Half of the blood sample was stored at 4 °C till the analysis of blood profile and heavy metals, while the other half was centrifuged at 3000 rpm for 10 min. After centrifugation, plasma was separated and stored at −20 °C until further analyzed. Girls and boys blood samples were pooled for all the studied parameters except for hormonal immunoassays.

Analysis of heavy metals

The method followed in this study was based on (Tripathi *et al*., 2017) with some modifications as described in chapter 1.

Blood profile on hematology analyzer

The blood collected in lavender vacutainer was used for blood count using an automated hematology analyzer at MultiLinks Laboratory, Rawalpindi as mentioned in chapter 2.

Biochemical studies

Antioxidant enzymes and total protein content were estimated in blood plasma of control and treated subjects. The stored blood plasma samples were thawed and used. The detailed procedure for measurement CAT (Reiter *et al*., 1995), POD (Chance & Maehly,

1955), SOD (Kakkar *et al*., 1995), ROS (Hayashi *et al*., 2007) and TBARs (Wright *et al*., 1981) have been mentioned in chapter 2.

The protein content in blood plasma of both groups was quantified using commercially available protein kit by AMEDA Labordiagnostik GmbH Krenngasse, whose procedure has been mentioned in chapter 2.

Enzyme Linked Immunosorbent Assay (ELISA)

Hormonal concentrations for growth hormone (GH) and cortisol were determined by different commercially available enzyme immune assay (EIA) tests kits.

Determination of serum Growth hormone (GH)

Growth hormone EIA kit provided by Amgenix Inc, USA was used. The assay was carried out as described in the protocol provided with the kit. The minimum detectable concentration of the assay was 0.5ng/ml. The principle and procedure of the assay is as follows:

Principle of the test:

The principle of solid phase ELSA assay is the basis for microLISA-HGH quantitative EIA test kit. The assay system uses polyclonal anti-hGH antibody for solid phase immobilization, while a mouse monoclonal anti-ferritin antibody was used in the antibody- enzyme conjugate solution. The sample reacts with both antibodies at the same time, leading to hGH molecules being sandwiched between the two types of antibodies. Washing of the wells with water following 60min incubation at room temperature is done, that removes all labeled unbound antibodies. It is followed by addition of TMB solution and incubation for 20min, resulting in blue color appearance (figure 43). The blue color changes to yellow as soon as 2N HCL was added, and absorbance is noted at 450nm wavelength. The color intensity of the test sample reflects the concentration of hGH.

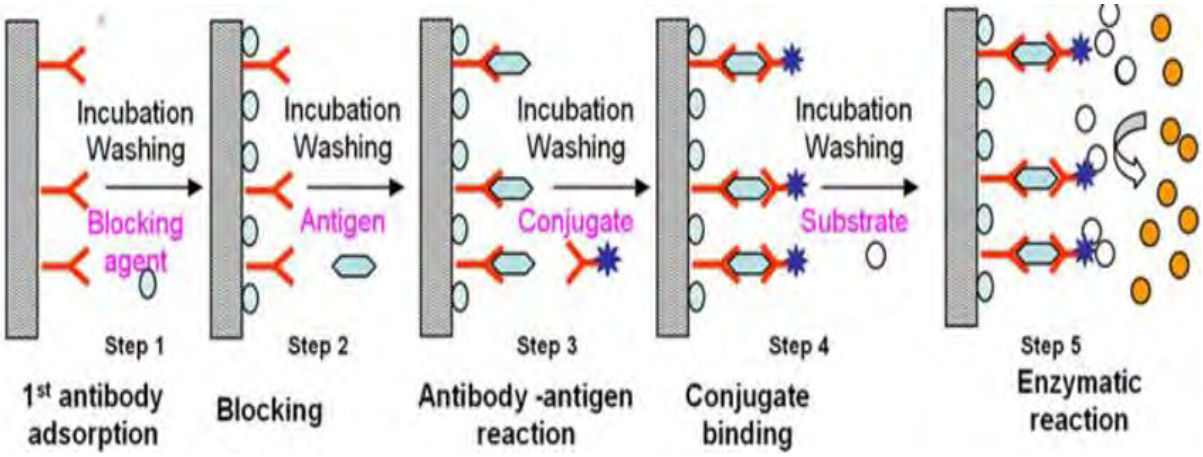


Figure 43. Principle of solid phase ELISA assay for human growth hormone (hGH)

Procedure

The required number of coated wells were held in the microtiter holder and dispensed with 50µl of provided standards, specimen, and controls into marked wells; followed by addition of 100µl of Enzyme Conjugate Reagent in all wells. A thorough and complete mixing for 30 seconds was done, and microtiter plate was incubated at 18-22°C for 60min. After incubation, the contents of plate were discarded by simple flipping plate. The wells were cleaned 5 times with washing buffer (1X). All the residual water droplets were completely removed from wells by striking microtiter well plate onto an absorbent paper. After washing, wells were administered with 100µl of TMB substrate, carefully mixed for 5sec, and kept at 18-22°C in the dark for 20min. At last, 100µl of stop solution was added in all the wells to stop the reaction, followed by gentle mixing for 30sec to ensure the conversion of blue color to yellow color. The OD was taken at 450nm with the help of microtiter plate reader within 30 minutes. The results were expressed in ng/ml.

Determination of serum Cortisol hormone

Cortisol hormone ELISA test kit for human (The Calbiotech, Inc. USA) was utilized to quantify cortisol in blood plasma. The assay was carried out as described in the protocol

provided with the kit, having sensitivity of 1.16ng/ml. The detailed protocol has been mentioned earlier (chapter 2)

Comet assay

This practice includes, implanting of single cell in agarose, lysing the cells and liberating out DNA by gel electrophoresis, forming a comet tail cleaving the intact DNA head. The whole and damaged DNA is pictured by fluorescence microscopy and computed by image analysis. The whole procedure was performed under low light in order to avoid induced damage to DNA.

Procedure

1. Frosted microscopic glass slides were slightly heated on slide warmer. Using 1% regular melting point agarose (RMPA) and low melting point agarose (1% LMPA), slides were prepared.
2. Cell lysis was performed by dipping the slides in a histology jar containing freshly prepared cold lysis buffer. Triton X-100 was supplemented just afore commencing the lysis. EDTA was dissolve with the aid of NaOH pellets at 0.2 g/ 10ml of solution. Slides were incubated with lysing solution for 24 hrs at room temperature, and later, were washed in the dark using distilled water 3X with 20min interval to eradicate salt traces and detergent.
3. For neutral electrophoresis, prepared slides were steadily kept in electrophoresis tray fronting towards anode containing distilled water and neutral electrophoresis buffer. Boric acid was dissolved at 45 ºC while stirring. Slides were then equilibrated with electrophoresis buffer for 20 min at 25V. Later, slides were protected from dark with aluminum foil and were air-dried overnight at 5°C.
4. Rehydration of the slides with distilled water for 60 min was maintained, followed by staining with Acridine orange and analysis using epi-fluorescent microscopy. Images were

taken for succeeding analyses/scoring with TRITEK software. The overall procedure of comet assay has been summarized as follows in figure 44.

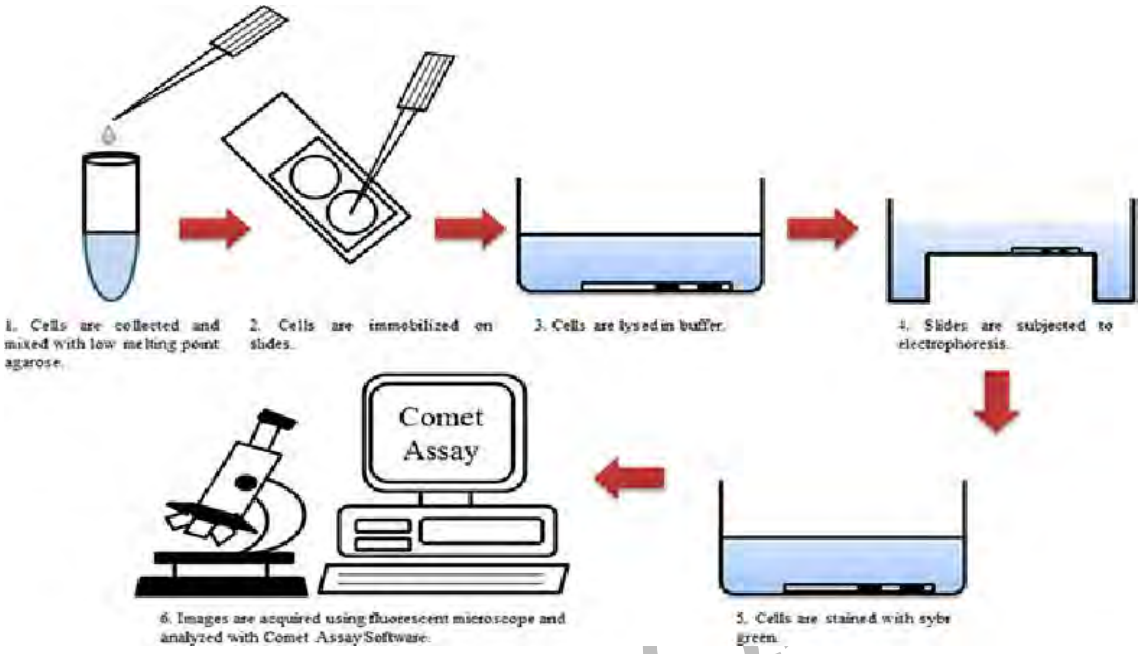


Figure 44. Summarized procedure of Comet assay

Statistical analysis

The data is expressed as mean and standard error of mean (mean±SEM). Student's sample t-test was performed for analysis of blood parameters, antioxidant enzymes and cortisol levels while, comet assay measurement were analyzed using TRITEK software. Correlation was determined among cortisol and GH in pairwise fashion using Pearson's correlation (r) and significance value (p) was measured. The significance level was decided to be $p<0.05$.

RESULTS

BMI and health characteristics of study population

Analysis of health characteristics and reproductive indicators from data obtained from brick kiln girls and boys showed that puberty was not achieved among maximum participants. The menarche age in girls was 10.5±0.5years in brick kiln workers as compared to control subjects (11.42±0.21). The number of girls who had attained puberty was only 9 among our studied subjects, out of which 3 girls were married. Physically girl's appearance was weak. 66% of boys and 50% of girls who participated in present study had underweight BMI<20. Prevalence of respiratory disorders, kidney problems, body weakness and bone pain were common among boys and girls (table 26). 70% of boys and 68% of girl were illiterate, and concept of early age marriage was prevalent among visited brick kiln communities. Following figure shows the average BMI of all the participant brick kiln children as compared to control.

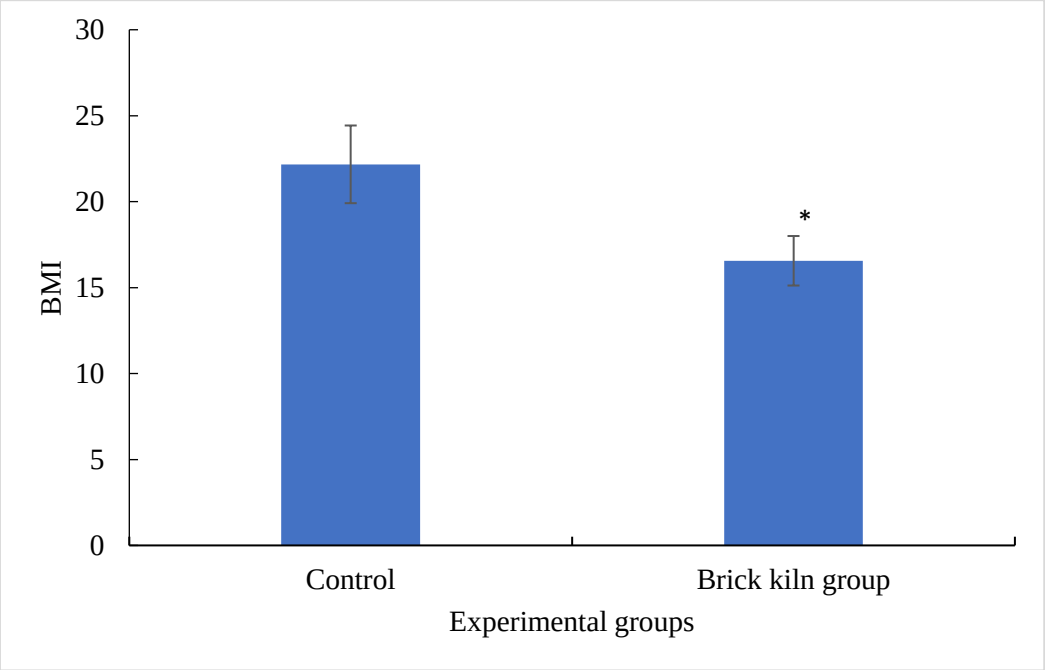


Figure 45. Figure shows the body mass index (BMI) of brick kiln and control children

Table 26. Health characteristics of study population of children working at brick kiln sites.

	Boys (n=85)		Girls (n=90)	
Age ranges (years)	**n**	**Percentage (%)**	**n**	**Percentage (%)**
4-8.0	10.00	11.76	12.00	13.33
9-12.0	36.00	42.35	16.00	17.78
13-15.0	23.00	27.06	42.00	46.67
16-17	16.00	18.82	20.00	22.22
Height (inches)				
15-39	4.00	4.71	8.00	8.89
40-45	22.00	25.88	21.00	23.33
46-55	51.00	60.00	36.00	40.00
56-60	8.00	9.41	25.00	27.78
Weight (Kg)				
15-25	35.00	41.18	36.00	40.00
26-35	28.00	32.94	30.00	33.33
36-45	7.00	8.24	12.00	13.33
45+	15.00	17.65	12.00	13.33
BMI (Kg/m^2)				
Underweight* BMI<20 (<5th percentile)	66.00	77.65	50.00	55.56
Healthy weight * BMI 20-<26 (5th percentile-< 85th percentile)	6.00	7.06	23.00	25.56
Overweight* BMI 26-<30 (85th - <95th percentile)	4.00	4.71	12.00	13.33
Obese	9.00	10.59	5.00	5.56

BMI>30 (=/>95th percentile)				
Health history				
Kidney problems	9.00	10.59	2.00	2.22
Asthma	4.00	4.71	4.00	4.44
Chest pain	4.00	4.71	1.00	1.11
Bone problems	3.00	3.53	4	4.44
Healthy	65.00	76.47	79.00	87.78
Education status				
Illiterate	70.00	82.35	68.00	75.56
Primary	15.00	17.65	22.00	24.44
Exposure to drug addiction				
Addiction to Naswar/Charas	8.00	9.41	0.00	0.00
Non addiction	77.00	90.59	90.00	100.00

*refers to percentiles as determined by center for disease control (*About Child & Teen BMI | Healthy Weight, Nutrition, and Physical Activity | CDC*, n.d.)

Heavy metals Analysis

The concentration of Cd, Cr and Ni was increased ($p<0.05$) in brick kiln children as compared to control group. There was seen a significant rise ($p<0.01$) in concentration of Zn among exposed group in contrast to control group (Table 27).

Table 27. Mean ±SEM heavy metals concentration in whole blood of control group and worker group.

Groups	**Heavy metal concentrations**			
	Zinc (µg/dl)	**Cadmium (µg/dl)**	**Chromium (µg/dl)**	**Nickel (µg/dl)**
Control (n=100)	17.97±1.99	1.40±0.22	0.87±0.05	0.54±0.07
Brick kiln group (n=175)	26.63±2.21**	2.16±0.15*	1.09±0.05*	0.96±0.15*

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (unpaired sample T test)

Hematological parameters

Table 28 represents the result of hematological parameters along with given reference ranges according to previous studies (Hughes & Kahl, 2018). There was observed noteworthy change ($p<0.05$) in the Hb level of brick kiln group as compared to control. The concentrations of HCT and MCH were significantly decreased ($p<0.01$) in kiln children than control. The level of MCHC was also significantly lowered ($p<0.05$) in exposed group than control. Number of WBCs was considerably higher ($p<0.05$) in heavy metal exposed group in contrast to control group. There was seen no significant change in platelet count.

Table 28. Mean ± SEM of hematological parameters of control and brick kiln children.

Hematological parameters	Control	Workers	Reference ranges [a]
Hb (g/dl)	12.05±0.18	12.79±0.14*	11.5-16
HCT (%)	38.02±0.21	35.14±0.26**	35-50
MCH (pg)	26.79±0.12	24.52±0.36**	25.9 – 31.0
MCHC (g/dl)	33.03±0.13	31.76±0.22*	31.0 – 37.0
WBC (1×10^3)	8.16±1.00	12.18±0.71*	4.5 – 13.5
Plt (1×10^3)	255.61±5.17	254.75±8.33	150 – 350

[a] Reference values of complete blood count for children samples (Hughes & Kahl, 2018)

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (unpaired sample T test)

Antioxidant enzymes

A significant decrease ($p<0.001$) in CAT and POD levels in brick kiln group in comparison with control was noted. The level of SOD also decreased ($p<0.01$) in kiln group in contrast to control. The concentration of TBARS was comparable among control and exposed group, however, the significant rise ($p<0.001$) in ROS production among kiln group was evident than control (figure 46). There was observed a noteworthy increase ($p<0.05$) in total protein content in the exposed group as shown in table 29 and figure 47.

Table 29. Mean ± SEM of biochemical variables in control and brick kiln children

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$

Biochemical parameters	Control	Workers
CAT (U/mg)	0.55±0.05	0.25±0.02***
POD (U/min)	6.24±0.95	1.01±0.52***
SOD (U/ mg)	4.79±1.03	1.44±0.24**
ROS (n/mol)	0.87±0.006	1.87±0.28***
TBAR (nM/mg protein)	1.39±0.08	2.66±0.11
Total Protein content (g/dl)	0.11±0.01	0.18±0.08*

compared to control (unpaired sample T test)

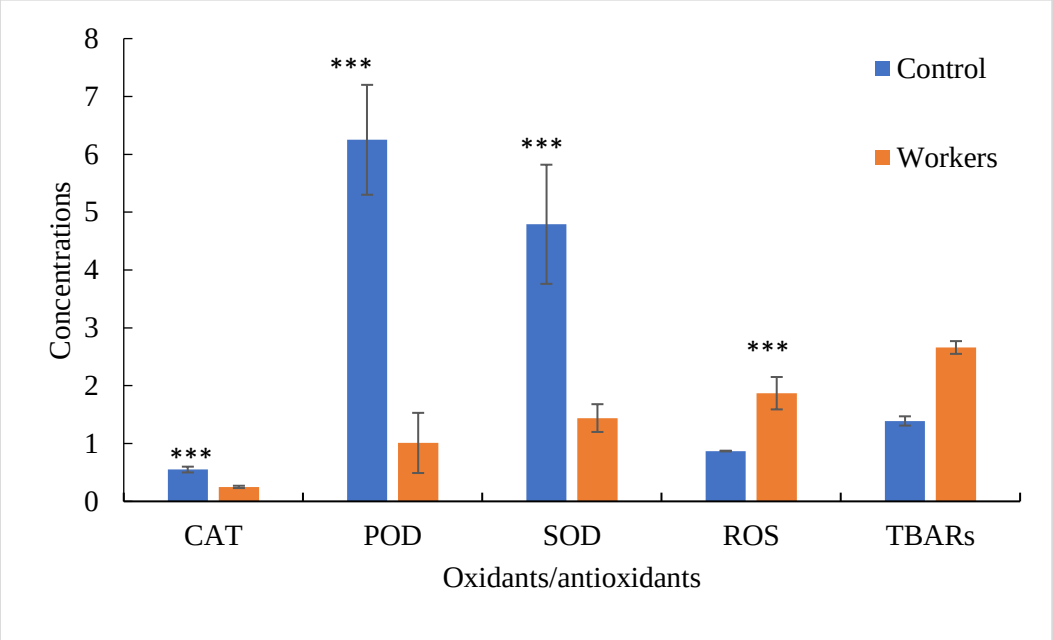


Figure 46. Comparison of percent Catalase (U/mg), Peroxidase (U/min), sodium dismutase (U/min), reactive oxygen species (n/mol) and Thiobarbaturic reactive oxygen species (nM/mg protein) in blood plasma of brick kiln emission exposed and control children

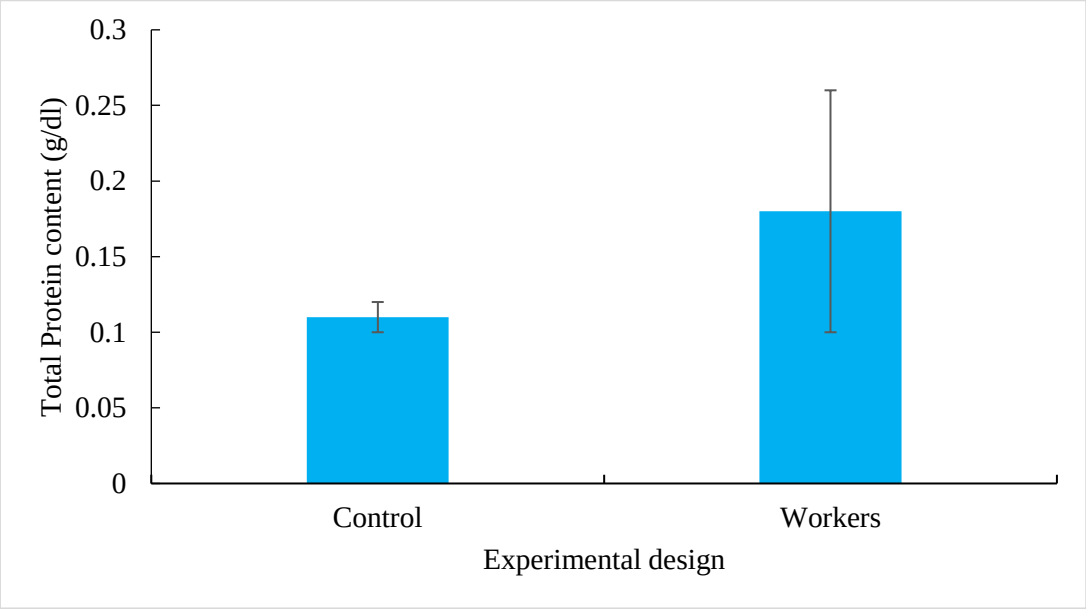


Figure 47. Effect of heavy metal burden on total protein content (g/dl) in blood plasma of brick kiln emission exposed and control children

Hormonal analysis

Table 30 shows the plasma concentrations of growth hormone and cortisol in children (boys and girls) of different age groups, while figure 48 presents overall significant change among both hormones in two groups.

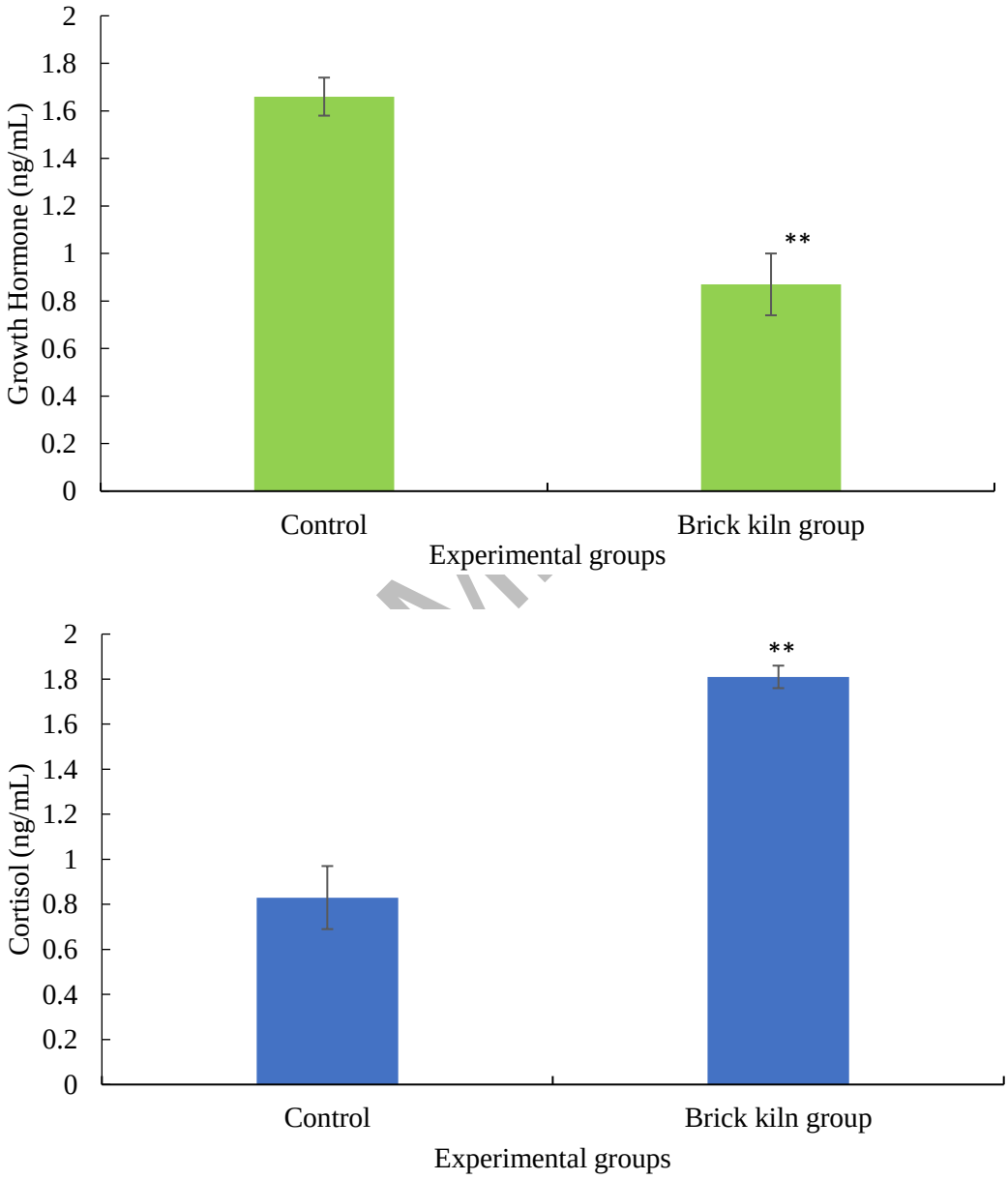


Figure 48. Effect of heavy metal burden on plasma concentration of Growth hormone (ng/mL) and cortisol (ng/mL) in brick kiln and control children

Table 30. Plasma concentrations of growth hormone and cortisol in children of different age groups.

	Growth hormone (ng/mL)				Cortisol (ng/mL)			
Gender	Girls		Boys		Girls		Boys	
Groups	Control (n=50)	Exposed (n=90)	Control (n=50)	Exposed (n=85)	Control (n=50)	Exposed (n=90)	Control (n=50)	Exposed (n=85)
Age (yrs)								
4-8	0.78 ± 0.03	0.07 ± 0.10	1.71 ± 0.01	0.32 ± 0.11	0.12 ± 0.13	1.01 ± 0.05	0.25 ± 0.19	1.23 ± 0.02
9-12	1.99 ± 0.05	1.80 ± 0.11	2.08 ± 0.01	1.38 ± 0.17	0.61 ± 0.14	1.81 ± 0.03	0.56 ± 0.11	1.97 ± 0.01
13-15	2.68 ± 0.01	2.09 ± 0.18	2.96 ± 0.06	2.01 ± 0.01	0.83 ± 0.19	2.07 ± 0.01	0.98 ± 0.14	2.86 ± 0.03
16-18	3.83 ± 0.03	2.95 ± 0.03	3.62 ± 0.02	2.87 ± 0.12	0.92 ± 0.09	2.54 ± 0.01	1.38 ± 0.11	3.00 ± 0.19

Correlation of hormones

The relationship between plasma concentrations of cortisol and growth hormone was determined through a Pearson's correlation in a pairwise fashion as shown in table 31 and figure 49. The findings of studies showed that blood plasma cortisol levels negatively correlated with GH (r=-0.284) with non-significant p value.

Table 31. A precise table showing Pearson's correlations among plasma GH and cortisol in children

	Correlation	
	Cortisol (ng/mL)	**Growth hormone (ng/mL)**
Cortisol (ng/mL)	1	-0.284
Growth hormone (ng/mL)	-0.284	1

** Correlation (r) is significant at the 0.01 level (2-tailed).

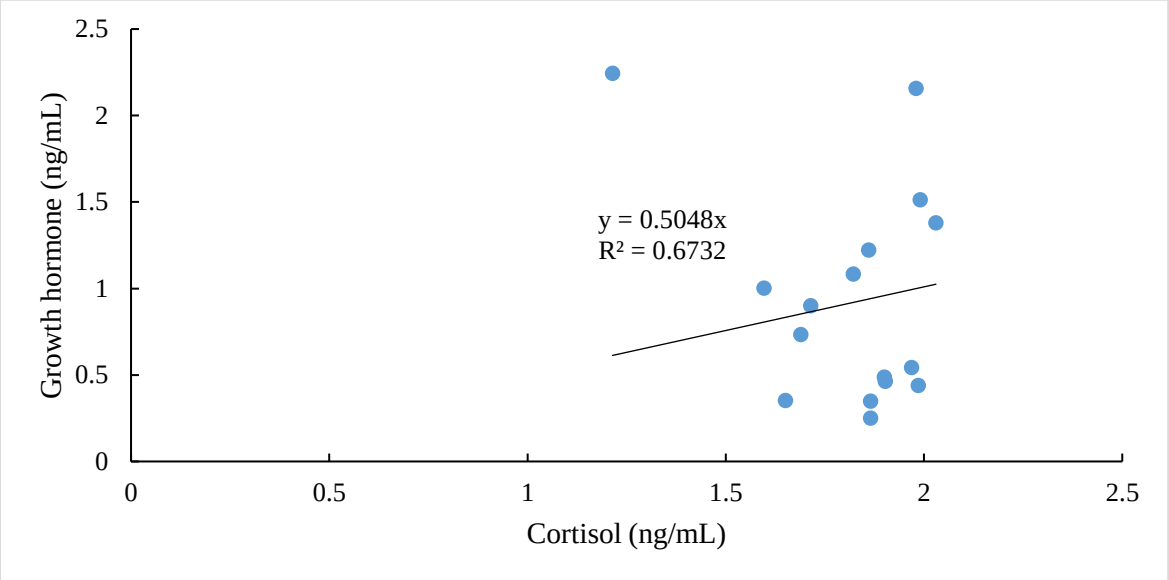


Figure 49. Correlation of plasma Cortisol (ng/mL) and GH levels among children samples

Comet assay

The results of comet analysis for DNA damage in control group and exposed group has been summarized in table 32 and statistical investigation showed non-significant (p=0.06) reduction in head length of exposed group in contrast to control, whereas tail length was significantly increased (p<0.01) in exposed group. The percentage DNA in head region presented substantial decrease (p<0.001), while significant rise (p<0.001) in percentage DNA in tail was evidenced in exposed group than control. Tail movement represent non-significant (p=0.15) difference among both groups (figure 50).

Table 32. Effect of brick kiln industrial working environment on cellular DNA of brick kiln children as compared to control.

Parameters	Control	Exposed
Head length (µm)	177.41±0.86	174.83±1.03
Tail length (µm)	25.16±0.45	28.90±0.80**
DNA in head (%)	90.41±0.48	83.27±0.95***
DNA in tail (%)	10.33±0.55	15.02±0.56***
Tail moment (µm)	3.96±0.19	4.40±0.23

*, **, *** indicates significant difference at probability p<0.05, p<0.01 and p<0.001 compared to control (unpaired sample T test)

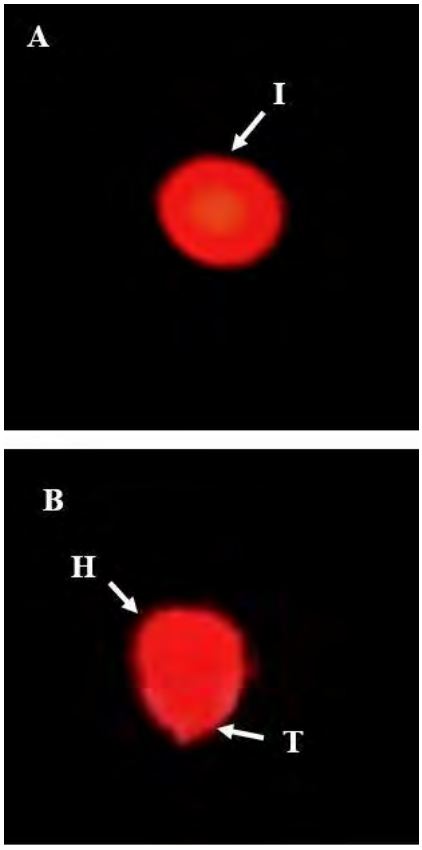


Figure 50. Effect of brick kiln industrial working environment on length of chromatin dispersion in the cellular DNA (A) Control group (B) Exposed group. 40 X. Head (H), Tail (T), Intact (I).

DISCUSSION

The present study focuses on the wellbeing status of children living/working at the brick kiln industries. A comparison of different demographic parameters between the two groups was made. Most of the children living at brick kiln sites were working unpaid, bare foot and were directly exposed to contaminated soil. The sanitary system was very underprivileged; the hygienic and nutritional conditions were quite poor. Education awareness and health situations were also pitiable. The education level also varied in control and exposed group; many of the exposed children were illiterate. Most of the children were facing skin problems, eye problem, and stomach problems, bones issues.

The present study reported presence of Cd, Cr, Zn, and Ni in blood of exposed group. In the environment, these metals are found in lower concentrations in soil, water, and air. They might be unsafe, when their levels increase from low to high (Martin & Griswold, 2009). In current study, decreased BMI was observed in children living in the vicinity of brick kiln sites. Some heavy metals such as cadmium is known to effect body fat mass and reduces BMI (Castagnetto *et al*., 2002). Decreased BMI in our results might be due to the high level of Cd detected in blood of exposed group.

In our survey reports, it was found that children living in the vicinity of brick kilns were having bones problems which might be due to the iron deficiency caused by elevated level of Cd detected in blood. Padilla *et al*. (2010) have reported that atmospheric introduction to heavy metals may induce changes in human weight (gain/loss) and may cause metabolic disorders such as obesity. The displacement of metals (Zn and Cr) may effect metabolic process such as production of energy, and carbohydrate tolerance (Padilla *et al*., 2010). Cd has the potential to attach itself with different amino acids such as cysteine, glutamate, histamine and aspartate ligands and ultimately, causing iron

deficiency (Castagnetto *et al*., 2002). Cd can also replace Zn, which is a part of metallothionein, hence, preventing its availability as free radical scavenger within the cell (Rzymski *et al*., 2014). The findings of present study showed high level of Zn in blood of exposed group. Previously, no such observations have been reported so far. The present results further showed higher concentrations of Cr and Ni in blood of brick kiln children. As Ni is known to possess haematotoxic, reprotoxic, immunotoxic, genotoxic, neurotoxic and carcinogenic effects, it is expected that there is a risk for the development of reproductive disorders and puberty interfering changes in exposed children (Lu *et al*., 2005).

Kamal *et al*. (2016) have shown in their respective study, that exposure to brick kiln emitted PAH affect the hematological parameters in blood such as serum c-reactive proteins (CRP), WBCs, Hb, RBC, and PLT counts (Kamal *et al*., 2015). The present study reported similar results, where we observed that children residing at brick kiln sites experience decreased concentrations of RBCs and Hb level in blood due to exposure to heavy metals. The current findings are in agreement with the previous work of Jahan *et al*. (2016), where decrease in RBCs number and Hb was evident among brick kiln male workers, whose blood was found to contain higher concentration of heavy metals (Cd, Cr, Ni) (Jahan *et al*., 2016). Heavy metals are known to inhibit haem and hemoglobin production by lowering δ-aminolevulinic acid dehydratase (ALAD) levels, which convert ALA into porphobilinogen (Gómez *et al*., 2012). Higher ALA and lowered prophobilinogen levels cause generation of reactive oxygen species, which disrupt RBCs morphology and survival (Gómez *et al*., 2012). The levels of MCH, MCHC, HCT and Hb were slightly decreased in present study that might be due to oxidative stress caused by

metals, leading to alteration in RBCs morphology and ultimately, decreasing level of Hb as previously suggested by Zhao *et al*. (2007) (Zhao *et al*., 2007).

Some heavy metals, including Cd and Cr induce the production of ROS, thus, increasing the oxidative stress and disrupting cell membrane in various tissues (Goyal *et al*., 2015; Kamal *et al*., 2015). The reaction between Cr and biological reductants (e.g thiols and ascorbate) results in ROS formation within cell, that ultimately results into DNA and proteins damage (Stohs & Bagchi, 1995). Ni is known to induce cellular toxicity and carcinogenicity via generation of ROS (Mohammadi *et al*., 2018). The present study showed a marked increase in number of ROS among children living at brick kiln sites. This increase in ROS production might be due to significantly high level of nickel, chromium and cadmium detected in their blood. A former study conducted by Jahan *et al.,* (2016) also presented similar results, where increase in number of ROS was evident due to increased heavy metal concentrations (Cd, Cr) in blood of brick kiln male workers (Jahan *et al*., 2016).

The oxidant/antioxidant system presents the sophisticated network within the human body, that neutralizes the toxic effects of ROS on macromolecules (Lucesoli *et al*., 1999). Cells produce various antioxidant enzymes such as SOD, CAT and GPx, and peptides with thiol groups, that react against the oxidant challenges utilizing molecular oxygen to initiate mechanisms that produce essential energy (Gómez *et al*., 2012). ROS can also be directly neutralized by small molecular weight antioxidants that includes Vit E, Vit C, TRX, NADPH, GSH, and trace metals (Dandekar *et al*., 2002). This intricate approach has the potential to sustain an redox balance as well as to reduce the molecular disruption caused by ROS within a cell (Bonnefont *et al*., 2001; Gómez *et al*., 2012). It is well documented that decreased level of antioxidant enzymes and increased level of ROS present the

homeostatic imbalance in the human body. In current study, lower level of SOD, POD and CAT were found. Chromium exerts its toxic effects on enzymes such as cytochromes oxidase, catalase and peroxidase having iron as their component (de-Angelis *et al*., 2017). Ni is also known to generate reactive oxygen species (Lippmann *et al*., 2006). Previously, Wąsowicz *et al*. (2001) found that work-related exposure to heavy metals e.g., cadmium or lead disturbs the normal homeostasis of the body by disturbing antioxidant capacity of the body (Wąsowicz *et al*., 2001). The decreased level of antioxidant enzymes in present study might be due to the increased production of ROS. Similar results have been previously reported (Gómez *et al*., 2012; Jahan *et al*., 2016).

Human reproduction is affected by exposure to severe stress challenges (Kalantaridou *et al*., 2010). Different experimental and clinical investigations have confirmed that hypothalamic pituitary adrenal axis (HPA) can be activated by severe physical, immune and psychological challenges and stress inputs, which ultimately leads to impeding of reproduction in humans as well as in other mammals (Ferin, 1999; Kalantaridou *et al*., 2010). Stress is believed to disturb the normal reproductive processes by reducing gonadotrophin secretion, which subsequently reduces the production of gonadal steroids (Ferin, 1999). This is mediated by stress activation of the HPA axis, which elicits the hypothalamic release of corticotrophin-releasing hormone (CRH) and arginine vasopressin (Kalantaridou *et al*., 2010). Increase in concentration of these neurohormones subsequently, increases pituitary ACTH levels and raises synthesis of adrenal cortisol (Ferin, 1999; Kalantaridou *et al*., 2010). The increased level of CRH negatively affects hypothalamic GnRH pulsatility and reduces sensitivity of GnRH at the pituitary, resulting in gonadotrophins reduction (Kalantaridou *et al*., 2010). In present study, increased level of cortisol was evident among boys and girls exposed to brick kiln emissions. We have

presented our findings on cortisol concentrations in different age ranges, however, there is not much data available on the age wise cortisol concentration in growing girls and boys due to involvement of multiple physiological and environmental factors, which affect pubertal onset. The physically active lifestyle and decrease levels of cortisol reflect the possible cause for precocious puberty (menarche at early age) in girls, while delayed puberty is expected to occur in boys under same circumstances. As children monitored were at pre-pubertal stage, the risk of progression of reproductive diseases is quite high in them and is thought to be mediated through stimulation of HPA axis.

The GH has essential role in determining puberty, gametogenesis and fertility of an individual (Le-Gac *et al*., 1993). It is responsible for determining the secondary sexual characteristics at pubertal development (Nathanson *et al*., 1941; Wanjari *et al*., 2019). In the present study, the level of GH was significantly decreased in brick kiln group in comparison with the control group. Decrease in GH may affect the reproductive potential of brick kiln workers by inducing precocious/delayed puberty. As puberty is determined by the interplay of adrenarche and pubarche, the increased levels of stress hormone (cortisol) might act along with decreased GH levels and may affect appearance of secondary sexual characters and puberty. We are reporting concentrations of growth hormone in different age groups for both girls and boys in brick kiln and control children for the first time in any industrial setup. No previous relevant reports are available on the age wise GH concentration in growing girls and boys for comparison of our results. Steroids act at the pituitary and hypothalamic levels and therefore, modulate the production of GH and support the sex related patterns of pulsatile GH secretions (Devesa *et al*., 1991; Eden *et al*., 1987). Increased levels of cortisol indicate the disturbance of normal HPG axis as well, that poses a risk for the delayed puberty in boys and precocious

puberty in girls due to their active and hectic lifestyle. Non significant negative correlation between cortisol and GH was seen. These results of correlation analysis have been supported by previous findings where GH and cortisol are known to have varied relationship (Duclos *et al.,* 2007).

As the immune system of children is most active against exposure of infectious agents/environmental pollutants, therefore, children represent the most vulnerable population of the community. The behavioral patterns of children such as playing in mud barefoot, increases their direct contact with soil/dust and raises inhalation rate per bodyweight unit, make their immune system exposed to environmental toxins, and therefore, risk assessment of this group has high priority (Landrigan, 1998). The results of current study have also shown similar results where, children with different age group living/working in brick kiln industries experienced more DNA damage in cells. We observed a significant reduction in head length and significant increase in tail length in brick kiln worker group as compared to control, where intact DNA was observed. Studies have confirmed that children who are occupationally as well as environmentally exposed to pollutants such as PAH, have increased level of DNA damage in blood cells (Cavallo *et al*., 2009; Wang *et al*., 2010). Our results also showed a substantial decrease in percentage DNA in head region, while significant rise in percentage DNA in tail was evidenced in brick kiln group than control. Tail movement represent non-significant ($p=0.15$) difference among both groups It might be due to different heavy metals that are incorporated and cause genotoxicity.

CONCLUSION

- It is concluded from the present study that brick kiln emissions which consist of environmental pollutants including heavy metals such as Cd, Cr, Zn and Ni, may become part of the circulation, and therefore affect the different body functions in growing boys and girls.
- The present study reported that children residing at brick kiln areas were underweight and had lowered BMI.
- The kiln children had altered hematology parameters, lowered antioxidant enzymes concentrations and increased oxidative stress response.
- Hormonal analysis showed decreased GH and increased cortisol levels along with induced DNA damage in blood cells.
- Increased levels of cortisol indicated the disturbance of normal HPG and HPA axis as well, that poses a risk for the reproductive problems in girls and boys

SUMMARY

Centrifugation at 13000 rpm and plasma separation

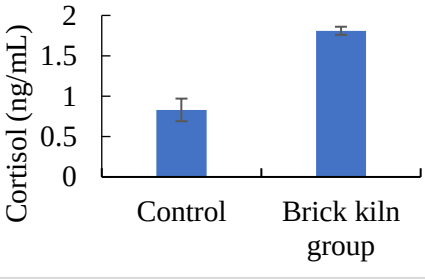
COMET ASSAY

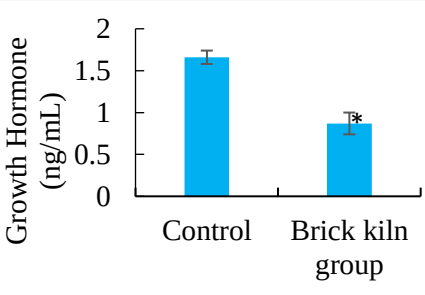
BIOCHEMICAL ANALYSIS

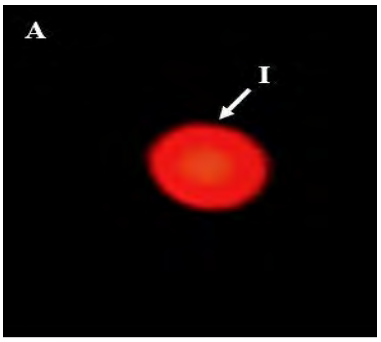
HORMONAL ANALYSIS

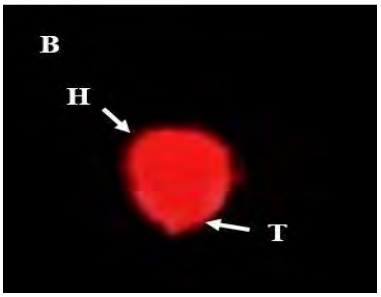
DNA DAMAGE ANALYSIS

Biochemical parameters	Control (n=100)	Exposed (n=175)
CAT (U/mg)	0.55±0.05	0.25±0.02***
POD (U/min)	6.24±0.95	1.01±0.52***
SOD (U/ mg)	4.79±1.03	1.44±0.24**
ROS (n/mol)	0.87±0.006	1.87±0.28***
TBARS (nM/mg protein)	1.39±1.28	2.66±1.10
Total Protein (g/dl)	0.11±0.01	0.18±0.08*









HEAVY METAL BURDEN IN BLOOD ALONG WITH COMPROMISED IMMUNE SYSTEM AND OXIDATIVE STRESS HINDERS WITH PHYSICAL AND REPRODUCTIVE GROWTH OF CHILDREN

GENERAL DISCUSSION

Sociodemographic data

The present study monitored and reported the socio-demographic trends and brick kiln occupant's health status from Rawat, Punjab. Our findings suggested prevalence of multiple health issues among workers including allergies, kidney, stomach disorder as well as respiratory illness and tuberculosis. Direct exposure to the toxic chemicals induce various types of musculoskeletal symptoms along with different types of cancers and therefore, they raise serious public health concerns among occupational workers (Rajesh & Niraj, 2010; Rzymski *et al*., 2014; Sanjel *et al*., 2016; Shaikh *et al*., 2012). The use of dust protective equipment (masks or gloves) was not found among workers and moulding of bricks using bare hands was practiced, this suggests exposure to various environmental pollutants through respiratory, digestive or dermal route that further increases health risks among them. As occupational workers are exposed to atmospheric pollutants such as poisonous gases, metals, trace element and particulate matter (P.M), occupational factors (such as job length, lack of PPE, work type) also play a key role in affecting the employees' physical and mental health as previous studies have suggested (Patil, 2017; Shaikh *et al*., 2012). It is known that work-related risk factors are a key source of respiratory illness (Shaikh *et al*., 2012). The findings of present study highlighted that no health or welfare facilities were provided to labour and working environment was open and poor. Likewise, findings have been reported by Patil. (2017) in their respective study, where cross sectional-observational research was conducted on brick workers from village Karad Taluka, India and likewise conditions at brick kiln sites were noticed (Patil, 2017). Of 346 men who participated in our study, 58% of them were addicted towards various drugs such as cigarette, naswaar (amalgamation of tobacco leaves, calcium oxide and

wood ash) and charras (hashish form of cannabis) as well as hukka (tobacco mixture). Direct exposure to these toxic chemicals induce various types of musculoskeletal symptoms along with different types of cancers and therefore, raise serious public health concerns among occupational workers (Rajesh & Niraj, 2010; Rzymski *et al*., 2014; Sanjel *et al*., 2016; Shaikh *et al*., 2012).

Body mass index

Environmental exposure to metals such as Cd negatively correlate with BMI as suggested by previous studies (Padilla *et al*., 2010). We found a significant lowered BMI among male workers, however, little decrease in BMI of female workers was observed but it was not significant and was near to normal (BMI= 23). The low BMI values of workers indicate generally poor health conditions of workers with poor immune system, making them prone to various allergies, musculoskeletal problems, respiratory disorders, and viral diseases as (Shahid *et al*., 2017). Maternal pre-pregnancy BMI genetic risk and gestational weight gain are an important determinant of fetal weight (Shrestha *et al*., 2019). In our study, most of the studied married female workers were either overweight or underweight despite of their BMI, which is near to normal (BMI 23). The number of underweight women working in brick kilns was found quite high as compared to control women that might be due to promising concentration of Cd circulating in blood. Previous studies have found that environmental exposure to low levels of Cd has significantly negative effect on body weight (Shirai *et al*., 2010). As most of the brick kiln workers are bonded labor and thus, they continue to work at kiln sites generation after generation. Therefore, among occupational men and women exposed to various environmental pollutants, it can be speculated that metals exposure through atmosphere may accord alterations in human weight (Padilla *et al*., 2010). However, ethnic differences have been noticed among Asian

and European peoples (Barba *et al*., 2004). Similarly, our results have shown that health risks associated with body fat are more increased in brick kiln male and female workers as contrasted with control subjects. Therefore, when planning a pregnancy, it is important for women to reach an optimal BMI before conception along with proper nutritional counseling by health care professionals (Papazian *et al*., 2017).

Heavy metal analysis in blood

In present study, analysis of heavy metals through AAS in whole blood revealed a remarkable increase in concentrations of Cd, Ni and Cr in both male and female workers. Multiple studies across Pakistan have reported that brick kiln emitted heavy metals prove to be an environmental pollutants affecting many life forms (David *et al*., 2020; Khan *et al*., 2019; Nasir *et al*., 2021; Shaikh *et al*., 2012). Exposure to the detected metals may impart adverse health risks by triggering disorders of the heart, liver, skin, brain, kidney, liver, and respiratory disorders (Ghosh *et al*., 2020). Ingestion of contaminated food or water as well as smoke inhalation makes human susceptible to Cd^{2+} ion toxicity. It is known to exert reprotoxic effects in males, through multiple processes (de-Angelis *et al*., 2017a), while in females, its concentration increases in ovaries usually with age, and hinders oocyte development. Gradual Cd accumulation in embryos prevents development to the blastocyst stage causing degeneration and decompaction with apoptosis and breakdown in cell adhesion (Thompson & Bannigan, 2008). The increased miscarriage/abortion rate in brick kiln workers observed in current study is correlated with previously reported literature (Thompson & Bannigan, 2008). Ni is another metal present in food, water and soil and is known to possess neurotoxic, immunotoxic, hematotoxic, genotoxic and reprotoxic potential. Ni is reported to accumulate in various tissues such as liver, lungs and kidneys of workers, who are exposed to nickel (Das & Das, 2008). The

findings of current study showed that women and men working at brick kilns develop metabolic and reproductive disorders that might be associated with Ni toxicity. It is known to induce cancers, respiratory disorders, neurotoxicity, epigenetic changes and reproductive diseases among males and females by various mechanisms (Rizvi *et al*., 2020). Growth and reproduction is found to be affected by Ni exposure that influence the health and survival of individual (Furness & Rainbow, 2018). The results of AAS revealed elevated levels of Cr in present study, which is thought to be associated with multiple pathologies in humans such as carcinogenicity (Pavesi & Moreira, 2020). It induces cellular toxicity via production of ROS with subsequent cellular damage (Seydi *et al*., 2020). Cr species are another major environment pollutants due to its extensive use in various industrial applications (Duran *et al*., 2011). The chronic exposure to Cr induces toxicity in liver and kidney (Cherfi *et al*., 2014). Thus, it is inferred that the presence of higher levels of these hazardous metals in blood serum may have detrimental effects on the health outcomes of kiln workers and participants residing in the close proximity.

External beam PIXE analysis for blood

Further non-destructive elemental examination of blood through PIXE analysis detected multiple elements with greater efficiency including Si, Ti, Mn, Fe, P, S, Cl, K, Ca, Co, Ni, Cu, and Zn. Cr that was only found in the blood samples retrieved from brick kiln workers and not in control participants, whilst control participants had significantly higher levels of Cu, Co, Mn, Ni, and Ti. No significant differences were detected between the control and brick kiln samples for essential (P, S, Cl, K, Ca) and non-essential elements (Si). In human body, Cu plays an important role in enzymatic reactions, however, Cu toxicity may occur resulting in multiple cellular changes (Zhou *et al*., 2018). Similarly, the presence of Co at elevated levels in blood serum causes erythrocytosis, and poses a risk to the development of heart and thyroid gland pathologies, and is responsible for

occupational asthma and dermatitis (Banza *et al*., 2009). Although manganese and Fe are naturally occurring metals which usually coexist in the environment such as in ground water, elevated levels of Mn in blood are usually most evident in Fe deficient children, causing anemia, and may compromise mental health (Rahman *et al*., 2013). Ni is another metal that exerts a negative effect on human health and is responsible for triggering the development of cancer and dermatitis (Zambelli *et al*., 2016). Similarly, even minute amounts of Ti in the body may exert toxic effects through altering cell cycle events and constriction of nuclear membranes inducing cell death (Baranowska *et al*., 2020). Interestingly, Cr was detected in the blood of brick kiln workers group and not in that of control participants. Previous work has also shown elevated blood levels of Cr in kiln workers that was associated with multiple health effects, including lung cancer, an altered immune response, and allergic reactions (Achmad *et al*., 2017; Paustenbach *et al*., 2003; Shrivastava *et al*., 2002).

Hair analysis using SEM

Interestingly, the non-destructive analysis of hair using SEM showed that scalp hair retrieved from brick kiln workers visually showed a higher and homogeneous density of particulate matter of various sizes either trapped or deposited on the hair surface compared to control participant hair samples. The particulate matter sized <2.5µm ($PM_{2.5}$) deposited on the hair of brick kiln workers may contain metals or other inorganic/organic solids, combustion particulate matter, or other aerial particulate matter (Galliano *et al*., 2017a; Qu *et al*., 2018). They are released during the activities performed at the brick kiln including the carriage, molding, and baking of bricks. SEM-EDX it is a semi-quantitative technique compared to PIXE, whilst the irregular surface of the hair may also negatively impact the data obtained using this technique, whilst operational, instrumental, and specimen errors (Rendón *et al*., 2017). In general, the deposition and adherence of

particulate matter from aerial pollution on the hair surface is poorly studied. Previous research has shown that a large number of particulate matter of various sizes can be deposited onto hair cuticle scales of individuals living in aerially polluted (e.g. brick kiln) environments, a process that may be mediated by the presence of sebum on the hair surface as well as the hair's physio-chemical properties including a multitude of environmental factors such as humidity (Galliano *et al*., 2017; Qu *et al*., 2018). The hair's physio-chemical properties may also be significantly altered by frictional forces (brushing and shampooing), solar UV ray exposure, and the extend of exposure to air pollutants that may contain oxidizing and per-oxidizing compounds that breaks down keratin and render the hair surface more amenable to particulate matter deposits increasing anionic binding sites by and lifting the hair cuticle (Galliano *et al*., 2017b; Qu *et al*., 2018; Rendón *et al*., 2017).

Heavy metals and Blood parameters

The findings of blood parameters in males and female worker showed a decrease in RBC and HGB levels that might be linked with heavy metals emitted from kilns. Our findings are further supported by previous work of Fazio *et al*. (2014) where lowered RBC and WBC levels were evident among fish exposed to metal pollution. As RBC are produced from the hematopoietic tissues of kidney and spleen, decrease in RBC number could result from the internal bleeding by damaged kidneys (Kori *et al*., 2006). Another study showed that in vitro incubation of erythrocytes and lymphocytes with different concentrations of K_2Cr_2O7 resulted in a dose-dependent increase in ROS number with reduction in antioxidant capacity of the cells (Husain & Mahmood, 2017). There was seen reduction in WBC levels of females, which is associated with metal toxicity; the molecular mechanism by which Cr (VI) induces cellular toxicity in human blood lymphocyte is through the formation of ROS with subsequent cellular damage (Seydi *et al*., 2020). Increase in platelet number in whole blood of female workers was also evidenced in our

results. Furthermore, decrease in percent hematocrit, MCV, MCH and MCHC was noted among brick kiln male workers that might be due to toxic effects of heavy metals. Comparable findings were previously reported by Jahan *et al*. (2016), where male brick kiln workers exposed to heavy metals Cd, Cr and Ni displayed significant decrease in RBC , HCT and MCHC levels as compared to unexposed males (Jahan *et al*., 2016). Kamal *et al*. (2014) have suggested that variety of brick kiln emitted particles may induce cellular toxicity in actively dividing cells such as bone marrow cells, further supporting our results (Kamal *et al*., 2014). These cells, later differentiate and constitute blood components (Kamal *et al*., 2014a, 2014b).

Heavy metals and Antioxidant enzyme concentrations

Wąsowicz *et al*. (2001) found that work-related exposure to heavy metals e.g., cadmium or lead disturbs the normal homeostasis of the body by upsetting antioxidant capacity of the body. This, in turns, alters the levels and activity of trace elements and other enzymes (Wąsowicz *et al*., 2001). Antioxidant enzymes such as CAT and POD are known to play an important role in reducing the threatening effects of heavy metals. Turkez *et al*. (2012) showed that exposure to heavy metals increases oxidants levels and reduces antioxidants concentrations (Turkez *et al*., 2012). In present study, significant decrease in SOD and POD level was observed in the men and women workers as compared to control. Our results are being supported by previous results of Jahan *et al*. (2016) where brick kiln workers, exposed to emitted heavy metals presented reduced levels of peroxidase enzyme (Jahan *et al*., 2016).

Heavy metals and oxidant production

Studies suggest that transition metals such as Cd, Ni and Cr act as catalysts in the oxidative reactions of biological macromolecules (Ercal *et al*., 2001). Due to their high degree of toxicity, some of them for instance, As, Cd, Cr, Pb, and Hg are considered as

systemic toxicants and are known to induce multiple organ damage, even at lower levels of exposure (Tchounwou *et al*., 2012). Our studies reported that brick kiln workers (men and women) had significantly increased number of ROS and MDA as compared to control. Redox-active metals, such as Cu and Cr act by redox cycling, whereas redox-inactive metals, such as Pb, Cd, Hg are involved in reducing antioxidants enzymes levels in the cell. Both of the mentioned redox reactions may induce an assembly of various reactive oxygen species (Ercal *et al*., 2001). Ni is known to generate reactive oxygen species as well (Lippmann *et al*., 2006). As body antioxidants are the major defensive mechanisms against ROS, and their reduced levels in exposed workers possibly make them prone to various health risks. However, further studies are required to determine the effect of antioxidant supplementation following heavy metal exposure.

Heavy metals and Gonadotropins profiles of males

Previous studies have reported that in male rodents, Cd changes the concentration of reproductive hormones such as FSH, LH and T and affects the steroidogenesis in the Leydig cells (Jahan *et al*., 2016). The results of present findings reported an increase in FSH and LH levels among brick kiln workers as compared to control. The increase in FSH and LH levels might be linked to blood metal burden in blood that may have significant effects on the level of this hormone (Rami *et al*., 2011). This suggests that presence of heavy metals in blood may cause some primary damage to the seminiferous tubules in the testes that hinders LH and FSH to bind to their respective receptors and mediate their actions (McGregor & Mason, 1990). Our findings are in contrast with the previous work of Lafuente *et al*. (2001), where rats exposed to cadmium chloride in drinking water experienced decrease in LH and increase in FSH concentrations (Lafuente *et al*., 2001). As testosterone produced by the testes is the key regulator of reproductive axis that controls the negative feedback actions, maintaining a regulated HPG axis; any disturbance

in its production at testicular site causes abnormal/reduced concentrations of testosterone in blood. We found decreased levels of testosterone in blood plasma of kiln workers as compared to control group that might be due to the presence of high concentration of heavy metals in blood that increased the oxidative stress. Lowered testosterone levels in blood indicate that occupational exposure to kiln pollutants might have deposited heavy metals in blood that can either inhibit steroidogenic activity at cellular level or interferes with gonadotropin binding on its receptors (Priya et al., 2004). Literature data also suggests that high metal concentration in blood may induce primary testicular defects, which reduced testosterone synthesis and hence, via lifting negative feedback, caused a rise in circulating gonadotropin levels.

Correlation of HPA and HPG axis- males

As it has been reported in previous studies that many components of the hypothalamic pituitary gonadal (HPG) axis are downregulated by plasma glucocorticoids such as cortisol. These effects are mediated either at hypothalamus and pituitary level, or by actions on the responsiveness of target tissues to gonadal hormones (Rehman *et al*., 2019; Thakore & Dinan, 1994). The findings of present study reported negative correlation of cortisol concentrations with pituitary gonadotropins LH and FSH levels in blood plasma of males. Multiple other studies have also reported the negative correlation of cortisol hormone and sex steroids (Chen *et al*., 1997; Liening & Josephs, 2010; Tsigos & Chrousos, 2002; Viau, 2002). The negative correlation is explained due to reciprocal relationship between the hypothalamic-pituitary-adrenal (HPA) and HPG axes wherein the activation of one affects the function of the other and vice versa (Toufexis *et al*., 2014). Previous studies showed that male monkeys subjected to restraints had experienced elevated plasma ACTH and cortisol, measured as indexes of stress, within 15 min after initiation of restraint and remained elevated for most of the restraint period; whereas LH

levels began to fall immediately after restraint and remained suppressed for several hours even after the removal of restraints (Norman & Smith, 1992). Similar results are obtained in our study, where negative correlation between cortisol concentration and testosterone level was observed. In humans, exposure to cortisol causes a significant decrease in testosterone production (Cumming *et al*., 1983). Another study suggested that animals subjected to stressors experience inhibited testosterone levels as compared to LH levels, a reflection of the fact that in some animals, testosterone remained low after the return of pulsatile LH secretion even after the removal of restraints (Norman & Smith, 1992). As the present study reported increase in concentration of cortisol among brick kiln workers, this increase in cortisol is thought to be responsible for decrease in sex steroid production from Leydig cells and therefore, can be considered accountable for reproductive function in men.

Correlation of HPA and HPG axis- females

Cortisol is known to negatively regulate the HPG axis by mediating its effects on pituitary gonadotropins LH, FSH, and ovarian steroids, estradiol and progesterone. Former study presented that increase in plasma cortisol level suppresses gonadotropin secretion from the pituitary and disrupts ovarian cyclicity (Oakley *et al*., 2009). Additionally, positive correlation was found among cortisol and prolactin levels as evidenced by previous studies in mice, where stress induced increase in prolactin levels through central and peripheral nervous system (Kirk *et al*., 2017). Correspondingly, evidence indicates that prolactin plays an important role in adrenal gland's response to stress in a way, that it not only stimulates the secretion of ACTH, but also increases the sensitivity of adrenal cortex, resulting in elevated corticosterone release (Levine & Delale, 2018). Further analysis showed that prolactin exhibits negative relationship with LH, FSH, estradiol and progesterone. Studies have suggested that prolactin mediates its direct effects on

reproductive axis through suppression of kisspeptin release at hypothalamic level, reducing activation of GnRH and lastly, decreasing gonadotropins secretion (Donato & Frazão, 2016). A positive relationship was found among LH, FSH and estradiol and progesterone justified by the fact that ovarian secretions are dependent on the pituitary gonadotropins. Estradiol and progesterone were found to have positive correlation between them.

Fertility indicators in female

For a healthy mother and baby, maternal undernutrition is considered as crucial determinant of healthy pregnancy outcomes. Undernutrition can be defined as depleted circulating or stored levels of nutriments, that shows dietary scantiness (Wells *et al*., 2020). It is reported by Khan *et al*. (2009) that women of rural areas suffer more from undernutrition due to lack of several vitamins and essential nutrients in their diets (Khan *et al*., 2009). It is speculated that among Pakistani women of reproductive age (25-44years), the prevalence of malnutrition accounts for 30% in rural women (Khan *et al*., 2009). Our findings suggested that brick kiln workers women who participated in our study, were 18 to 45 years of age and most of them were malnourished. The maternal malnutrition is known to enhance the risk in developing offspring for metabolic and neurodevelopmental disorders, affecting immune responses and bringing neuroimmune consequences (Smith & Reyes, 2017). As malnutrition may bring hazardous and life threatening risks for mothers and baby during childbirth, therefore, it is speculated that factor of malnutrition among brick workers may reduce reproductive potential and may increase the risk of blocked labor (Wells *et al*., 2020).

A regular menstrual cycle is an indication of healthy body with response to all the hormonal and physiological changes. However, various conditions may cause menstrual irregularities. Our findings suggested that menstrual irregularities were experienced among women working at kiln sites. During adolescence, variations in the menstrual cyclicity is a result of immature and disturbed hypothalamic-pituitary-ovarian (HPO) axis (Deligeoroglou & Creatsas, 2012). The menstrual irregularities may result into anemia, osteoporosis and ultimately, infertility (Sherly *et al*., 2017). The present study further reported that the trend of early age marriage and bonded labor was quite common among the brick kiln community. The rate of abortion was quite high among brick workers, as they had to do work, even during the conception that might be the major cause of simultaneous abortion and miscarriages. During conception, exposure to environmental pollution, occupational heavy metal and smoke may bring pathophysiological consequences and may accumulate in fetus by translocating through the placenta (Zhao *et al*., 2017). Cd inhaled via smoking integrates into the body tissues and is transported through the bloodstream to the ovaries, where it represses oocyte development, reduces steroid synthesis, and increases ovarian hemorrhage and necrosis (Thompson & Bannigan, 2008). Therefore, it is expected that menstrual irregularities, early age marriages poor hygiene conditions and increased abortion rate among brick kiln workers impart a serious reproductive health concern on the maternal and child health of whole community and there is a need to address these issues at government and private level.

Protein content

Different study groups have shown that decrease in levels of plasma total protein is considered as a conceded functioning of liver (Alam *et al*., 2017; Karakilcik *et al*., 2004). Liver damage may impart detrimental effects on cellular homeostasis through

peroxidation of polyunsaturated fatty acids and with the formation of aldehydes, that may result in multiple pathological conditions (Yamaguchi *et al*., 2007). The present study showed decrease in plasma proteins among kiln workers when compared with control. This decrease in total protein in blood plasma suggests liver malfunctioning as experienced in our study subjects. Other liver enzymes and antioxidants concentrations have also been measured in present study and are discussed further.

Lipid profile in females

Studies have also suggested that increase in production of fatty acids results in elevated cholesterol level and triglyceride level, that may result in liver dysfunction (Sayed, 2012). Cholesterol is an essential component of mammalian cell membranes, which plays major roles in membrane permeability and fluidity and also serves as a precursor of bile acids, steroid hormones and fat-soluble vitamins (Ihedioha *et al*., 2013). Thus, disturbed levels of cholesterol may interfere with other physiological functions such as metabolic and reproductive malfunctions. The current findings for lipid profile analysis showed an increase in plasma total cholesterol, LDL, and triglyceride (TG), and HDL levels in workers exposed to environmental pollutants; these findings are authenticated with our observed surge in BMI and abdominal fat. Our results are being supported by previous reports where heavy metal (nickel and chromium) induced rise in serum TC, LDL, and TG (Gupta *et al*., 2008). The ratio of TG to HDL is used clinically for the identification of metabolic disorders (Murguía *et al*., 2013). As LDL is considered as a bad cholesterol, therefore, elevated levels of plasma LDL in present findings could be due to its receptors overactivation, as suggested before (Karacaoĝlu & Selmanoĝlu, 2010). Rehman *et al*., (2019) have proposed that rats exposed to food toxicant had increased LDL levels because their receptors permit cholesterol to enter hepatocytes, releasing its free cholesterol and triglycerides that in turn, inhibit cholesterol and formation of new LDL

receptor, reducing LDL uptake, and promoting cholesterol storage (Rehman *et al*., 2019). The decrease in LDL uptake and loss of receptors function boost the cholesterol levels in serum (Adkison, 2012). The results of HDL cholesterol showed a comparable concentration among the control and workers groups; one of the possible explanations for these findings might be the active lifestyle of brick kiln women, who were engaged in prolonged strenuous activity that might have contributed in the production/accumulation of HDL, which is considered as a good cholesterol. Our results are being supported by previous studies which stated that HDL is inversely linked to BMI (Njelekela *et al*., 2002), this has been shown here that BMI (results from chapter 1) is near to normal, while little increase in HDL is found. Extensive literature review on public health and reproductive health concerning women subjects proposed that lipid profile analysis is important for determining the health status and reproductive studies of occupational women because components of lipid profile may vary during different phases of menstrual cycle and is an important indicator of reproductive health (Hatma, 2011; Knauff *et al*., 2008; Shohaimi *et al*., 2014; Wamala *et al.*, 1997). As we studied some of the reproductive parameters in women in this chapter, therefore lipid profile was performed for women samples but not in chapter 3 (men).

Heavy metals and Gonadotropins profiles of females

Previous studies have suggested that environmental exposure to even low levels of metals might be associated with variation in hormonal levels in women of reproductive age (Pollack *et al*., 2011). These variation in women are contributing risk factors for breast and ovarian cancers (Brinton *et al*., 1988; Kelsey *et al*., 1993). Literature data suggests that heavy metals such as Cd is known to have inverse relation with FSH (Dutta *et al*., 2021). In present study, reduced LH and FSH levels were observed among occupational brick workers, that is consistent with earlier study of Lafuente *et al*. (2003), which

concluded that Cd exerts dose dependent effects on the secretory patterns of the pituitary gonadotropins LH and FSH (Lafuente *et al*., 2003). Our findings are also supported by previous study of Pollack *et al*., (2011), where Cd exposure among healthy menstruating women resulted in reduced levels of FSH amplitude (Kawai *et al*., 2002; Pollack *et al*., 2011). This in turns, disrupts the overall ovarian function of hormone synthesis. The results of metal toxicity on LH and FSH concentrations here contrast with our previous results obtained for men (mentioned in chapter 2) where heavy metals induced elevated gonadotropin (LH, FSH) concentrations in blood, which might be due to lack of negative feedback mechanism controlling HPT axis or the presence of blood testes barrier (BTB) which prevented the direct interaction of testis with metals. Furthermore, previous reports also found that concentration and longevity of exposure of the rats under the influence of metals such as Pb, damage the signaling systems in the hypothalamus and pituitary gland, including their hyperfunction, leading to over-production of gonadotropin hormone GnRH and LH (Morphology, 2005). Whereas in case of females HPG axis is directly affected prior to metal toxicity, for example Cd is considered as a metallo-estrogen and is capable to join the oestrogen receptors alpha and beta and stimulate it (Rzymski *et al*., 2015).

Heavy metals and Prolactin

Metals are known to exert dose-dependent effects on both prolactin and ACTH secretion. Studies indicate that exposure to minute levels of Cd may induce increase in plasma prolactin levels (Lafuente *et al*., 2003). Our findings presented that with circulation of heavy metals in blood, the occurrence of hyperprolactinemia among women working at brick kiln sites was also witnessed. The increase in prolactin levels might be due to induction by cortisol hormone (Bridges & Bridges, 2018). Our study has also reported increased cortisol levels in blood of female workers that might be due to occupational exposure to environmental stress. It has been reported that exposure to physical or

psychological stress results in activation of the hypothalamic pituitary system (HPA) system causing secretion of corticotropin releasing hormone (CRH) from the hypothalamus, which in turn stimulates adrenocorticotropin's to release adrenocorticotropic hormone (ACTH) and beta-endorphin from the hypothalamus, and eventually the release of corticosteroids from the adrenal cortex (Einarsson *et al*., 2008). We found decrease in estradiol and progesterone levels among brick workers that might be due to increased level of cortisol in blood plasma, which is known to decrease sex steroids production such as estradiol, by mediating its effects through malfunctioning of granulosa cell within the follicle, which ultimately results in deterioration of oocyte quality (Baker *et al*., 2013; Prasad *et al*., 2016).

Heavy metals and genotoxicity

Brick kilns release enormous amounts of metals into the atmosphere, which are deposited in the human body and bring genotoxic reactions in cells, affecting gene function. In the present study, we found multiple disease-causing mutations and polymorphisms in two selected exons of the ABCG2 gene, which is a protein-coding gene using female samples. Although this gene is expressed in BBB, liver, and intestine as well, however, significant expression of this protein has been observed in the placenta, which may suggest a potential role for this molecule in placenta tissue (Mao, 2008; To *et al*., 2020). Multiple variants encoding different isoforms have been found for this gene in the intestine and placenta (Kobayashi *et al*., 2005; Zamber *et al*., 2003). The polymorphisms in ABCG2 gene expression that resulted in inhibition of these transporters might bring feto-toxicity in human placenta (Karttunen *et al*., 2017). Studies reported that genetic polymorphisms in ABCG2 expression disrupt their normal function, resulting in pathological conditions. Previous studies have found that an epistatic interaction pair of polycystin-2 (PKD2) and ABCG2

gene (rs2728121: rs2231137) is found among Chinese individuals; that is associated with increased urate levels since it acts as a urate transporter. This SNP pair is identified to influence the development of gout from both hyperuricemia and healthy individuals males (Dong *et al*., 2020). Another study conducted by Nie *et al*. (2018) showed that ABCG2 exhibit protective role against oxidative stress in colorectal cancer. ABCG2 mediates its actions by inhibition of expression of inflammatory genes and reducing oxidative stress (Nie *et al*., 2018). Former studies also reported that toxicity via chemotherapy induces genetic polymorphisms in cells, bringing adverse actions. The found SNP rs2231137 is also associated with ischemic stroke, as testified in Chinese population (Liu *et al*., 2018). Further, literature review suggests that polymorphisms in different regions of the ABCG2 gene are associated with ischemic strokes, hyperuricemia, and gout (Dong *et al*., 2020; Liu *et al*., 2018; Yang *et al*., 2021).

The first single nucleotide variation was found in the brick kiln worker sample at genomic position g.91361G>A where a single substitution of G with A was present, which resulted in a replacement of valine (V) with methionine (M) shift at position 12 in the protein structure of ABCG2 protein. This observed change presents a missense variation that brings alleles to change C>T and is a naturally occurring nonsynonymous SNP (Itoda *et al*., 2003). This variation has been reported in humans 43 times and assigned reference ID as rs2231137. This variant (V12M) has been studied worldwide and its high prevalence has been reported in South and East Asia, America, Europe, Japan and Africa (Bäckström *et al*., 2003; Honjo *et al*., 2002; Iida *et al*., 2002; Zamber *et al*., 2003). As ABCG2 acts as a G protein coupled receptor, analysis of protein structure shows that Val12Met (G>A) is present near amino terminal of ABCG2 receptor protein within the cell. Previous literature suggested that the novel ABCG2 mutation (ABCG2-M71V) is also involved in the removal

of uric acid, causing build-up in serum uric acid levels resulting in development of gout. Studies have indicated that polymorphism that result in variant rs2231137 is linked with decrease in protein expression of ABCG2 in vitro as well as altered substrate specificity (Maekawa *et al*., 2006). The rs2231137 variant (G>A) has been recently reported in patients with esophageal squamous cell carcinoma, exposed to chemotherapy radiation (Yang *et al*., 2021). The study depicted that chemotherapy toxicity brings genetic polymorphisms in cells, bringing adverse actions. The mentioned SNP rs2231137 is also associated with ischemic stroke, seen in Chinese population (Liu *et al*., 2018). Other studies found that an epistatic interaction pair of polycystin-2 (PKD2) and ABCG2 gene (rs2728121: rs2231137) is found among Chinese individuals; that is associated with urate levels, because ABCG2 gene acts as a urate transporter. This SNP pair is identified to influence the development of gout from both hyperuricemia and healthy individuals males (Dong *et al*., 2020).

Two more novel variations were found in sample **AF2**, these included disease-causing mutation at position g.91474G>A that did not affected the amino acid sequence, leaving normal protein structure; and a novel frameshift mutation (F52*) at genomic position g.91482delinsAA, where events of deletion followed by insertion were evident. The amino acid chain was shifted with the substitution of phenylalanine codon to stop codon at position 52 in protein structure, that may hinder with the normal function of ABCG2 protein. Analysis of worker sample **AF58** depicted 11 novel polymorphisms at genomic positions g.91410T>A, g.91414G>A, g.91416C>A, g.91419T>A, g.91427G>A, g.91436T>A, g.91450C>T, g.91454T>A, g.91456C>A, g.91459T>A, g.91461G>A (rs142634180), and g.91463G>A. One of the above-mentioned polymorphisms has been reported earlier at g.91461G>A and has assigned rsID as rs142634180, it presents a single nucleotide substitution which brings protein change from arginine to glutamine at position

45 in protein structure (R45Q). This variant (rs142634180) of ABCG2 is a missense variant and has been cited nowhere so far. Novel disease-causing mutations were also found at g.91414G>A and g.91450C>T, where single substitution of G>A and C>T occurred respectively, however, no change in AA sequence was present and normal protein was produced. Three novel polymorphisms were observed in worker sample **AF59**, present at genomic positions g.91416C>A, g.91427G>C, and g.91430G>C where single substitution of nucleotide in coding sequence of gene were identified. A novel disease-causing mutation was also seen at position g.91440_91441delinsAA where deletion and insertion in nucleotide sequence shifted AA sequence as well from serine to lysine at AA position 38.

The sequence analysis of sample **BF16** (control) showed presence of two novel disease-causing mutations; at genomic position g.91474G>A and g.91507G>A, where a single substitution at both spots did not bring protein structural change through AA substitution. the variant g.91521C>T has been reported before but no rsID has been assigned yet. Analysis of another control sample **AF50** revealed presence of four novel variants at g.91346G>C, g.91416C>A, g.91430G>A, and g.80676T>A. A disease-causing mutation was seen at position g.91446A>T where a single base exchange caused replacement of histidine with leucine at position 40.

Previous studies also reported ABCG2 polymorphisms, linked with development of risk for metabolic diseases such as gout, hyperuricemia, and other ischemic strokes. Our results found the presence of multiple single nucleotide polymorphisms and disease-causing mutations, that alter the function of ABCG2 gene and may affect the normal healthy pregnancy.

Health status of brick kiln girls and boys

Investigating the wellbeing status of children living/working at the brick kiln industries, a comparison of different demographic parameters between the two groups were made. Most of the children living at brick kiln sites were working unpaid, bare foot and were directly exposed to contaminated soil. The sanitary system was very underprivileged; the hygienic and nutritional conditions were quite poor. Education awareness and health situations were also pitiable. The education level also varied in control and exposed group; many of the exposed children were illiterate. Most of the children were facing skin problems, eye problem, and stomach problems, bones issues.

Heavy metal associated health problems in children

There was found a significant presence of heavy metals, Cd, Cr, Zn, and Ni in blood of brick kiln children. In the environment, these metals are found in lower concentrations in soil, water, and air. They might be unsafe, when their levels increase from low to high (Martin & Griswold, 2009). In current study, decreased BMI was observed in children living in the vicinity of brick kiln sites. Some heavy metals such as Cd is known to effect body fat mass and reduces BMI (Castagnetto *et al*., 2002). Decreased BMI in our results might be due to the high level of Cd detected in blood of exposed group. In our survey reports, we found that children living in the vicinity of brick kilns were having bones problems which might be due to the iron deficiency caused by elevated level of Cd detected in blood. Padilla *et al*. (2010) have reported that atmospheric introduction to heavy metals may induce changes in human weight (gain/loss) and cause metabolic disorders such as obesity. The displacement of metals (Zn and Cr) may effect metabolic process such as production of energy, and carbohydrate tolerance (Padilla *et al*., 2010). Cd is potent to attach itself with different amino acids such as cysteine, glutamate, histamine and aspartate ligands and ultimately, causing iron deficiency (Castagnetto *et al*., 2002). Cd can also replace Zn, which is a part of metallothionein, hence, preventing its

availability as free radical scavenger within the cell (Rzymski *et al*., 2014). The findings of present study showed high level of Zn in blood of exposed group. Previously, no such observations have been reported so far. The present results further showed higher concentrations of Cr and Ni in blood of brick kiln children. As Ni is known to possess haematotoxic, reprotoxic, immunotoxic, genotoxic, neurotoxic and carcinogenic effects, it is expected that there is a risk for the development of reproductive disorders and puberty interfering changes in exposed children (H. Lu *et al*., 2005).

Heavy metal linked hematological disturbance in children

Kamal *et al*. (2016) have shown in their respective study, that exposure to brick kiln emitted PAH affect the hematological parameters in blood such as serum c-reactive proteins (CRP), WBCs, Hb, RBC, and PLT counts (Kamal *et al*., 2015). The present study reported similar results, where we observed that children residing at brick kiln sites experience decreased concentrations of RBCs and Hb level in blood due to exposure to heavy metals. The current findings are in agreement with the previous work of Jahan *et al*. (2016), where decrease in RBCs number and Hb was evident among brick kiln male workers, whose blood was found to contain higher concentration of heavy metals (Cd, Cr, Ni) (Jahan *et al*., 2016). Heavy metals are known to inhibit haem and hemoglobin production by lowering δ-aminolevulinic acid dehydratase (ALAD) levels, which convert ALA into porphobilinogen (Gómez *et al*., 2012). Higher ALA and lowered prophobilinogen levels cause generation of reactive oxygen species, which disrupt RBCs morphology and survival (Gómez *et al*., 2012). The levels of MCH, MCHC, HCT and Hb were slightly decreased in present study that might be due to oxidative stress caused by metals, leading to alteration in RBCs morphology and ultimately, decreasing level of Hb as previously suggested by Zhao *et al*. (2007) (Zhao *et al*., 2007).

Heavy metals and oxidant/antioxidant system

The oxidant/antioxidant system presents the sophisticated network within the human body, that neutralizes the toxic effects of ROS on macromolecules (Lucesoli *et al*., 1999). Cells produce various antioxidant enzymes such as SOD, CAT and GPx, and peptides with thiol groups, that react against the oxidant challenges utilizing molecular oxygen to initiate mechanisms that produce essential energy (Gómez *et al*., 2012). ROS can also be directly neutralized by small molecular weight antioxidants that includes Vit E, Vit C, TRX, NADPH, GSH, and trace metals (Dandekar *et al*., 2002). This intricate approach has the potential to sustain an redox balance as well as to reduce the molecular disruption caused by ROS within a cell (Bonnefont *et al*., 2001; Gómez *et al*., 2012). It is well documented that decreased level of antioxidant enzymes and increased level of ROS present the homeostatic imbalance in the human body. In current study, lowered levels of SOD, POD and CAT were found. Cr exerts its toxic effects on enzymes such as cytochromes oxidase, catalase and peroxidase having iron as their component (de-Angelis *et al*., 2017). Previously, Wąsowicz *et al*. (2001) found that work-related exposure to heavy metals e.g., Cd or Pb disturbs the normal homeostasis of the body by disturbing antioxidant capacity of the body (Wąsowicz *et al*., 2001). The decreased level of antioxidant enzymes in present study might be due to the increased production of ROS, supported by previous reports as well (Gómez *et al*., 2012; Jahan *et al*., 2016).

Some heavy metals, including Cd, Ni and Cr induce the production of ROS, increasing the oxidative stress and disrupting cell membrane in various tissues (Goyal *et al*., 2015; Kamal *et al*., 2015). The reaction between Cr and biological reductants (e.g thiols and ascorbate) results in ROS formation within cell, that ultimately results into DNA and proteins damage (Stohs & Bagchi, 1995). Ni is known to induce cellular toxicity and carcinogenicity via generation of ROS (Mohammadi *et al*., 2018). The present study showed a marked increase in number of ROS among children living at brick kiln sites.

This increase in ROS production might be due to significantly high level of Ni, Cr and Cd detected in their blood.

Stress and reproduction

Human reproduction is affected by exposure to severe stress challenges (Kalantaridou *et al*., 2010). Stress is believed to disturb the normal reproductive processes by reducing gonadotrophin secretion, which subsequently reduces the production of gonadal steroids (Ferin, 1999). This is mediated by stress activation of the HPA axis, which elicits the hypothalamic release of corticotrophin-releasing hormone (CRH) and arginine vasopressin (Kalantaridou *et al*., 2010). Increase in concentration of these neurohormones subsequently, increases pituitary ACTH levels and raises synthesis of adrenal cortisol (Ferin, 1999; Kalantaridou *et al*., 2010). The increased level of CRH negatively affects hypothalamic GnRH pulsatility and reduces sensitivity of GnRH at the pituitary, resulting in gonadotrophins reduction (Kalantaridou *et al*., 2010). In present study, increased level of cortisol was evident among boys and girls exposed to brick kiln emissions. We have presented our findings on cortisol concentrations in different age ranges, however, there is not much data available on the age wise cortisol concentration in growing girls and boys due to involvement of multiple physiological and environmental factors, which affect pubertal onset. The physically active lifestyle and decrease levels of cortisol reflect the possible cause for precocious puberty (menarche at early age) in girls, while delayed puberty is expected to occur in boys under same circumstances. As children monitored were at pre-pubertal stage, the risk of progression of reproductive diseases is quite high in them and is thought to be mediated through stimulation of HPA axis.

Puberty, Growth and role of HPA axis

The GH has essential role in determining puberty, gametogenesis and fertility of an individual (Le-Gac *et al*., 1993). It is responsible for determining the secondary sexual

characteristics at pubertal development (Nathanson *et al*., 1941; Wanjari *et al*., 2019). In the present study, the level of GH was significantly decreased in brick kiln group in comparison with the control group. Decrease in GH may affect the reproductive potential of brick kiln workers by inducing precocious/delayed puberty. As puberty is determined by the interplay of adrenarche and pubarche, the increased levels of stress hormone (cortisol) might act along with decreased GH levels and may affect appearance of secondary sexual characters and puberty. We are reporting concentrations of growth hormone in different age groups for both girls and boys in brick kiln and control children for the first time in any industrial setup. No previous relevant reports are available on the age wise GH concentration in growing girls and boys for comparison of our results. Steroids act at the pituitary and hypothalamic levels and therefore, modulate the production of GH and support the sex related patterns of pulsatile GH secretions (Devesa *et al*., 1991; Eden *et al*., 1987). Increased levels of cortisol indicate the disturbance of normal HPG axis as well, that poses a risk for the delayed puberty in boys and precocious puberty in girls due to their active and hectic lifestyle. Non-significant negative correlation between cortisol and GH was seen. These results of correlation analysis have been supported by previous findings where GH and cortisol are known to have varied relationship (Duclos *et al.,* 2007).

DNA damage

Studies have confirmed that children who are occupationally as well as environmentally exposed to pollutants such as PAH, have increased level of DNA damage in blood cells (Cavallo *et al*., 2009; Wang *et al*., 2010). The findings of current study showed that children of different age group living/working in brick kiln industries experienced more DNA damage in cells. We observed a significant reduction in head length and notable increase in tail length among brick kiln worker group as compared to

control, where intact DNA was observed. Our results also showed a substantial decrease in percentage DNA in head region, and significant rise in percentage DNA in tail. It might be due to different heavy metals that are incorporated and cause genotoxicity.

GENERAL CONCLUSION

The results from first chapter concluded that brick kiln individuals experience lowered body weight, disturbed BMI and had multiple health issues including skin allergies, asthma, stomach and kidney disorder, and other diseases, where occupational factors played their role; elemental data on hair and blood samples showed that the average values of metals Co, Ni, Ti, Cr, Mn and Fe in blood were found higher than permissible limits recommended by FDA; SEM/EDS analysis of hair depicted the presence of macro-element with average levels in the order of: K > S > Ca > P > Cl and a micro-element profile in the order of: Rb > Fe > Mn > Cu > Sr > Zn. The PIXE analysis of soil showed the presence of trace elements and heavy metals including Si, S, Cl, Ar, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, and Zn. The results here confirmed that blood and human scalp hair represent an excellent screening biomatrices, in which xenobiotic compounds such as metals and airborne particulate matter can be incorporated. Conclusively, it can be stated that external and internal deposition of heavy metal on body of brick kiln workers is much higher than that of normal population that poses health risks among occupational individuals and may raise health problems and diseases.

The findings of second chapter, where men subjects were included showed that heavy metal burden in blood of workers caused alteration in blood parameters, decreased antioxidant enzyme levels, increased oxidant production; elevated stress response resulting in high cortisol concentrations, which affected HPG axis by altered production of pituitary gonadotropins (LH, FSH); and lowered production of testosterone. Therefore, it is determined that long-term compromised antioxidant enzyme levels in blood, increased production of ROS, higher oxidative stress and disturbed production of gonadotropins and sex steroids serve as contributing factors in disturbing the metabolic health of brick kiln

men, putting them at the risk for the development of other health problems that ultimately affect their reproductive outcomes.

The outcomes obtained from chapter 3 concluded that occupational exposure to environmental pollutants increased heavy metal burden in blood of labor; observations of reproductive health and fertility indicators suggested that the female workers experienced issues of malnutrition, poor hygiene, irregular menstruation and missed abortions along with other pathological conditions and were at the verge of developing major reproductive disorders with complication in conception and pregnancy; moreover, blood parameters, oxidant levels, antioxidant enzymes concentrations, total protein content, lipid profile, and plasma concentrations of reproductive hormones/stress hormones were compromised and disturbed. There was also found a negative correlation of cortisol with the reproductive hormones (LH, FSH, E, P), while positive correlation with prolactin was seen.

The genetic analysis performed in chapter 4 was based on the previous results depicted earlier in fertility analysis (from chapter 3), where the rate of miscarriage in brick kiln workers was greater than control. We found the presence of 28 novel SNPs in two studied exons of ABCG2 gene from brick kiln and control population, where most of the mutations were disease causing and others were polymorphisms. Therefore, it can be inferred from the present findings that occurrence of these polymorphisms might interfere with the normal function of ABCG2 protein expressed in placental tissue, bringing fetal exposure to multidrug metabolites and xenobiotics compounds and increasing chances of miscarriage.

It is determined from the outcomes of fifth chapter that brick kiln emitters including heavy metal burden in blood may affect different body functions in growing boys and girls; it is reported that children residing at brick kiln areas were underweight and had

lowered BMI; altered hematology parameters, lowered antioxidant enzymes concentrations and increased oxidative stress response; hormonal analysis showed decreased GH and increased cortisol levels along with induced DNA damage in blood cells. Increased levels of cortisol indicated the disturbance of normal HPG and HPA axis as well, that poses a risk for the reproductive problems in girls and boys.

RECOMMENDATIONS AND FUTURE PERSPECTIVES

Brick kiln industries release enormous amounts of environmental pollutants and hazardous gases, which affect the surrounding atmosphere (Khan *et al*., 2019). The present study reported that occupational exposure to environmental pollutants increases heavy metal burden in blood of working subjects including men, women, and children; and might impart deleterious effects on general health as well as reproductive health as depicted by multiple studied variables (BMI, blood, biochemical and hormonal analysis). We also found that long-term prevalence of heavy metal burden in blood, compromised antioxidant enzyme levels in blood, increased production of ROS, higher oxidative stress and disturbed production of gonadotropins and sex steroids might disturb the metabolic and biochemical health of brick kiln residents, putting them at the risk for the development of other metabolic and reproductive disorders. Thus, it is recommended that there should be proper monitoring of health status of working individuals at kiln sites, and it is a need of the hour for the higher authorities to ensure the availability of primary health care facilities in all the industrial setups especially at brick kilns.

Further, based on our survey reports, it is suggested that there must be accessibility to proper sanitation, proper hygiene, and health care facilities with availability of registered medical staff for monitoring the health status of labor on regular basis, so that problems associated with maternal and child health prevailing at kilns could be addressed on time.

There is a need to take actions at the government level to design, implement and monitor the defined rules and regulations so that adverse effects of brick kilns on human health and environment can be inhibited as suggested previously (Khan *et al*., 2019a). The government of Pakistan has developed a data sheet for recording statistical data for labor

working at Punjab province. However, other provinces should be monitored as well. Moreover, it is recommended that new technologies for making bricks should be introduced and practiced that will decrease the environmental effects of brick kiln emissions on public health and will improve lifestyle habits of local community.

Lastly, it is stated that as hair and blood are considered as good predictors of health, disease and socio-demographic factors, further similar studies can be planned to investigate the correlation of metal deposition in different biological tissues and their induced effects over prolonged deposition through use of powerful techniques such as PIXE and SEM/EDS. Additional such studies are needed to evaluate the molecular basis of the risks associated with heavy metals burden and other pollutants in blood.

REFERENCES

About Child & Teen BMI | Healthy Weight, Nutrition, and Physical Activity | CDC. (n.d.). Retrieved May 9, 2022, from https://www.cdc.gov/healthyweight/assessing/bmi/childrens_bmi/about_childrens_bmi.html

Achakzai, K., Khalid, S., Adrees, M., Bibi, A., Ali, S., Nawaz, R., & Rizwan, M. (2017). Air pollution tolerance index of plants around brick kilns in Rawalpindi, Pakistan. *Journal of Environmental Management*, *190*, 252–258. https://doi.org/10.1016/j.jenvman.2016.12.072

Achakzai, K., Khalid, S., & Bibi, A. (2015). Determination of Heavy Metals in Agricultural Soil Adjacent to Functional Brick Kilns: A Case Study of Rawalpindi. *Science, Technology and Development*, *34*(3), 122–129. https://doi.org/10.3923/std.2015.122.129

Achmad, R. T., Budiawan, & Auerkari, E. I. (2017). Effects of chromium on human body. In *Annual Research and Review in Biology*. https://doi.org/10.9734/ARRB/2017/33462

Ackova, D. G. (2018). *Plant Science Today*. *5*, 15–19.

Adkison, L. R. (2012). Genetics of Metabolic Disorders. In *Elsevier's Integrated Review Genetics*. https://doi.org/10.1016/b978-0-323-07448-3.00004-2

Ahmad, N., Hussain, J., Ahmad, I., & Ahmad, N. (2020). Estimation of health risk to humans from heavy metals in soil of coal mines in Harnai , Balochistan of coal mines in Harnai , Balochistan. *International Journal of Environmental Analytical Chemistry*, *00*(00), 1–12. https://doi.org/10.1080/03067319.2020.1776863

Akesson, A., Berglund, M., Schütz, A., Bjellerup, P., Bremme, K., & Vahter, M. (2002). Cadmium exposure in pregnancy and lactation in relation to iron status. *American*

Journal of Public Health. https://doi.org/10.2105/AJPH.92.2.284

Alaee, S. (2018). *Air Pollution and Infertility – A letter to Editor*. *6*(4), 72–73.

Alam, R. T., Zeid, E. H. A., & Imam, T. S. (2017). Protective role of quercetin against hematotoxic and immunotoxic effects of furan in rats. *Environmental Science and Pollution Research*. https://doi.org/10.1007/s11356-016-8108-9

Alaraidh, I. A., Alsahli, A. A., & Abdel Razik, E. S. (2018). Alteration of antioxidant gene expression in response to heavy metal stress in Trigonella foenum-graecum L. *South African Journal of Botany*, *115*, 90–93. https://doi.org/10.1016/J.SAJB.2018.01.012

Ali, I. (2010). The quest for active carbon adsorbent substitutes: Inexpensive adsorbents for toxic metal ions removal from wastewater. *Separation and Purification Reviews*, *39*(3–4), 95–171. https://doi.org/10.1080/15422119.2010.527802

Alsanosi, S. M. M., Skiffington, C., & Padmanabhan, S. (2014). Pharmacokinetic Pharmacogenomics. *Handbook of Pharmacogenomics and Stratified Medicine*, 341–364. https://doi.org/10.1016/B978-0-12-386882-4.00017-7

Amadi, C. N., Igweze, Z. N., & Orisakwe, O. E. (2017). Heavy metals in miscarriages and stillbirths in developing nations. In *Middle East Fertility Society Journal*. https://doi.org/10.1016/j.mefs.2017.03.003

Andreão, W. L., Albuquerque, T. T. A., & Kumar, P. (2018). Excess deaths associated with fine particulate matter in Brazilian cities. *Atmospheric Environment*, *194*, 71–81. https://doi.org/10.1016/j.atmosenv.2018.09.034

Annangi, B., Bonassi, S., Marcos, R., & Hernández, A. (2016). Biomonitoring of humans exposed to arsenic, chromium, nickel, vanadium, and complex mixtures of metals by using the micronucleus test in lymphocytes. *Mutation Research - Reviews in Mutation*

Research, *770*, 140–161. https://doi.org/10.1016/j.mrrev.2016.03.003

Apykhtina, O. L., Dybkova, S. M., Sokurenko, L. M., & Chaikovsky, Y. B. (2018). Cytotoxic and genotoxic effects of cadmium sulfide nanoparticles. *Experimental Oncology*, *40*(3), 194–199. https://doi.org/10.31768/2312-8852.2018.40(3):194-199

Asia, S. (n.d.). *Human Labour & Animal Welfare*.

Aslam, B., Khalil, U., Azam, U., & Maqsoom, A. (2021). A correlation study between weather and atmosphere with COVID-19 pandemic in Islamabad, Pakistan. *Spatial Information Research*, *29*(4), 605–613. https://doi.org/10.1007/S41324-020-00366-2/TABLES/3

Azeh Engwa, G., Udoka Ferdinand, P., Nweke Nwalo, F., & N. Unachukwu, M. (2019). Mechanism and Health Effects of Heavy Metal Toxicity in Humans. *Poisoning in the Modern World - New Tricks for an Old Dog?* https://doi.org/10.5772/intechopen.82511

Bäckström, G., Taipalensuu, J., Melhus, H., Brändström, H., Svensson, A. C., Artursson, P., & Kindmark, A. (2003). Genetic variation in the ATP-binding Cassette Transporter gene ABCG2 (BCRP) in a Swedish population. *European Journal of Pharmaceutical Sciences*, *18*(5), 359–364. https://doi.org/10.1016/S0928-0987(03)00038-1

Bailey-Dell, K. J., Hassel, B., Doyle, L. A., & Ross, D. D. (2001). Promoter characterization and genomic organization of the human breast cancer resistance protein (ATP-binding cassette transporter G2) gene. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression*, *1520*(3), 234–241. https://doi.org/10.1016/S0167-4781(01)00270-6

Baker, M. R., Gobush, K. S., & Vynne, C. H. (2013). Review of factors influencing stress hormones in fish and wildlife. In *Journal for Nature Conservation*. https://doi.org/10.1016/j.jnc.2013.03.003

Banza, C. L. N., Nawrot, T. S., Haufroid, V., Decrée, S., De Putter, T., Smolders, E., Kabyla, B. I., Luboya, O. N., Ilunga, A. N., Mutombo, A. M., & Nemery, B. (2009). High human exposure to cobalt and other metals in Katanga, a mining area of the Democratic Republic of Congo. *Environmental Research*. https://doi.org/10.1016/j.envres.2009.04.012

Baranowska-wójcik, E., Szwajgier, D., Oleszczuk, P., & Winiarska-mieczan, A. (2020). *Baranowska-Wójcik2020_Article_EffectsOfTitaniumDioxideNanopa.pdf*. 118–129.

Barba, C., Cavalli-Sforza, T., Cutter, J., Darnton-Hill, I., Deurenberg, P., Gill, T., James, P., & Ko, G. (2004). Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. *Lancet*. https://doi.org/10.1016/S0140-6736(03)15268-3

Baum, E. (2010). Black Carbon From Brick Kilns Brick Making Summary. *Clean Air (Task Force)*. file:///Users/yangyang/YANGYANGXU/work/NewAllreference_other/Black_Carbon_from_Brick_Kilns.pdf

Begum, B. A., Biswas, S. K., & Hopke, P. K. (2011). Key issues in controlling air pollutants in Dhaka, Bangladesh. *Atmospheric Environment*. https://doi.org/10.1016/j.atmosenv.2010.10.022

Bhat, M. S., Afeefa, Q. S., Ashok, K. P., & Bashir, A. G. (2014). Brick kiln emissions and its environmental impact: A Review. *Journal of Ecology and The Natural Environment*, *6*(1), 1–11. https://doi.org/10.5897/JENE2013.0423

Bhatt, R. V. (2000). Environmental influence on reproductive health. *International Journal of Gynecology and Obstetrics*, *70*(1), 69–75. https://doi.org/10.1016/S0020-

7292(00)00221-6

Bhattacharya, S. (2020). The Role of Probiotics in the Amelioration of Cadmium Toxicity. *Biological Trace Element Research*. https://doi.org/10.1007/s12011-020-02025-x

Bhutta, Z. A., & Hafeez, A. (2015). What can Pakistan do to address maternal and child health over the next decade? In *Health Research Policy and Systems*. https://doi.org/10.1186/s12961-015-0036-5

Bhutta, Z. A., Hafeez, A., Rizvi, A., Ali, N., Khan, A., Ahmad, F., Bhutta, S., Hazir, T., Zaidi, A., & Jafarey, S. N. (2013). Reproductive, maternal, newborn, and child health in Pakistan: Challenges and opportunities. *The Lancet*, *381*(9884), 2207–2218. https://doi.org/10.1016/S0140-6736(12)61999-0

Bircsak, K. M., Moscovitz, J. E., Wen, X., Archer, F., Yuen, P. Y. S., Mohammed, M., Memon, N., Weinberger, B. I., Saba, L. M., Vetrano, A. M., & Aleksunes, L. M. (2018). Interindividual regulation of the breast cancer resistance protein/abcg2 transporter in term human placentas s. *Drug Metabolism and Disposition*, *46*(5), 619–627. https://doi.org/10.1124/DMD.117.079228/-/DC1

Bonde, J. P. E. (2013). Occupational causes of male infertility. In *Current Opinion in Endocrinology, Diabetes and Obesity*. https://doi.org/10.1097/MED.0b013e32835f3d4b

Bonnefont-Rousselot, D., Therond, P., Beaudeux, J.-L., Peynet, J., Legrand, A., & Delattre, J. (2001). Aging and oxidative stress. Which potential markers? *Annales de Biologie Clinique*, *59*(4), 453–459.

Bozkurt, Z., Üzmez, Ö. Ö., Döğeroğlu, T., Artun, G., & Gaga, E. O. (2018). Atmospheric concentrations of SO2, NO2, ozone and VOCs in Düzce, Turkey using passive air samplers: Sources, spatial and seasonal variations and health risk estimation.

Atmospheric Pollution Research, *9*(6), 1146–1156. https://doi.org/10.1016/j.apr.2018.05.001

Bridges, R. S., & Bridges, R. S. (2018). Prolactin: Regulation and Actions. In *Oxford Research Encyclopedia of Neuroscience*. https://doi.org/10.1093/acrefore/9780190264086.013.61

Brinton, L. A., Schairer, C., Hoover, R. N., & Fraumeni, J. F. (1988). Menstrual factors and risk of breast cancer. *Cancer Investigation*. https://doi.org/10.3109/07357908809080645

Bromberg, P. A. (2016). Mechanisms of the acute effects of inhaled ozone in humans. *Biochimica et Biophysica Acta - General Subjects*, *1860*(12), 2771–2781. https://doi.org/10.1016/j.bbagen.2016.07.015

Castagnetto, J. M., Hennessy, S. W., Roberts, V. A., Getzoff, E. D., Tainer, J. A., & Pique, M. E. (2002). MDB: the metalloprotein database and browser at the Scripps Research Institute. *Nucleic Acids Research*, *30*(1), 379–382.

Cavallo, D., Ursini, C. L., Rondinone, B., & Iavicoli, S. (2009). Evaluation of a suitable DNA damage biomarker for human biomonitoring of exposed workers. *Environmental and Molecular Mutagenesis*, *50*(9), 781–790.

CCAC secretariat. (2018). *Climate and clean air coalition*. https://doi.org/http://www.ccacoalition.org/en/news/pakistan-moves-toward-environmentally-friendly-andcost-effective-brick-kilns

Chance, B., & Maehly, A. C. (1955). [136] Assay of catalases and peroxidases. {black small square}. *Methods in Enzymology*. https://doi.org/10.1016/S0076-6879(55)02300-8

Chen, S. Y., Wang, J., Yu, G. Q., Liu, W., & Pearce, D. (1997). Androgen and glucocorticoid receptor heterodimer formation: A possible mechanism for mutual inhibition of

transcriptional activity. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.272.22.14087

Chen, X., Li, Y., Zhang, B., Zhou, A., Zheng, T., Huang, Z., Pan, X., Liu, W., Liu, H., Jiang, Y., Sun, X., Hu, C., Xing, Y., Xia, W., & Xu, S. (2018). Maternal exposure to nickel in relation to preterm delivery. *Chemosphere*, *193*, 1157–1163. https://doi.org/10.1016/j.chemosphere.2017.11.121

Cherfi, A., Abdoun, S., & Gaci, O. (2014a). Food survey : Levels and potential health risks of chromium , lead , zinc and copper content in fruits and vegetables consumed in Algeria. *FOOD AND CHEMICAL TOXICOLOGY*, *70*, 48–53. https://doi.org/10.1016/j.fct.2014.04.044

Cherfi, A., Abdoun, S., & Gaci, O. (2014b). Food survey: Levels and potential health risks of chromium, lead, zinc and copper content in fruits and vegetables consumed in Algeria. *Food and Chemical Toxicology*. https://doi.org/10.1016/j.fct.2014.04.044

Chiroma, T. M., & Ebewele, O. R. (2014). Comparative Assessement Of Heavy Metal Levels In Soil, Vegetables And Urban Grey Waste Water Used For Irrigation In Yola And Kano. *International Refereed Journal of Engineering and Science*, *3*(2), 1–09. www.irjes.com

Cohen, A. J., Brauer, M., Burnett, R., Anderson, H. R., Frostad, J., Estep, K., Balakrishnan, K., Brunekreef, B., Dandona, L., Dandona, R., Feigin, V., Freedman, G., Hubbell, B., Jobling, A., Kan, H., Knibbs, L., Liu, Y., Martin, R., Morawska, L., … Forouzanfar, M. H. (2017). Estimates and 25-year trends of the global burden of disease attributable to ambient air pollution: an analysis of data from the Global Burden of Diseases Study 2015. *The Lancet*, *389*(10082), 1907–1918. https://doi.org/10.1016/S0140-

6736(17)30505-6

Cohen, M. D., Kargacin, B., Klein, C. B., & Costa, M. (1993). Mechanisms of chromium carcinogenicity and toxicity. *Critical Reviews in Toxicology*. https://doi.org/10.3109/10408449309105012

Cousens, S., Blencowe, H., Stanton, C., Chou, D., Ahmed, S., Steinhardt, L., Creanga, A. A., Tunalp, Ö., Balsara, Z. P., Gupta, S., Say, L., & Lawn, J. E. (2011). National, regional, and worldwide estimates of stillbirth rates in 2009 with trends since 1995: A systematic analysis. *The Lancet*. https://doi.org/10.1016/S0140-6736(10)62310-0

Cumming, D. C., Quigley, M. E., & Yen, S. S. C. (1983). Acute suppression of circulating testosterone levels by cortisol in men. *Journal of Clinical Endocrinology and Metabolism*. https://doi.org/10.1210/jcem-57-3-671

Curtis, L., Rea, W., Smith-Willis, P., Fenyves, E., & Pan, Y. (2006). Adverse health effects of outdoor air pollutants. In *Environment International* (Vol. 32, Issue 6, pp. 815–830). Elsevier Ltd. https://doi.org/10.1016/j.envint.2006.03.012

Dandekar, S. P., Nadkarni, G. D., Kulkarni, V. S., & Punekar, S. (2002). Lipid peroxidation and antioxidant enzymes in male infertility. *Journal of Postgraduate Medicine*.

Das, K. K., & Das, S. N. (2008). *Nickel, its adverse health effects & oxidative stress - ProQuest*. *January 2017*.

Das, K. K., Reddy, R. C., Bagoji, I. B., Das, S., Bagali, S., Mullur, L., Khodnapur, J. P., & Biradar, M. S. (2019). Primary concept of nickel toxicity - An overview. *Journal of Basic and Clinical Physiology and Pharmacology*, *30*(2), 141–152. https://doi.org/10.1515/jbcpp-2017-0171

David, M., Afzal, M., Shoaib, M., Aman, F., Cloete, K. J., Turi, N., & Jahan, S. (2020). *Study*

of occupational exposure to brick kiln emissions on heavy metal burden , biochemical profile , cortisol level and reproductive health risks among female workers at Rawat , Pakistan.

David, M., Qurat-Ul-Ain, Afzal, M., Shoaib, M., Aman, F., Cloete, K. J., Turi, N., & Jahan, S. (2020). Study of occupational exposure to brick kiln emissions on heavy metal burden, biochemical profile, cortisol level and reproductive health risks among female workers at Rawat, Pakistan. *Environmental Science and Pollution Research*, *27*(35), 44073–44088. https://doi.org/10.1007/s11356-020-10275-4

David, M., Turi, N., Ain, Q. ul, Rahman, H., & Jahan, S. (2020). Evaluation of environmental effects of heavy metals on biochemical profile and oxidative stress among children at brick kiln sites. *Archives of Environmental and Occupational Health*, *0*(0), 1–9. https://doi.org/10.1080/19338244.2020.1854645

Davis, P. G., & Briggs, D. E. G. (1995). Fossilization of feathers. *Geology*. https://doi.org/10.1130/0091-7613(1995)023<0783:FOF>2.3.CO;2

de Angelis, C., Galdiero, M., Pivonello, C., Salzano, C., Gianfrilli, D., Piscitelli, P., Lenzi, A., Colao, A., & Pivonello, R. (2017a). The environment and male reproduction: The effect of cadmium exposure on reproductive functions and its implication in fertility. *Reproductive Toxicology*, *73*, 105–127. https://doi.org/10.1016/j.reprotox.2017.07.021

Deligeoroglou, E., & Creatsas, G. (2012). Menstrual disorders. *Endocrine Development*. https://doi.org/10.1159/000331697

Devesa, J., Lois, N., Arce, V., Diaz, M. J., Lima, L., & Tresguerres, J. A. F. (1991). The role of sexual steroids in the modulation of growth hormone (GH) secretion in humans. *The Journal of Steroid Biochemistry and Molecular Biology*, *40*(1–3), 165–173.

Domingo, J. L., & Rovira, J. (2020). Effects of air pollutants on the transmission and severity of respiratory viral infections. In *Environmental Research* (Vol. 187, p. 109650). Academic Press Inc. https://doi.org/10.1016/j.envres.2020.109650

Donato, J., & Frazão, R. (2016). Interactions between prolactin and kisspeptin to control reproduction. *Archives of Endocrinology and Metabolism*. https://doi.org/10.1590/2359-3997000000230

Dong, Z., Zhou, J., Jiang, S., Li, Y., Zhao, D., Yang, C., Ma, Y., He, H., Ji, H., Jin, L., Zou, H., & Wang, J. (2020). Epistatic interaction between PKD2 and ABCG2 influences the pathogenesis of hyperuricemia and gout. *Hereditas*, *157*(1). https://doi.org/10.1186/S41065-020-0116-6

Duclos, M., Guinot, M., & Bouc, Y. Le. (2007). Cortisol and GH: odd and controversial ideas. *Https://Doi.Org/10.1139/H07-064*, *32*(5), 895–903. https://doi.org/10.1139/H07-064

Duran, A., Tuzen, M., & Soylak, M. (2011). Speciation of Cr(III) and Cr(VI) in geological and water samples by ytterbium(III) hydroxide coprecipitation system and atomic absorption spectrometry. *Food and Chemical Toxicology*. https://doi.org/10.1016/j.fct.2011.04.016

Dutta, S., Gorain, B., Choudhury, H., Roychoudhury, S., & Sengupta, P. (2021). Environmental and occupational exposure of metals and female reproductive health. *Environmental Science and Pollution Research 2021 29:41*, *29*(41), 62067–62092. https://doi.org/10.1007/S11356-021-16581-9

Eden, S., Jansson, J. O., & Oscarsson, J. (1987). Sexual dimorphism of growth hormone secretion. *Growth Hormone—Basic and Clinical Aspects*, 129–151.

Einarsson, S., Brandt, Y., Lundeheim, N., & Madej, A. (2008). Stress and its influence on reproduction in pigs: A review. In *Acta Veterinaria Scandinavica*. https://doi.org/10.1186/1751-0147-50-48

Eljarrat, E., Li, F., Jahangir Alam, M., Stavropoulou, E., Manisalidis, I., Stavropoulos, A., & Bezirtzoglou, E. (2020). Environmental and Health Impacts of Air Pollution: A Review. *Frontiers in Public Health | Www.Frontiersin.Org*, *8*, 14. https://doi.org/10.3389/fpubh.2020.00014

Ercal, N., Gurer-Orhan, H., & Aykin-Burns, N. (2001). Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. *Current Topics in Medicinal Chemistry*.

Ercelawn, A., & Nauman, M. (2004). Unfree Labour in South Asia: Debt Bondage at Brick Kilns in Pakistan. *Economic and Political Weekly*. https://doi.org/10.2307/4415093

Fatima, Irm, G. A. Bhat, and A. N. G. K. (2011). *Impact of brick kiln emissions on the ambient air quality and vegetation: A case study of district Budgam*.

Fazio, F., Piccione, G., Tribulato, K., Ferrantelli, V., Giangrosso, G., Arfuso, F., & Faggio, C. (2014). Bioaccumulation of heavy metals in blood and tissue of striped mullet in two Italian lakes. *Journal of Aquatic Animal Health*, *26*(4), 278–284. https://doi.org/10.1080/08997659.2014.938872

Ferin, M. (1999). Stress and the Reproductive Cycle. *The Journal of Clinical Endocrinology & Metabolism*. https://doi.org/10.1210/jcem.84.6.5367

Flora, S. J. S., Mittal, M., & Mehta, A. (2008). Heavy metal induced oxidative stress & its possible reversal by chelation therapy. In *Indian Journal of Medical Research*. https://doi.org/10.1093/jexbot/53.366.1

Furness, R. W., & Rainbow, P. S. (2018). Heavy metals in the marine environment. In *Heavy Metals in the Marine Environment*. https://doi.org/10.1201/9781351073158

Galliano, A., Ye, C., Su, F., Wang, C., Wang, Y., Liu, C., Wagle, A., Guerin, M., Flament, F., & Steel, A. (2017a). Particulate matter adheres to human hair exposed to severe aerial pollution: consequences for certain hair surface properties. *International Journal of Cosmetic Science*. https://doi.org/10.1111/ics.12416

Galliano, A., Ye, C., Su, F., Wang, C., Wang, Y., Liu, C., Wagle, A., Guerin, M., Flament, F., & Steel, A. (2017b). Particulate matter adheres to human hair exposed to severe aerial pollution: consequences for certain hair surface properties. *International Journal of Cosmetic Science*, *39*(6), 610–616. https://doi.org/10.1111/ICS.12416

Georgescu, B., Georgescu, C., Dărăban, S., Bouaru, A., & Paşcalău, S. (2011). Heavy Metals Acting as Endocrine Disrupters. *Scientific Papers Animal Science and Biotechnologies*.

Geravandi, S., Goudarzi, G., & Mohammadi, M. J. (2015). Sulfur and Nitrogen Dioxide Exposure and the Incidence of Health Endpoints in Ahvaz , Iran Sadat Taghavirad ; Shokrollah Salmanzadeh. *Health Scope*, *4*(2), 2–7.

Gerber, M., Brand, S., Lindwall, M., Elliot, C., Kalak, N., Christian, H., Pühse, U., & Jonsdottir, I. H. (2012). Concerns regarding hair cortisol as a biomarker of chronic stress in exercise and sport science. In *Journal of Sports Science and Medicine*.

Ghosh, G. C., Khan, M. J. H., Chakraborty, T. K., Zaman, S., Kabir, A. H. M. E., & Tanaka, H. (2020). Human health risk assessment of elevated and variable iron and manganese intake with arsenic-safe groundwater in Jashore, Bangladesh. *Scientific Reports*, *10*(1), 1–9. https://doi.org/10.1038/s41598-020-62187-5

Gómez-Oliván, L. M., Corral-Avitia, A. Y., Carrasco-Urrutia, K. A., González-Granados, N. A., Galar-Martínez, M., Neri-Cruz, N., Islas-Flores, H., & Dublan-García, O. (2012). Oxidative stress in brickmakers of Juárez City, Chihuahua, México: Case-control study. *Advances in Bioscience and Biotechnology*, *3*(07), 1051.

Goyal, M., Demchuk, A. M., Menon, B. K., Eesa, M., Rempel, J. L., Thornton, J., Roy, D., Jovin, T. G., Willinsky, R. A., & Sapkota, B. L. (2015). Randomized assessment of rapid endovascular treatment of ischemic stroke. *New England Journal of Medicine*, *372*(11), 1019–1030.

Gunshin, H., Mackenzie, B., Berger, U. V., Gunshin, Y., Romero, M. F., Boron, W. F., Nussberger, S., Gollan, J. L., & Hediger, M. A. (1997). Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature*. https://doi.org/10.1038/41343

Gupta, A. Das, Das, S. N., Dhundasi, S. A., & Das, K. K. (2008). Effect of Garlic (Allium sativum) on Heavy Metal (Nickel II and ChromiumVI) Induced Alteration of Serum Lipid Profile in Male Albino Rats. *International Journal of Environmental Research and Public Health 2008, Vol. 5, Pages 147-151*, *5*(3), 147–151. https://doi.org/10.3390/IJERPH5030147

Guttikunda, S. K., Begum, B. A., & Wadud, Z. (2013a). Particulate pollution from brick kiln clusters in the Greater Dhaka region, Bangladesh. *Air Quality, Atmosphere and Health*, *6*(2), 357–365. https://doi.org/10.1007/s11869-012-0187-2

Ha, B. J. (2011). Spectroscopic evaluation on the chemical damage of hair by hydrogen peroxide. *Applied Chemistry for Engineering*.

Hanif, H., Waseem, A., Kali, S., Qureshi, N. A., Majid, M., Iqbal, M., Ur-Rehman, T., Tahir,

M., Yousaf, S., Iqbal, M. M., Khan, I. A., & Zafar, M. I. (2020). Environmental risk assessment of diclofenac residues in surface waters and wastewater: a hidden global threat to aquatic ecosystem. *Environmental Monitoring and Assessment*, *192*(4). https://doi.org/10.1007/s10661-020-8151-3

Hatma, R. D. (2011). *Lipid Profiles Among Diverse Ethnic Groups in Indonesia*.

Hauser, R., Skakkebaek, N. E., Hass, U., Toppari, J., Juul, A., Andersson, A. M., Kortenkamp, A., Heindel, J. J., & Trasande, L. (2015). Male reproductive disorders, diseases, and costs of exposure to endocrine-disrupting chemicals in the European Union. *Journal of Clinical Endocrinology and Metabolism*, *100*(4), 1267–1277. https://doi.org/10.1210/jc.2014-4325

Hayashi, I., Morishita, Y., Imai, K., Nakamura, M., Nakachi, K., & Hayashi, T. (2007). High-throughput spectrophotometric assay of reactive oxygen species in serum. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*. https://doi.org/10.1016/j.mrgentox.2007.04.006

He, L., Zhang, S., Hu, J., Li, Z., Zheng, X., Cao, Y., Xu, G., Yan, M., & Wu, Y. (2020). *On-road emission measurements of reactive nitrogen compounds from heavy-duty diesel trucks in China* *. https://doi.org/10.1016/j.envpol.2020.114280

Hollman, A. L., Tchounwou, P. B., & Huang, H.-C. (2016). *The Association between Gene-Environment Interactions and Diseases Involving the Human GST Superfamily with SNP Variants*. https://doi.org/10.3390/ijerph13040379

Honjo, Y., Morisaki, K., Mickley Huff, L., Robey, R. W., Hung, J., Dean, M., & Bates, S. E. (2002). Single-Nucleotide Polymorphism (SNP) Analysis in the ABC Half-Transporter ABCG2 (MXR/BCRP/ABCP1). *Http://Dx.Doi.Org/10.4161/Cbt.322*, *1*(6), 696–702.

https://doi.org/10.4161/CBT.322

Hu, H. (2002). HUMAN HEALTH AND HEAVY METALS EXPOSURE Howard. In *Life Support: The Environment and Human Health*.

Hughes, H. K., & Kahl, L. K. (2018). *A Manual for Pediatric House Officers The Harriet Lane Handbook.*

Humbert, L., Hoizey, G., & Lhermitte, M. (2014). Drugs Involved in Drug-Facilitated Crimes (DFC). Analytical Aspects: 1-Blood and Urine. In *Toxicological Aspects of Drug-Facilitated Crimes*. https://doi.org/10.1016/B978-0-12-416748-3.00007-4

Husain, N., & Mahmood, R. (2017). Hexavalent chromium induces reactive oxygen species and impairs the antioxidant power of human erythrocytes and lymphocytes: Decreased metal reducing and free radical quenching ability of the cells. *Toxicology and Industrial Health*. https://doi.org/10.1177/0748233717703892

Ihedioha, J. I., Noel-Uneke, O. A., & Ihedioha, T. E. (2013). Reference values for the serum lipid profile of albino rats (Rattus norvegicus) of varied ages and sexes. *Comparative Clinical Pathology*, *22*(1), 93–99. https://doi.org/10.1007/S00580-011-1372-7/TABLES/5

Iida, A., Saito, S., Sekine, A., Mishima, C., Kitamura, Y., Kondo, K., Harigae, S., Osawa, S., & Nakamura, Y. (2002). Catalog of 605 single-nucleotide polymorphisms (SNPs) among 13 genes encoding human ATP-binding cassette transporters: ABCA4, ABCA7, ABCA8, ABCD1, ABCD3, ABCD4, ABCE1, ABCF1, ABCG1, ABCG2, ABCG4, ABCG5, and ABCG8. *Journal of Human Genetics*, *47*(6), 285–310. https://doi.org/10.1007/s100380200041

Imai, Y., Nakane, M., Kage, K., Tsukahara, S., Ishikawa, E., Tsuruo, T., Miki, Y., &

Sugimoto, Y. (2002). C421A Polymorphism in the Human Breast Cancer Resistance Protein Gene Is Associated with Low Expression of Q141K Protein and Low-Level Drug Resistance 1 Supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology, …. *Molecular Cancer Therapeutics*, *1*(8).

Inbaraj, L., Haebar, O., Saj, F., Dawson, S., Paul, P., Prabhakar, A. K., Mohan, V., & Alex, R. (2013). Prevalence of musculoskeletal disorders among brick kiln workers in rural southern India. *Indian Journal of Occupational and Environmental Medicine*, *17*(2), 71–75. https://doi.org/10.4103/0019-5278.123170

Intergovernmental Panel on Climate Change. (2014). Climate Change 2014 Mitigation of Climate Change. In *Climate Change 2014 Mitigation of Climate Change*. https://doi.org/10.1017/cbo9781107415416

Iqbal, S., Wasim, M., Tufail, M., Arif, M., & Chaudhry, M. M. (2012). Elemental contamination in urban parks of Rawalpindi/Islamabad - A source identification and pollution level assessment study. *Environmental Monitoring and Assessment*, *184*(9), 5497–5510. https://doi.org/10.1007/S10661-011-2356-4

Ishaq, M., Khan, M. A., Jan, F. A., & Ahmad, I. (2010a). Heavy metals in brick kiln located area using atomic absorption spectrophotometer: A case study from the city of Peshawar, Pakistan. *Environmental Monitoring and Assessment*. https://doi.org/10.1007/s10661-009-1011-9

Ishaq, M., Khan, M. A., Jan, F. A., & Ahmad, I. (2010b). Heavy metals in brick kiln located area using atomic absorption spectrophotometer: A case study from the city of Peshawar, Pakistan. *Environmental Monitoring and Assessment*, *166*(1–4), 409–420. https://doi.org/10.1007/s10661-009-1011-9

Ismail, H., Muniandy, K., & Othman, N. (2012). Fatigue life, morphological studies, and thermal aging of rattan powder-filled natural rubber composites as a function of filler loading and a silane coupling agent. *BioResources, 7*(1), 841–858.

Ismail, M., Muhammad, D., Khan, F. U., Munsif, F., Ahmad, T., Ali, S., Khalid, M., Ul Haq, N., & Ahmad, M. (2012). Effect of Brick Kilns ' Emissions on Heavy Metal (Cd And Cr) Content of Contiguous Soil and Plants. *Sarhad. J. Agric*, *28*(3), 403–409.

Itoda, M., Saito, Y., Shirao, K., Minami, H., Ohtsu, A., Yoshida, T., Saijo, N., Suzuki, H., Ozawa, S., Sugiyama, Y., & Sawada, J. I. (2003). Eight Novel Single Nucleotide Polymorphisms in ABCG2 /BCRP in Japanese Cancer Patients Administered Irinotacan. *Drug Metabolism And Pharmacokinetics*, *18*(3), 212–217. https://doi.org/10.2133/dmpk.18.212

Jahan, S., Falah, S., Ullah, H., Ullah, A., & Rauf, N. (2016a). Antioxidant enzymes status and reproductive health of adult male workers exposed to brick kiln pollutants in Pakistan. *Environmental Science and Pollution Research*, *23*(13), 12932–12940. https://doi.org/10.1007/s11356-016-6454-2

Jahan, S., Falah, S., Ullah, H., Ullah, A., & Rauf, N. (2016b). Antioxidant enzymes status and reproductive health of adult male workers exposed to brick kiln pollutants in Pakistan. *Environmental Science and Pollution Research*, *23*(13), 12932–12940. https://doi.org/10.1007/s11356-016-6454-2

Jaiswal, A., Verma, A., & Jaiswal, P. (2018). Detrimental effects of heavy metals in soil, plants, and aquatic ecosystems and in humans. *Journal of Environmental Pathology, Toxicology and Oncology*, *37*(3), 183–197. https://doi.org/10.1615/JEnvironPatholToxicolOncol.2018025348

Jan, A. T., Azam, M., Siddiqui, K., Ali, A., Choi, I., & Haq, Q. M. R. (2015). Heavy metals and human health: Mechanistic insight into toxicity and counter defense system of antioxidants. In *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms161226183

Jan, F. A., Khan, S., Ishaq, M., Naeem, M., Ahmad, I., & Hussain, S. (2014). Brick kiln exhaust as a source of polycyclic aromatic hydrocarbons (PAHs) in the surrounding soil and plants: A case study from the city of Peshawar, Pakistan. *Arabian Journal of Geosciences*, *7*(1), 13–19. https://doi.org/10.1007/s12517-013-0901-x

Järup, L. (2003). Hazards of heavy metal contamination. *British Medical Bulletin*, *68*, 167–182. https://doi.org/10.1093/bmb/ldg032

Jenkins, D. W. (1979). Toxic trace metals in mammalian hair and nails. *Environmental Monitoring and Support Laboratory, Office of Research and Development, US Environmental Protection Agency*, *1*.

Johansson, T. B., Akselsson, R., & Johansson, S. A. E. (1970). X-ray analysis: Elemental trace analysis at the 10-12 g level. In *Nuclear Instruments and Methods*. https://doi.org/10.1016/0029-554X(70)90751-2

Joshi, S. K., & Dudani, I. (2008). Environmental health effects of brick kilns in Kathmandu valley. *Kathmandu University Medical Journal (KUMJ)*.

Junaid, M., Hashmi, M. Z., & Malik, R. N. (2016). Evaluating levels and health risk of heavy metals in exposed workers from surgical instrument manufacturing industries of Sialkot, Pakistan. *Environmental Science and Pollution Research*, *23*(18), 18010–18026. https://doi.org/10.1007/s11356-016-6849-0

Jurewicz, J., Dziewirska, E., Radwan, M., & Hanke, W. (2018). Air pollution from natural and anthropic sources and male fertility. *Reproductive Biology and Endocrinology*, *16*(1). https://doi.org/10.1186/s12958-018-0430-2

Kakkar, R., Kalra, J., Mantha, S. V, & Prasad, K. (1995). Lipid peroxidation and activity of antioxidant enzymes in diabetic rats. *Molecular and Cellular Biochemistry*, *151*(2), 113–119.

Kalantaridou, S. N., Zoumakis, E., Makrigiannakis, A., Lavasidis, L. G., Vrekoussis, T., & Chrousos, G. P. (2010). Corticotropin-releasing hormone, stress and human reproduction: An update. In *Journal of Reproductive Immunology*. https://doi.org/10.1016/j.jri.2010.02.005

Kamal, A., Cincinelli, A., Martellini, T., & Malik, R. N. (2015). A review of PAH exposure from the combustion of biomass fuel and their less surveyed effect on the blood parameters. *Environmental Science and Pollution Research*, *22*(6), 4076–4098.

Kamal, A., Malik, R. N., Martellini, T., & Cincinelli, A. (2014a). Cancer risk evaluation of brick kiln workers exposed to dust bound PAHs in Punjab province (Pakistan). *Science of the Total Environment*, *493*, 562–570. https://doi.org/10.1016/j.scitotenv.2014.05.140

Kamal, A., Qamar, K., Gulfraz, M., Anwar, M. A., & Malik, R. N. (2015). PAH exposure and oxidative stress indicators of human cohorts exposed to traffic pollution in Lahore city (Pakistan). *Chemosphere*. https://doi.org/10.1016/j.chemosphere.2014.05.021

Kampa, M., Pelekanou, V., & Castanas, E. (2008). Membrane-initiated steroid action in breast and prostate cancer. *Steroids*. https://doi.org/10.1016/j.steroids.2007.12.009

Karacaoğlu, E., & Selmanoğlu, G. (2010). Effects of heat-induced food contaminant furan on reproductive system of male rats from weaning through postpuberty. *Food and*

Chemical Toxicology. https://doi.org/10.1016/j.fct.2010.02.026

Karakilcik, A. Z., Zerin, M., Arslan, O., Nazligul, Y., & Vural, H. (2004). Effects of vitamin C and E on liver enzymes and biochemical parameters of rabbits exposed to aflatoxin B1. *Veterinary and Human Toxicology*.

Karimi, G., Shahar, S., Homayouni, N., Rajikan, R., Bakar, F. A., & Othman, M. S. (2012). *Association between Trace Element and Heavy Metal Levels in Hair and Nail with Prostate Cancer*. *13*, 4249–4253.

Karttunen, V., Mohammed, A. M., & Vähäkangas, K. (2017). The Significance of ABC Transporters in Human Placenta for the Exposure of Fetus to Xenobiotics. *Reproductive and Developmental Toxicology*, 1275–1300. https://doi.org/10.1016/B978-0-12-804239-7.00067-6

Kaushik, R., Khaliq, F., Subramaneyaan, M., & Ahmed, R. (2012). Pulmonary dysfunctions, oxidative stress and DNA damage in brick kiln workers. *Human & Experimental Toxicology*. https://doi.org/10.1177/0960327112450899

Kawai, M., Swan, K. F., Green, A. E., Edwards, D. E., Anderson, M. B., & Henson, M. C. (2002). Placental Endocrine Disruption Induced by Cadmium: Effects on P450 Cholesterol Side-Chain Cleavage and 3β-Hydroxysteroid Dehydrogenase Enzymes in Cultured Human Trophoblasts. *Biology of Reproduction*. https://doi.org/10.1095/biolreprod67.1.178

Kelsey, J. L., Gammon, M. D., & John, E. M. (1993). Reproductive Factors and Breast Cancer. *Epidemiologic Reviews*. https://doi.org/10.1093/oxfordjournals.epirev.a036115

Kempson, I. M., Skinner, W. M., & Martin, R. R. (2010). *CHANGES IN THE METAL CONTENT OF HUMAN HAIR DURING DIAGENESIS FROM 500 YEARS ,*

EXPOSURE TO GLACIAL AND AQUEOUS ENVIRONMENTS *. *3*(February 2009), 450–466. https://doi.org/10.1111/j.1475-4754.2009.00489.x

Khalid, S. (n.d.). *USING URINARY FLUORIDE AND DENTAL FLUOROSIS AS BIOMARKERS OF FLUORIDE EXPOSURE IN BRICK KILN WORKERS IN BALOCHISTAN, PAKISTAN*. Retrieved October 9, 2021, from www.fluorideresearch.online/epub/files/033.pdf

Khan, M. W., Ali, Y., De Felice, F., Salman, A., & Petrillo, A. (2019a). Impact of brick kilns industry on environment and human health in Pakistan. *Science of the Total Environment*. https://doi.org/10.1016/j.scitotenv.2019.04.369

Khan, M. W., Ali, Y., De Felice, F., Salman, A., & Petrillo, A. (2019b). Impact of brick kilns industry on environment and human health in Pakistan. *Science of the Total Environment*, *678*, 383–389. https://doi.org/10.1016/j.scitotenv.2019.04.369

Khan, M. W., Ali, Y., De Felice, F., Salman, A., & Petrillo, A. (2019c). Impact of brick kilns industry on environment and human health in Pakistan. *Science of the Total Environment*, *678*, 383–389. https://doi.org/10.1016/j.scitotenv.2019.04.369

Khan, R., & Vyas, H. (2008). A Study of Impact of Brick Industries on Environment and Human Health in Ujjain city, India. *Journal of Environmental Research And Development*.

Khan, Y. P., Bhutta, S. Z., Munim, S., & Bhutta, Z. A. (2009). Maternal Health and Survival in Pakistan: Issues and Options. *Journal of Obstetrics and Gynaecology Canada*. https://doi.org/10.1016/S1701-2163(16)34321-3

Khisroon, M., Khan, A., Imran, M., Zaidi, F., Ahmadullah, F., & Fatima, S. H. (2018). Biomonitoring of DNA damage in individuals exposed to brick kiln pollution from

Peshawar, Khyber Pakhtunkhwa, Pakistan. *Archives of Environmental and Occupational Health*, *73*(2), 115–120. https://doi.org/10.1080/19338244.2017.1304881

Kim, K. H., Kabir, E., & Kabir, S. (2015). A review on the human health impact of airborne particulate matter. *Environment International*, *74*, 136–143. https://doi.org/10.1016/j.envint.2014.10.005

Kirk, S. E., Xie, T. Y., Steyn, F. J., Grattan, D. R., & Bunn, S. J. (2017). Restraint stress increases prolactin-mediated phosphorylation of signal transducer and activator of transcription 5 in the hypothalamus and adrenal cortex in the male mouse. *Journal of Neuroendocrinology*. https://doi.org/10.1111/jne.12477

Knauff, E. A. H., Westerveld, H. E., Goverde, A. J., Eijkemans, M. J., Valkenburg, O., Van Santbrink, E. J. P., Fauser, B. C. J. M., & Van Der Schouw, Y. T. (2008). Lipid profile of women with premature ovarian failure. *Menopause*, *15*(5), 919–923. https://doi.org/10.1097/GME.0B013E31816B4509

Kobayashi, D., Ieiri, I., Hirota, T., Takane, H., Maegawa, S., Kigawa, J., Suzuki, H., Nanba, E., Oshimura, M., Terakawa, N., Otsubo, K., Mine, K., & Sugiyama, Y. (2005). FUNCTIONAL ASSESSMENT OF ABCG2 (BCRP) GENE POLYMORPHISMS TO PROTEIN EXPRESSION IN HUMAN PLACENTA. *Drug Metabolism and Disposition*, *33*(1), 94–101. https://doi.org/10.1124/DMD.104.001628

Kobayashi, D., Ieri, I., Hirota, T., Takane, H., & Sugiyama, Y. (2005). FUNCTIONAL ASSESSMENT OF ABCG2 (BCRP) GENE POLYMORPHISMS TO PROTEIN EXPRESSION IN HUMAN PLACENTA Daisuke Kobayashi , Ichiro Ieiri , Takeshi Hirota , Hiroshi Takane , Shinji Maegawa , Junzo Kigawa , and Yuichi Sugiyama ABSTRACT : *Drug Metabolism and Disposition*, *33*(1), 94–101.

https://doi.org/10.1124/dmd.104.001628.speculated

Kori-Siakpere, O., Ake, J. E. G., & Avworo, U. M. (2006). Sublethal effects of cadmium on some selected haematological parameters of Heteroclarias (a hybrid of Heterobranchus bidorsalis and Clarias gariepinus). *International Journal of Zoological Research*. https://doi.org/10.3923/ijzr.2006.77.83

Kumar, S. (2018). Occupational and environmental exposure to lead and reproductive health impairment: An overview. *Indian Journal of Occupational and Environmental Medicine*. https://doi.org/10.4103/ijoem.IJOEM-126-18

Lafuente, A., Cano, P., & Esquifino, A. I. (2003). Are cadmium effects on plasma gonadotropins, prolactin, ACTH, GH and TSH levels, dose-dependent? *BioMetals*, *16*(2), 243–250. https://doi.org/10.1023/A:1020658128413

Lafuente, A., Márquez, N., Pérez-Lorenzo, M., Pazo, D., & Esquifino, A. I. (2001). Cadmium effects on hypothalamic-pituitary-testicular axis in male rats. *Experimental Biology and Medicine*. https://doi.org/10.1177/153537020122600615

Lahiry, S., Khanra, S., Kumar, R., & Chakraborty, A. (2017). Particle induced X-ray emission study of blood samples of Indian Kala-azar patients. *Journal of Parasitic Diseases*, *41*(1), 193–198. https://doi.org/10.1007/s12639-016-0775-4

Landrigan, P. J. (1998). Environmental hazards for children in USA. *International Journal of Occupational Medicine and Environmental Health*, *11*(2), 189–194.

Laxmi Priya, P. N., Pillai, A. I., & Gupta, S. (2004). Effect of simultaneous exposure to lead and cadmium on gonadotropin binding and steroidogenesis on granulosa cells: An in vitro study. *Indian Journal of Experimental Biology*, *42,* 143–148.

Le Gac, F., Blaise, O., Fostier, A., Le Bail, P.-Y., Loir, M., Mourot, B., & Weil, C. (1993).

Growth hormone (GH) and reproduction: a review. *Fish Physiology and Biochemistry*, *11*(1–6), 219–232.

Levine, S., & Muneyyirci-Delale, O. (2018). Stress-induced hyperprolactinemia: Pathophysiology and clinical approach. *Obstetrics and Gynecology International*. https://doi.org/10.1155/2018/9253083

Levy, R. J. (2015). Carbon monoxide pollution and neurodevelopment: A public health concern. In *Neurotoxicology and Teratology*. https://doi.org/10.1016/j.ntt.2015.03.001

Li, H., Chen, L., Guo, Z., Sang, N., & Li, G. (2012). In vivo screening to determine neurological hazards of nitrogen dioxide (NO 2) using Wistar rats. *Journal of Hazardous Materials*, *225–226*, 46–53. https://doi.org/10.1016/j.jhazmat.2012.04.063

Li, J., Cen, D., Huang, D., Li, X., Xu, J., Fu, S., Cai, R., Wu, X., Tang, M., Sun, Y., Zhang, J., & Zheng, J. (2014). Detection and Analysis of 12 Heavy Metals in Blood and Hair Sample from a General Population of Pearl River Delta Area. *Cell Biochemistry and Biophysics*, *70*(3), 1663–1669. https://doi.org/10.1007/s12013-014-0110-6

Li, T., Zhang, Y., Wang, J., Xu, D., Yin, Z., Chen, H., Lv, Y., Luo, J., Zeng, Y., Liu, Y., Kinney, P. L., & Shi, X. (2018). All-cause mortality risk associated with long-term exposure to ambient PM2·5 in China: a cohort study. *The Lancet Public Health*, *3*(10), e470–e477. https://doi.org/10.1016/S2468-2667(18)30144-0

Li, X., Gu, A. Z., Zhang, Y., Li, D., & Chen, J. (2019). *Sub-lethal concentrations of heavy metals induce antibiotic resistance via mutagenesis*. https://doi.org/10.1016/j.jhazmat.2019.02.006

Liening, S. H., & Josephs, R. A. (2010). It Is Not Just About Testosterone: Physiological Mediators and Moderators of Testosterone's Behavioral Effects. *Social and Personality*

Psychology Compass. https://doi.org/10.1111/j.1751-9004.2010.00316.x

Lin, Y., Huang, R., & Yao, X. (2021). Air pollution and environmental information disclosure: An empirical study based on heavy polluting industries. *Journal of Cleaner Production*, *278*, 124313. https://doi.org/10.1016/j.jclepro.2020.124313

Lippmann, M., Ito, K., Hwang, J. S., Maciejczyk, P., & Chen, L. C. (2006). Cardiovascular effects of nickel in ambient air. *Environmental Health Perspectives*. https://doi.org/10.1289/ehp.9150

Liu, L., Oza, S., Hogan, D., Perin, J., Rudan, I., Lawn, J. E., Cousens, S., Mathers, C., & Black, R. E. (2015). Global, regional, and national causes of child mortality in 2000-13, with projections to inform post-2015 priorities: An updated systematic analysis. *The Lancet*. https://doi.org/10.1016/S0140-6736(14)61698-6

Liu, T., Jiang, F., Liu, X., Zhang, H., Wang, L., Liu, W., & Wang, X. (2018). Association of ABCG2 polymorphisms with ischemic stroke in a Chinese population. *Annals of Human Genetics*, *82*(5), 325–330. https://doi.org/10.1111/AHG.12258

Lopez, A., Lyoda, N., Segal, R., & Tsai, T. (2012). Building Materials: Pathways To Efficiency in the South Asia Brickmaking Industry. *John Hopkins University SAIS Research Report*, 6–10. http://carbonwarroom.com/sites/default/files/reports/Pathways to Efficiency in the South Asia Brickmaking Industry %28Carbon War Room%29_0.pdf

Lu, H., Shi, X., Costa, M., & Huang, C. (2005). Carcinogenic effect of nickel compounds. *Molecular and Cellular Biochemistry*, *279*(1–2), 45–67.

Lu, P., Zhang, Y., Xia, G., Zhang, W., Li, S., & Guo, Y. (2019). Short-term exposure to air pollution and conjunctivitis outpatient visits: A multi-city study in China. *Environmental Pollution*, *254*, 113030. https://doi.org/10.1016/j.envpol.2019.113030

Lucesoli, F., Caligiuri, M., Roberti, M. F., Perazzo, J. C., & Fraga, C. G. (1999). Dose-dependent increase of oxidative damage in the testes of rats subjected to acute iron overload. *Archives of Biochemistry and Biophysics*. https://doi.org/10.1006/abbi.1999.1476

Luka, Y., Highina, B. K., Zubairu, A. (2018). Bioremediation : A Solution to Environmental Pollution-A Review American Journal of Engineering Research (AJER). *American Journal of Engineering Research*, *7*(2), 101–109.

M., I., M.A., K., F.A., J., & I., A. (2010). Heavy metals in brick kiln located area using atomic absorption spectrophotometer: A case study from the city of Peshawar, Pakistan. In *Environmental Monitoring and Assessment*.

Madaniyazi, L., Nagashima, T., Guo, Y., Pan, X., & Tong, S. (2016). Projecting ozone-related mortality in East China. *Environment International*, *92–93*, 165–172. https://doi.org/10.1016/j.envint.2016.03.040

Madden, E. F., & Fowler, B. A. (2000). Mechanisms of nephrotoxicity from metal combinations: A review. *Drug and Chemical Toxicology*. https://doi.org/10.1081/DCT-100100098

Maekawa, K., Itoda, M., Sai, K., Saito, Y., Kaniwa, N., Shirao, K., Hamaguchi, T., Kunitoh, H., Yamamoto, N., Tamura, T., Minami, H., Kubota, K., Ohtsu, A., Yoshida, T., Saijo, N., Kamatani, N., Ozawa, S., & Sawada, J. ichi. (2006). Genetic variation and haplotype structure of the ABC transporter gene ABCG2 in a Japanese population. *Drug Metabolism and Pharmacokinetics*, *21*(2), 109–121. https://doi.org/10.2133/dmpk.21.109

Malik, M. F. A., & Kayani, M. A. (2014a). Issues of maternal health in Pakistan: Trends towards millennium development goal 5. *Journal of the Pakistan Medical Association*, *64*(6), 690–693.

Malik, M. F. A., & Kayani, M. A. (2014b). Issues of maternal health in Pakistan: Trends towards millennium development goal 5. *Journal of the Pakistan Medical Association*.

Manisalidis, I., Stavropoulou, E., Stavropoulos, A., & Bezirtzoglou, E. (2020). Environmental and Health Impacts of Air Pollution: A Review. *Frontiers in Public Health*, *8*(February), 1–13. https://doi.org/10.3389/fpubh.2020.00014

Mao, Q. (2008). BCRP/ABCG2 in the placenta: Expression, function and regulation. *Pharmaceutical Research*, *25*(6), 1244–1255. https://doi.org/10.1007/s11095-008-9537-z

Martin, S., & Griswold, W. (2009). Human health effects of heavy metals. *Environmental Science and Technology Briefs for Citizens*, *15*, 1–6.

Matsuo, H., Takada, T., Nakayama, A., Shimizu, T., Sakiyama, M., Shimizu, S., Chiba, T., Nakashima, H., Nakamura, T., Takada, Y., Sakurai, Y., Hosoya, T., Shinomiya, N., & Ichida, K. (2014). ABCG2 dysfunction increases the risk of renal overload hyperuricemia. *Nucleosides, Nucleotides and Nucleic Acids*, *33*(4–6), 266–274. https://doi.org/10.1080/15257770.2013.866679

Mauro, R. Di, Cantarella, G., Bernardini, R., Rosa, M. Di, Barbagallo, I., Distefano, A., Longhitano, L., Vicario, N., Nicolosi, D., Lazzarino, G., Tibullo, D., Gulino, M. E., Spampinato, M., Avola, R., & Volti, G. L. (2019). The biochemical and pharmacological properties of ozone: The smell of protection in acute and chronic diseases. *International Journal of Molecular Sciences*, *20*(3).

https://doi.org/10.3390/ijms20030634

McGregor, A. J., & Mason, H. J. (1990). Chronic Occupational Lead Exposure and Testicular Endocrine Function. *Http://Dx.Doi.Org/10.1177/096032719000900602*, *9*(6), 371–376. https://doi.org/10.1177/096032719000900602

Mehra, R., & Thakur, A. (2010). Assessment of metal hazard taking hair as an indicator of trace element exposure to workers in occupational environment. *Journal of Elemntology*. https://doi.org/10.5601/jelem.2010.15.4.671-678

Modgil, S., Lahiri, D. K., Sharma, V. L., & Anand, A. (2014). Role of early life exposure and environment on neurodegeneration: Implications on brain disorders. *Translational Neurodegeneration*, *3*(1), 1–14. https://doi.org/10.1186/2047-9158-3-9

Mohammadi, S., Gholamin, M., Mansouri, A., Mahmoodian, R. S., Babazadeh, B., Kebriaei, S. M., Zibaei, B., Roshanaei, M., Daneshvar, F., Khandehro, M., Khodadadegan, M. A., Delshad, A., Mohammadzadeh, F., Mohammadi, M., Sadeghi, S., Shoeibi, S., Boroumand-Noughabi, S., Ghayour-Mobarhan, M., Tavallaie, S., … Ferns, G. A. A. (2018a). Effect of cadmium and nickel on expression of CatSper 1 and 2 genes in mice. *Toxin Reviews*, *37*(3), 216–222. https://doi.org/10.1080/15569543.2017.1350192

Montero-Montoya, R., López-Vargas, R., & Arellano-Aguilar, O. (2018). Volatile organic compounds in air: Sources, distribution, exposure and associated illnesses in children. *Annals of Global Health*, *84*(2), 225–238. https://doi.org/10.29024/aogh.910

Morphology, E. (2005). *Acta morphologica л \ et anthropological A " /.*

Mostafavi, S. A., Safikhani, H., & Salehfard, S. (2021). Air pollution distribution in Arak city considering the effects of neighboring pollutant industries and urban traffics. *International Journal of Energy and Environmental Engineering*, *12*(2), 307–333.

https://doi.org/10.1007/s40095-020-00379-5

Mózner, O., Bartos, Z., Zámbó, B., Homolya, L., Heged, T., Us, ″, & Sarkadi, B. (n.d.). *cells Cellular Processing of the ABCG2 Transporter-Potential Effects on Gout and Drug Metabolism*. https://doi.org/10.3390/cells8101215

Murguía-Romero, M., Jiménez-Flores, J. R., Sigrist-Flores, S. C., Espinoza-Camacho, M. A., Jiménez-Morales, M., Piña, E., Méndez-Cruz, A. R., Villalobos-Molina, R., & Reaven, G. M. (2013). Plasma triglyceride/HDL-cholesterol ratio, insulin resistance, and cardiometabolic risk in young adults. *Journal of Lipid Research*. https://doi.org/10.1194/jlr.M040584

Nadeem, K., Hussain, J., Ul Haq, N., Ul Haq, A., Akram, W., & Ahmad, I. (2019). A proton induced X-ray emission (PIXE) analysis of concentration of major/trace and toxic elements in broiler gizzard and flesh of Tehsil Gujar Khan area in Pakistan. *Nuclear Engineering and Technology*, *51*(8), 2042–2049. https://doi.org/10.1016/j.net.2019.06.005

Naga, P. S. G. J., & Pradeep, R. A. S. (2012). *Analysis of trace elements in blood sera of breast cancer patients by particle induced X-ray emission*. 355–361. https://doi.org/10.1007/s10967-011-1505-0

Naga, P. S. G. J., & Ravi, R. M. (2013). *Analysis of blood serum of lung cancer patients using particle induced X-ray emission*. 431–436. https://doi.org/10.1007/s10967-012-2398-2

Nasir, M., Rehman, F. U., Kishwar, S., Bashir, S., & Adil, M. (2021). Air pollution and child health: the impact of brick kiln pollution on children's cognitive abilities and physical health in Pakistan. *Environment, Development and Sustainability*, *0123456789*.

https://doi.org/10.1007/s10668-021-01229-y

Nathanson, I. T., Towne, L. E., & Aub, J. C. (1941). Normal Excretion of Sex Hormones in Childhood. *Endocrinology*, *28*(6), 851–865.

National Institute of Population Studies. (2008). Pakistan Demographic and Health Survey 2006-07. In *Studies in Family Planning*. https://doi.org/10.1111/j.1728-4465.2009.00208.x

Ng, E., Lind, P. M., Lindgren, C., Ingelsson, E., Mahajan, A., Morris, A., & Lind, L. (2015). *Genome-wide association study of toxic metals and trace elements reveals novel associations*. https://doi.org/10.1093/hmg/ddv190

Nie, S., Huang, Y., Shi, M., Qian, X., Li, H., Peng, C., Kong, B., Zou, X., & Shen, S. (2018). Protective role of ABCG2 against oxidative stress in colorectal cancer and its potential underlying mechanism. *Oncology Reports*, *40*(4), 2137–2146. https://doi.org/10.3892/or.2018.6594

Njelekela, M. A., Negishi, H., Nara, Y., Sato, T., Tomohiro, M., Kuga, S., Noguchi, T., Kanda, T., Yamori, M., Mashalla, Y., Liu, L. J., Ikeda, K., Mtabaji, J., & Yamori, Y. (2002). Obesity and lipid profiles in middle aged men and women in Tanzania. *East African Medical Journal*, *79*(2), 58–64. https://doi.org/10.4314/eamj.v79i2.8901

Norman, R. L., & Smith, C. J. (1992). Restraint Inhibits Luteinizing Hormone and Testosterone Secretion in Intact Male Rhesus Macaques: Effects of Concurrent Naloxone Administration. *Neuroendocrinology*, *55*(4), 405–415. https://doi.org/10.1159/000126151

Oakley, A. E., Breen, K. M., Clarke, I. J., Karsch, F. J., Wagenmaker, E. R., & Tilbrook, A. J. (2009). Cortisol reduces gonadotropin-releasing hormone pulse frequency in follicular

phase ewes: Influence of ovarian steroids. *Endocrinology*. https://doi.org/10.1210/en.2008-0587

Ogbodo, E. C., Okpogba, A. N., Amah, U. K., Mounmbegna, E. P., & Obi-Ezeani, C. N. (2020). Evaluation of some heavy metal levels in blood of lead acid battery manufacturing factory workers in Nnewi, Nigeria. *Indian Journal of Pharmacy and Pharmacology*. https://doi.org/10.18231/j.ijpp.2020.017

Ogen, Y. (2020). Assessing nitrogen dioxide (NO2) levels as a contributing factor to coronavirus (COVID-19) fatality. *Science of the Total Environment*, *726*, 138605. https://doi.org/10.1016/j.scitotenv.2020.138605

Ortiz, C., Linares, C., Carmona, R., & Díaz, J. (2017). *Evaluation of short-term mortality attributable to particulate matter pollution in Spain Health impact assessment*. https://doi.org/10.1016/j.envpol.2017.02.037

Ouyang, X., Shao, Q., Zhu, X., He, Q., Xiang, C., & Wei, G. (2018). *Environmental regulation, economic growth and air pollution: Panel threshold analysis for OECD countries*. https://doi.org/10.1016/j.scitotenv.2018.12.056

Pacheco, H., Díaz-López, S., Jarre, E., Pacheco, H., Méndez, W., & Zamora-Ledezma, E. (2020). NO2 levels after the COVID-19 lockdown in Ecuador: A trade-off between environment and human health. *Urban Climate*, *34*, 100674. https://doi.org/10.1016/j.uclim.2020.100674

Padilla, M. A., Elobeid, M., Ruden, D. M., & Allison, D. B. (2010). An examination of the association of selected toxic metals with total and central obesity indices: NHANES 99-02. *International Journal of Environmental Research and Public Health*. https://doi.org/10.3390/ijerph7093332

Pal, S., & Shil, K. (2019). Metabolic Toxicity and Alteration of Cellular Bioenergetics by Hexavalent Chromium. *Handbook of Environmental Materials Management*, *February 2019*, 2397–2424. https://doi.org/10.1007/978-3-319-73645-7_58

Papazian, T., Tayeh, G. A., Sibai, D., Hout, H., Melki, I., & Khabbaz, L. R. (2017). Impact of maternal body mass index and gestational weight gain on neonatal outcomes among healthy Middle-Eastern females. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0181255

Paschoalini, A. L., Savassi, L. A., Arantes, F. P., Rizzo, E., & Bazzoli, N. (2019). Heavy metals accumulation and endocrine disruption in Prochilodus argenteus from a polluted neotropical river. *Ecotoxicology and Environmental Safety*, *169*, 539–550. https://doi.org/10.1016/j.ecoenv.2018.11.047

Patil, D. S. (2017). A Cross Sectional Study of Socio – Demographic and Morbidity Profile of Brick Kiln Workers in Rural Area of Karad, in Satara District. *Journal of Medical Science And Clinical Research*. https://doi.org/10.18535/jmscr/v5i1.20

Paustenbach, D. J., Finley, B. L., Mowat, F. S., & Kerger, B. D. (2003). Human health risk and exposure assessment of chromium (VI) in tap water. In *Journal of Toxicology and Environmental Health - Part A*. https://doi.org/10.1080/15287390306388

Pavesi, T., & Moreira, J. C. (2020). Mechanisms and individuality in chromium toxicity in humans. In *Journal of Applied Toxicology*. https://doi.org/10.1002/jat.3965

Pedersen, J. M., Khan, E. K., Bergström, C. A. S., Palm, J., Hoogstraate, J., & Artursson, P. (2017). Substrate and method dependent inhibition of three ABC-transporters (MDR1, BCRP, and MRP2). *European Journal of Pharmaceutical Sciences*, *103*, 70–76. https://doi.org/10.1016/j.ejps.2017.03.002

Pollack, A. Z., Schisterman, E. F., Goldman, L. R., Mumford, S. L., Albert, P. S., Jones, R. L., & Wactawski-Wende, J. (2011). Cadmium, lead, and mercury in relation to reproductive hormones and anovulation in premenopausal women. *Environmental Health Perspectives*, *119*(8), 1156–1161. https://doi.org/10.1289/ehp.1003284

Prasad, S., Tiwari, M., Pandey, A. N., Shrivastav, T. G., & Chaube, S. K. (2016). Impact of stress on oocyte quality and reproductive outcome. In *Journal of Biomedical Science*. https://doi.org/10.1186/s12929-016-0253-4

Progesterone - Health Encyclopedia - University of Rochester Medical Center. (n.d.). Retrieved May 1, 2022, from https://www.urmc.rochester.edu/encyclopedia/content.aspx?ContentTypeID=167&ContentID=progesterone

Qu, X., Niu, L., Kroon, B., & Foltis, L. (2018). *cosmetics Pollution Damage and Protection of Asian Hair*. https://doi.org/10.3390/cosmetics5010017

Raaschou-Nielsen, O., Andersen, Z. J., Hvidberg, M., Jensen, S. S., Ketzel, M., Sørensen, M., Hansen, J., Loft, S., Overvad, K., & Tjønneland, A. (2011). Air pollution from traffic and cancer incidence: A Danish cohort study. *Environmental Health: A Global Access Science Source*. https://doi.org/10.1186/1476-069X-10-67

Rachel, J. E., Mee, J. K., Robin, S., Nadav, A., & Deanna, L. K. (2018). Rare variants in the ABCG2 promoter modulate in vivo activity. *Drug Metabolism and Disposition*, *46*(5), 636–642. https://doi.org/10.1124/dmd.117.079541

Rahman, A., Kumarathasan, P., & Gomes, J. (2016). Infant and mother related outcomes from exposure to metals with endocrine disrupting properties during pregnancy. In *Science of the Total Environment*. https://doi.org/10.1016/j.scitotenv.2016.06.134

Rahman, M. A., Rahman, B., & Ahmed, N. (2013). High blood manganese in iron-deficient children in Karachi. *Public Health Nutrition*, *16*(9), 1677–1683. https://doi.org/10.1017/S1368980013000839

Rajarathnam, U., Athalye, V., Ragavan, S., Maithel, S., Lalchandani, D., Kumar, S., Baum, E., Weyant, C., & Bond, T. (2014). Assessment of air pollutant emissions from brick kilns. *Atmospheric Environment*, *98*, 549–553. https://doi.org/10.1016/j.atmosenv.2014.08.075

Rajaratnam, J. K., Marcus, J. R., Flaxman, A. D., Wang, H., Levin-Rector, A., Dwyer, L., Costa, M., Lopez, A. D., & Murray, C. J. (2010). Neonatal, postneonatal, childhood, and under-5 mortality for 187 countries, 1970-2010: a systematic analysis of progress towards Millennium Development Goal 4. *The Lancet*. https://doi.org/10.1016/S0140-6736(10)60703-9

Rajesh, M., & Niraj, P. (2010). Morbidity profile of brick kiln workers around Ahmedabad city, Gujarat. *Healthline, Journal of Indian Association of Preventive and Social Medicine*, *1*(1), 41–44.

Rajonee, A. A., & Uddin, M. J. (2018). Changes in Soil Properties with Distance in Brick Kiln Areas around Barisal. *Open Journal of Soil Science*, *08*(03), 118–128. https://doi.org/10.4236/ojss.2018.83009

Rami, Y., Karim Ebrahimpour, ·, Maghami, M., Shoshtari-Yeganeh, B., & Kelishadi, · Roya. (2011). *The Association Between Heavy Metals Exposure and Sex Hormones: a Systematic Review on Current Evidence*. *1*, 3. https://doi.org/10.1007/s12011-021-02947-0

Rao, A. N. (2005). Trace element estimation - Methods & clinical context. In *Online Journal of Health and Allied Sciences*.

Rao, V. K., Wangsa, D., Robey, R. W., Huff, L., Honjo, Y., Hung, J., Knutsen, T., Ried, T., & Bates, S. E. (2005). Characterization of ABCG2 gene amplification manifesting as extrachromosomal DNA in mitoxantrone-selected SF295 human glioblastoma cells. *Cancer Genetics and Cytogenetics*, *160*, 126–133. https://doi.org/10.1016/j.cancergencyto.2004.12.013

Raza, A., Qamer, M. F., Afsheen, S., Adnan, M., Naeem, S., & Atiq, M. (2014). Particulate matter associated lung function decline in brick kiln workers of Jalalpur Jattan, Pakistan. *Pakistan Journal of Zoology*, *46*(1), 237–243.

Rehman, H., Ullah, I., David, M., Ullah, A., & Jahan, S. (2019a). Neonatal exposure to furan alters the development of reproductive systems in adult male Sprague Dawley rats. *Food and Chemical Toxicology*, *130*. https://doi.org/10.1016/j.fct.2019.05.020

Reiter, R. J., Melchiorri, D., Sewerynek, E., Poeggeler, B., Barlow-Walden, L., Chuang, J., Ortiz, G. G., & AcuñaCastroviejo, D. (1995). A review of the evidence supporting melatonin's role as an antioxidant. *Journal of Pineal Research*, *18*(1), 1–11.

Release, P., Set, D., This, I., Lbxfsh, A., Sas, L., Follicle, L., & Follicle, D. (2002). *Follicle Stimulating Hormone (FSH) in Serum – NHANES 2001-2002 Follicle Stimulating Hormone (FSH) in Serum – NHANES 2001-2002*. 1–9.

Release, P., Set, D., This, I., Lbxlh, A., Sas, L., Luteinizing, L., & Luteinizing, D. (2002). *Luteinizing Hormone in Serum – NHANES 2001-2002 White Sands Research Center Luteinizing Hormone in Serum – NHANES 2001-2002 White Sands Research Center*. 1–12.

Ren, Y., Qu, Z., Du, Y., Xu, R., Ma, D., Yang, G., Shi, Y., Fan, X., Tani, A., Guo, P., Ge, Y., & Chang, J. (2017). Air quality and health effects of biogenic volatile organic compounds emissions from urban green spaces and the mitigation strategies. *Environmental Pollution*, *230*, 849–861. https://doi.org/10.1016/j.envpol.2017.06.049

Rendón-Lugo, A. N., Santiago, P., Puente-Lee, I., & León-Paniagua, L. (2017). Permeability of hair to cadmium, copper and lead in five species of terrestrial mammals and implications in biomonitoring. *Environmental Monitoring and Assessment, 189*(12). https://doi.org/10.1007/s10661-017-6338-z

Resistance, I. (2020). *Role of Minerals and Trace Elements in Diabetes and Insulin Resistance*. 1–17.

Rizvi, A., Parveen, S., Khan, S., & Naseem, I. (2020). Nickel toxicology with reference to male molecular reproductive physiology. In *Reproductive Biology*. https://doi.org/10.1016/j.repbio.2019.11.005

Robey, R. W., To, K. K. K., Polgar, O., Dohse, M., Fetsch, P., Dean, M., & Bates, S. E. (2009). ABCG2: A perspective. *Advanced Drug Delivery Reviews*, *61*(1), 3–13. https://doi.org/10.1016/j.addr.2008.11.003

Rohde, R. A., & Muller, R. A. (2015). *Air Pollution in China: Mapping of Concentrations and Sources*. https://doi.org/10.1371/journal.pone.0135749

Ruano, P., Delgado, L. L., Picco, S., Villegas, L., Tonelli, F., Merlo, M., Rigau, J., Diaz, D., & Masuelli, M. (2016). We are IntechOpen , the world ’ s leading publisher of Open Access books Built by scientists , for scientists TOP 1 %. *Intech*, *tourism*, 13. https://www.intechopen.com/books/advanced-biometric-technologies/liveness-detection-in-biometrics

Rutstein, S. O., & Rojas, G. (2006). Guide to DHS Statistics. Demographic and Health Surveys Methodology. In *DHS Publication*.

Rzymski, P., Rzymski, P., Tomczyk, K., Niedzielski, P., Jakubowski, K., Poniedziałek, B., & Opala, T. (2014). Metal status in human endometrium: Relation to cigarette smoking and histological lesions. *Rzymski, P., Rzymski, P., Tomczyk, K., Niedzielski, P., Jakubowski, K., Poniedziałek, B., & Opala, T. (2014). Metal Status in Human Endometrium: Relation to Cigarette Smoking and Histological Lesions. Environmental Research. Https://Doi.Org/10.1016/j.Envr*. https://doi.org/10.1016/j.envres.2014.04.025

Rzymski, P., Tomczyk, K., Rzymski, P., Poniedziałek, B., Opala, T., & Wilczak, M. (2015). Impact of heavy metals on the female reproductive system. In *Annals of Agricultural and Environmental Medicine*. https://doi.org/10.5604/12321966.1152077

Saeed, A. (2017). Pakistan Third Largest Brick-producing Country in South Asia. *Business Recorder*, 2017–2018.

Sain, M. K., & Meena, M. L. (2018). Exploring the musculoskeletal problems and associated risk-factors among brick kiln workers. *International Journal of Workplace Health Management*. https://doi.org/10.1108/IJWHM-05-2018-0061

Saju, J. A., Debnath, P., & Nayan, S. B. (2020). *Impacts of Air Pollution on Human Health and Environment Due to Brick Kilns Emission : A Review IMPACTS OF AIR POLLUTION ON HUMAN HEALTH AND ENVIRONMENT DUE TO BRICK KILNS EMISSION : A REVIEW*. *January*, 0–9.

Sanjel, S., Thygerson, S. M., Khanal, S. N., & Joshi, S. K. (2016). Environmental and Occupational Pollutants and Their Effects on Health among Brick Kiln Workers. *Open Journal of Safety Science and Technology*, *06*(04), 81–98.

https://doi.org/10.4236/ojsst.2016.64008

Satoh-Takayama, N., Vosshenrich, C. A. J., Lesjean-Pottier, S., Sawa, S., Lochner, M., Rattis, F., Mention, J.-J., Thiam, K., Cerf-Bensussan, N., & Mandelboim, O. (2008). Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity*, *29*(6), 958–970.

Sayed M. Rawi. (2012). Hazardous effects of acrylamide on immature male and female rats. *African Journal of Pharmacy and Pharmacology*, *6*(18). https://doi.org/10.5897/ajpp12.148

Sett, M., & Sahu, S. (2014). Effects of occupational heat exposure on female brick workers in West Bengal, India. *Global Health Action*. https://doi.org/10.3402/gha.v7.21923

Seydi, E., Mahzari, F., Hadi, M., Ramazani, M., & Pourahmad, J. (2020). *Hexavalent Chromium Induced Oxidative Stress and Toxicity on isolated human lymphocytes*. *3*(1), 1–7.

Sfakianakis, D. G., Renieri, E., Kentouri, M., & Tsatsakis, A. M. (2015). Effect of heavy metals on fish larvae deformities: A review. *Environmental Research*, *137*, 246–255. https://doi.org/10.1016/j.envres.2014.12.014

Shah, A. S. V., Langrish, J. P., Nair, H., McAllister, D. A., Hunter, A. L., Donaldson, K., Newby, D. E., & Mills, N. L. (2013). Global association of air pollution and heart failure: A systematic review and meta-analysis. *The Lancet*, *382*(9897), 1039–1048. https://doi.org/10.1016/S0140-6736(13)60898-3

Shahid, S., Zafar, A., & Jahan, S. (2017). Environmental Impact of Coal Clay Brick Kiln Emission on the Reproductive Health of Children and Adult Male Workers. *International Journal of Environmental Science and Development*, *8*(7), 521–525.

https://doi.org/10.18178/ijesd.2017.8.7.1008

Shaikh, S., Nafees, A. A., Khetpal, V., Jamali, A. A., Arain, A. M., & Yousuf, A. (2012). Respiratory symptoms and illnesses among brick kiln workers: a cross sectional study from rural districts of Pakistan. *BMC Public Health*, *12*, 999. https://doi.org/10.1186/1471-2458-12-999

Sharif, F., & Nasim, S. (2018). To Adopt, or Not to Adopt, Why is the Question: An Environmental and Economic Case for Zigzag Kilns Ψ. *Cdpr.Org.Pk*, undefined-undefined. https://www.mendeley.com/catalogue/93cd83d5-6bd4-35f9-a7ae-5f82d1a0cbbd/?utm_source=desktop&utm_medium=1.19.8&utm_campaign=open_catalog&userDocumentId=%7Bf7bae80d-f1b4-4dc1-9f31-5420e007c7cb%7D%0Ahttps://cdpr.org.pk/wp-content/uploads/2019/04/010819-SN-IG

Sheet, F. (n.d.). *Brick sector in Pakistan Sector overview*.

Shen, X., Chi, Y., & Xiong, K. (2019). The effect of heavy metal contamination on humans and animals in the vicinity of a zinc smelting facility. *PLoS ONE*, *14*(10), 1–15. https://doi.org/10.1371/journal.pone.0207423

Shen, Y., Zhang, H., Jiang, Y., Mzayek, F., Arshad, H., & Karmaus, W. (2020). Maternal Birth Weight and BMI Mediate the Transgenerational Effect of Grandmaternal BMI on Grandchild's Birth Weight. *Obesity*. https://doi.org/10.1002/oby.22680

Sherly Deborah, G., Siva Priya, D. V., & Rama Swamy, C. (2017). Prevalence of menstrual irregularities in correlation with body fat among students of selected colleges in a district of Tamil Nadu, India. *National Journal of Physiology, Pharmacy and Pharmacology*. https://doi.org/10.5455/njppp.2017.7.0307422032017

Shirai, S., Suzuki, Y., Yoshinaga, J., & Mizumoto, Y. (2010). Maternal exposure to low-level heavy metals during pregnancy and birth size. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*. https://doi.org/10.1080/10934529.2010.500942

Shohaimi, S., Boekholdt, M. S., Luben, R., Wareham, N. J., & Khaw, K. T. (2014). Distribution of lipid parameters according to different socio-economic indicators- the EPIC-Norfolk prospective population study. *BMC Public Health*, *14*(1), 1–10. https://doi.org/10.1186/1471-2458-14-782/TABLES/7

Shrestha, D., Rahman, M. L., Hinkle, S. N., Workalemahu, T., & Tekola-Ayele, F. (2019). Maternal BMI-Increasing Genetic Risk Score and Fetal Weights among Diverse US Ethnic Groups. *Obesity*. https://doi.org/10.1002/oby.22499

Shrivastava, R., Upreti, R. K., Seth, P. K., & Chaturvedi, U. C. (2002). Effects of chromium on the immune system. In *FEMS Immunology and Medical Microbiology*. https://doi.org/10.1016/S0928-8244(02)00345-0

Silver, B., Reddington, C. L., Arnold, S. R., & Spracklen, D. V. (2018). LETTER • OPEN ACCESS Recent citations Substantial changes in air pollution across China during 2015-2017. *Environ. Res. Lett*, *13*, 114012. https://doi.org/10.1088/1748-9326/aae718

Sirotkin, A. V., & Harrath, A. H. (2017). Influence of oil-related environmental pollutants on female reproduction. In *Reproductive Toxicology* (Vol. 71, pp. 142–145). Elsevier Inc. https://doi.org/10.1016/j.reprotox.2017.05.007

Skinder, B. M. (2014). Brick kilns: Cause of Atmospheric Pollution. *Journal of Pollution Effects & Control*. https://doi.org/10.4172/2375-4397.1000112

Smith, B. L., & Reyes, T. M. (2017). Offspring neuroimmune consequences of maternal malnutrition: Potential mechanism for behavioral impairments that underlie metabolic and neurodevelopmental disorders. In *Frontiers in Neuroendocrinology*. https://doi.org/10.1016/j.yfrne.2017.07.007

Song, X., Fiati Kenston, S. S., Kong, L., & Zhao, J. (2017). Molecular mechanisms of nickel induced neurotoxicity and chemoprevention. *Toxicology*, *392*(October), 47–54. https://doi.org/10.1016/j.tox.2017.10.006

Stohs, S. J., & Bagchi, D. (1995). Oxidative mechanisms in the toxicity of metal ions. *Free Radical Biology and Medicine*, *18*(2), 321–336.

Tabrez, S., Priyadarshini, M., Priyamvada, S., Shahnawaz Khan, M., & Kashif Zaidi, S. (2014). Gene-environment interactions in heavy metal and pesticide carcinogenesis. *Mutation Research*, *760*, 1–9. https://doi.org/10.1016/j.mrgentox.2013.11.002

Tamura, A., Watanabe, M., Saito, H., Nakagawa, H., Kamachi, T., Okura, I., & Ishikawa, T. (2006). Functional Validation of the Genetic Polymorphisms of Human ATP-Binding Cassette (ABC) Transporter ABCG2: Identification of Alleles That Are Defective in Porphyrin Transport. *Molecular Pharmacology*, *70*(1), 287–296. https://doi.org/10.1124/MOL.106.023556

Tan, X., Han, L., Zhang, X., Zhou, W., Li, W., & Qian, Y. (2021). A review of current air quality indexes and improvements under the multi-contaminant air pollution exposure. *Journal of Environmental Management*, *279*, 111681. https://doi.org/10.1016/j.jenvman.2020.111681

Taylor, N. M. I., Manolaridis, I., Jackson, S. M., Kowal, J., Stahlberg, H., & Locher, K. P. (2017). Structure of the human multidrug transporter ABCG2. *Nature*, *546*(7659), 504–

509. https://doi.org/10.1038/nature22345

Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K., & Sutton, D. J. (2012). Heavy metal toxicity and the environment. In *EXS*. https://doi.org/10.1007/978-3-7643-8340-4_6

Thakore, J. H., & Dinan, T. G. (1994). Growth hormone secretion: The role of glucocorticoids. In *Life Sciences*. https://doi.org/10.1016/0024-3205(94)00237-1

Thévenod, F. (2009). Cadmium and cellular signaling cascades: To be or not to be? *Toxicology and Applied Pharmacology*, *238*(3), 221–239. https://doi.org/10.1016/j.taap.2009.01.013

Thompson, J., & Bannigan, J. (2008a). Cadmium: Toxic effects on the reproductive system and the embryo. In *Reproductive Toxicology*. https://doi.org/10.1016/j.reprotox.2008.02.001

Thompson, J., & Bannigan, J. (2008b). Cadmium: Toxic effects on the reproductive system and the embryo. *Reproductive Toxicology*, *25*(3), 304–315. https://doi.org/10.1016/j.reprotox.2008.02.001

To, K. K. W., Wu, M., Tong, C. W. S., & Yan, W. (2020). Drug transporters in the development of multidrug resistance in colorectal cancer. *Drug Resistance in Colorectal Cancer: Molecular Mechanisms and Therapeutic Strategies*, 35–55. https://doi.org/10.1016/B978-0-12-819937-4.00002-9

Tomei, G., Tomao, E., Ciarrocca, M., Rosati, M., Caciari, T., Gamberale, D., Palermo, P., De Sio, S., Tria, M., Panfili, A., & Tomei, F. (2009). Follicle-stimulating hormone levels in male workers exposed to urban chemical, physical, and psychosocial stressors. *Toxicology and Industrial Health*. https://doi.org/10.1177/0748233709106466

Toufexis, D., Rivarola, M. A., Lara, H., & Viau, V. (2014). Stress and the Reproductive Axis. *Journal of Neuroendocrinology*, *26*(9), 573. https://doi.org/10.1111/JNE.12179

Tripathi, R. M., Raghunath, R., & Krishnamoorthy, T. M. (1997). Dietary intake of heavy metals in Bombay city, India. *Science of the Total Environment*. https://doi.org/10.1016/S0048-9697(97)00290-8

Tseng, C. H., Lei, C., & Chen, Y. C. (2018). Evaluating the health costs of oral hexavalent chromium exposure from water pollution: A case study in Taiwan. *Journal of Cleaner Production*, *172*, 819–826. https://doi.org/10.1016/j.jclepro.2017.10.177

Tsigos, C., & Chrousos, G. P. (2002). Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *Journal of Psychosomatic Research*. https://doi.org/10.1016/S0022-3999(02)00429-4

Turkez, H., Geyikoglu, F., Tatar, A., Keles, M. S., & Kaplan, I. (2012). The effects of some boron compounds against heavy metal toxicity in human blood. *Experimental and Toxicologic Pathology*. https://doi.org/10.1016/j.etp.2010.06.011

U.S. Department of Health and Human Services. (2010). How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease. In *How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General*.

Ukaogo, P. O., Ewuzie, U., & Onwuka, C. V. (2020). Environmental pollution: causes, effects, and the remedies. In *Microorganisms for Sustainable Environment and Health*. Elsevier. https://doi.org/10.1016/b978-0-12-819001-2.00021-8

Unicel, B. (2012). *Laboratory Procedure Manual*. 1–9.

Vahter, M., Berglund, M., Åkesson, A., & Lidén, C. (2002). Metals and women's health. *Environmental Research*. https://doi.org/10.1006/enrs.2002.4338

Vaiserman, A. (2014). Early-life exposure to endocrine disrupting chemicals and later-life health outcomes: An Epigenetic Bridge? In *Aging and Disease*. https://doi.org/10.14336/AD.2014.0500419

Viau, V. (2002). Functional cross-talk between the hypothalamic-pituitary-gonadal and -Adrenal axes. *Journal of Neuroendocrinology*. https://doi.org/10.1046/j.1365-2826.2002.00798.x

Vijayan, V., & Marg, S. (2002). *Pixe analysis of human blood in cancer*. *12*, 47–52.

Vogel, S., Contois, J. H., Tucker, K. L., Wilson, P. W. F., Schaefer, E. J., & Lammi-Keefe, C. J. (1997). Plasma retinol and plasma and lipoprotein tocopherol and carotenoid concentrations in healthy elderly participants of the Framingham Heart Study. *American Journal of Clinical Nutrition*, *66*(4), 950–958. https://doi.org/10.1093/ajcn/66.4.950

Wamala, S. P., Wolk, A., Schenck-Gustafsson, K., & Orth-Gomer, K. (1997). Lipid profile and socioeconomic status in healthy middle aged women in Sweden. *Epidemiology and Community Health*, *51*, 400–407. https://doi.org/10.1136/jech.51.4.400

Wang, F., He, Y., Guo, H., Li, J., Yang, Y., Wu, Z., Zheng, H., & Wu, T. (2010). Genetic variants of nucleotide excision repair genes are associated with DNA damage in coke oven workers. *Cancer Epidemiology and Prevention Biomarkers*, *19*(1), 211–218.

Wanjari, R., Wanjari, S., Agrawal, H., & Kumari, P. (2019). *PRIMARY HYPOTHYROIDISM PRESENTING AS SHORT STATURE AND DELAYED MENARCHE*.

Wąsowicz, W., Gromadzińska, J., & Rydzyński, K. (2001). Blood Concentration of Essential Trace Elements and Heavy Metals in Workers Exposed To Lead and Cadmium.

International Journal of Occupational Medicine and Environmental Health.

Wells, J. C., Sawaya, A. L., Wibaek, R., Mwangome, M., Poullas, M. S., Yajnik, C. S., & Demaio, A. (2020). The double burden of malnutrition: aetiological pathways and consequences for health. In *The Lancet*. https://doi.org/10.1016/S0140-6736(19)32472-9

WHO. (2018). *World Health Organization. "Clean Air for Health: Geneva Action Agenda." In First WHO global conference on air pollution and health-Summary Report.*

Wicke, D., Cochrane, T. A., & O'Sullivan, A. (2012). Build-up dynamics of heavy metals deposited on impermeable urban surfaces. *Journal of Environmental Management*. https://doi.org/10.1016/j.jenvman.2012.09.005

Woodruff, T. J., Carlson, A., Schwartz, J. M., & Giudice, L. C. (2008). Proceedings of the Summit on Environmental Challenges to Reproductive Health and Fertility: executive summary. *Fertility and Sterility*, *89*(2), 281–300. https://doi.org/10.1016/j.fertnstert.2007.10.002

Wright, J. R., Colby, H. D., & Miles, P. R. (1981). Cytosolic factors which affect microsomal lipid peroxidation in lung and liver. *Archives of Biochemistry and Biophysics*. https://doi.org/10.1016/0003-9861(81)90095-3

Yamaguchi, K., Yang, L., McCall, S., Huang, J., Xing, X. Y., Pandey, S. K., Bhanot, S., Monia, B. P., Li, Y. X., & Diehl, A. M. (2007). Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. *Hepatology*. https://doi.org/10.1002/hep.21655

Yang, Y., Jia, J., Sun, Z., Liu, C., Li, Z., Xiao, Y., Yu, J., Du, F., Shi, Y., Sun, J., Shui, J., & Zhang, X. (2021). Polymorphism of FGD4 and myelosuppression in patients with esophageal squamous cell carcinoma. *Future Oncology (London, England)*, *17*(18),

2351–2363. https://doi.org/10.2217/FON-2020-1191

Zambelli, B., Uversky, V. N., & Ciurli, S. (2016). Nickel impact on human health: An intrinsic disorder perspective. *Biochimica et Biophysica Acta - Proteins and Proteomics*, *1864*(12), 1714–1731. https://doi.org/10.1016/j.bbapap.2016.09.008

Zamber, C. P., Lamba, J. K., Yasuda, K., Farnum, J., Thummel, K., Schuetz, J. D., & Schuetz, E. G. (2003). Natural allelic variants of breast cancer resistance protein (BCRP) and their relationship to BCRP expression in human intestine. *Pharmacogenetics*, *13*(1), 19–28. https://doi.org/10.1097/00008571-200301000-00004

Zhang, J., Li, Z., Qie, M., Zheng, R., Shetty, J., & Wang, J. (2016). Sodium fluoride and sulfur dioxide affected male reproduction by disturbing blood-testis barrier in mice. *Food and Chemical Toxicology*, *94*, 103–111. https://doi.org/10.1016/j.fct.2016.05.017

Zhang, W., Sun, S., Zhang, W., & Shi, Z. (2018). Polymorphisms of ABCG2 and its impact on clinical relevance. *Biochemical and Biophysical Research Communications*, *503*(2), 408–413. https://doi.org/10.1016/j.bbrc.2018.06.157

Zhang, Z., Wong, Y. C., Arulrajah, A., & Horpibulsuk, S. (2018). A review of studies on bricks using alternative materials and approaches. In *Construction and Building Materials*. https://doi.org/10.1016/j.conbuildmat.2018.08.152

Zhao, R., Wu, Y., Zhao, F., Lv, Y., Huang, D., Wei, J., Ruan, C., Huang, M., Deng, J., Huang, D., & Qiu, X. (2017). The risk of missed abortion associated with the levels of tobacco, heavy metals and phthalate in hair of pregnant woman. *Medicine (United States)*. https://doi.org/10.1097/MD.0000000000009388

Zhao, Y., Wang, L., Shen, H.-B., Wang, Z.-X., Wei, Q.-Y., & Chen, F. (2007). Association between δ-aminolevulinic acid dehydratase (ALAD) polymorphism and blood lead levels: A meta-regression analysis. *Journal of Toxicology and Environmental Health, Part A*, *70*(23), 1986–1994.

Zhou, J., Liang, J., Hu, Y., Zhang, W., Liu, H., You, L., Zhang, W., Gao, M., & Zhou, J. (2018). Exposure risk of local residents to copper near the largest flash copper smelter in China. *Science of The Total Environment*, *630*, 453–461. https://doi.org/10.1016/J.SCITOTENV.2018.02.211

Zoran, M. A., Savastru, R. S., Savastru, D. M., & Tautan, M. N. (2020). Assessing the relationship between ground levels of ozone (O3) and nitrogen dioxide (NO2) with coronavirus (COVID-19) in Milan, Italy. *Science of the Total Environment*, *740*, 140005. https://doi.org/10.1016/j.scitotenv.2020.140005

Date of sample collection		Background area		Sample no Location/City			
Ethnicity/ Language	Participant's name		Age	Marital status			Gender ☐ M ☐ F
Height	Weight		Menarche age	Period duration			Regularity
Age at marriage		Age at 1st baby birth		Sleep duration/day			
Physical appearance	Deep voice Facial hair appearance Acne Hair growth		Education Status	Illiterate Primary Matriculate Intermediate	Monthly Income	Less than 10,000 10,000-15,000 15,000-20,000 20,000-30,000	
Earlier health history	T.B/fever/cough/cold/stomach problems/any allergy/asthma/any type of bone pain/hepatitis/kidney problem (urination problem) Operation (if any)						
Family history	Family size	Children No.	Dead children	Number of child birth/abortion		Diseased child No	Pregnancy no and status
Food preference				Medication (if any)			
Type of work at brick Kiln	Carriage and Placement Molders Bakers			Exposure material		1.Fuel 2.Smoke 3.Radiations 4.Smog	
Work history	- in Years			Exposure hours/day		Years of living in vicinity	
Any addiction	Cigarette/naswaar/huqqa/Charas/any other?			Exposure type		1.Fuel 2.Smoke 3.Radiations	
Smoking Status:	Age at Starting Smoking/addiction				Intake/day		

Consent letter for participation in research project related to health issues in brick kiln workers

Institute: Quaid-i-azam University Islamabad

We are conducting a research related to **health issues in brick kiln** in Quaid-i-Azam University Islamabad. Your involvement in this research is worth important.

As you know the Industry workers are exposed to different health issues that are getting complicated day by day but proper management can control this situation. Especially coming generations can be protected from these complications.

Our research team will collect information from you and your family members, only with help of your consent. Blood sample of about 5-10ml will be collected and medical reports will be shared as asked. We will keep your precious information confidential.

Consent

I hereby certify that after reading this letter and some queries, I willingly want to be a part of this research.

موضوع: اینٹ بنو کارکنوں میں صحت کے مسائل سے متعلق ریسرچ پروجیکٹ میں حصہ لینے کے لئے رضامندی کا خط.

ہم قائداعظم یونیورسٹی اسلام آباد میں اینٹ بٹ میں صحت کے مسائل سے متعلق تحقیقات کر رہے ہیں. اس تحقیق میں آپ کی شمولیت اہم ہے.

جیسا کہ آپ جانتے ہیں کہ صنعت کار کارکن مختلف صحت کے مسائل پر مبنی ہیں جو دن دن پیچیدہ حاصل کررہے ہیں لیکن مناسب انتظام اس صورت حال کو کنٹرول کرسکتے ہیں. خاص طور پر آنے والی نسلوں کو ان پیچیدگیوں سے محفوظ کیا جا سکتا ہے.

ہماری تحقیقاتی ٹیم آپ اور آپ کے خاندان کے ارکان سے معلومات جمع کرے گی، صرف آپ کی رضامندي کی مدد سے. تقریبا 5-10 ملی میٹر کے خون کے نمونہ جمع کیے جائیں گے اور طبی رپورٹوں سے پوچھا جائے گا. ہم آپ کی قیمتی معلومات خفیہ رکھیں گے.

Subject participating in research/Guardian`s signature:

Signature of researcher

Turnitin Originality Report

Effect of Environmental and Occupational Pollutants on Health Status of Brick Kiln Workers: A Molecular and Cellular Based Study by Mehwish David .

From CL QAU (DRSML)

- Processed on 12-Aug-2022 14:42 PKT
- ID: 1881702512
- Word Count: 46512

Similarity Index
14%
Similarity by Source

Internet Sources:
9%
Publications:
6%
Student Papers:
6%

sources:

1 2% match (student papers from 26-Dec-2017)
Submitted to Higher Education Commission Pakistan on 2017-12-26

2 1% match (publications)
Nisar Ahmad, Niamatullah, Javed Hussain, Ishaq Ahmad, Muhammad Asif. "Estimation of health risk to humans from heavy metals in soil of coal mines in Harnai, Balochistan", International Journal of Environmental Analytical Chemistry, 2020

3 1% match (Internet from 13-Feb-2020)
https://link.springer.com/article/10.1007%2Fs11356-016-6454-2

4 1% match (Internet from 02-Apr-2022)
https://www.pubfacts.com/author/N+A+Turi

5 1% match (Internet from 24-Jul-2022)
https://www.gcwus.edu.pk/wp-content/uploads/2021/12/Manuscript-No-PRJBS-2021-MS-02.pdf

6 < 1% match (student papers from 04-Feb-2017)
Submitted to Higher Education Commission Pakistan on 2017-02-04

7 < 1% match (student papers from 13-Feb-2012)
Submitted to Higher Education Commission Pakistan on 2012-02-13

8 < 1% match (student papers from 29-Jan-2011)
Submitted to Higher Education Commission Pakistan on 2011-01-29

9 < 1% match (student papers from 03-Aug-2022)
Submitted to Higher Education Commission Pakistan on 2022-08-03

Environmental Science and Pollution Research (2020) 27:44073–44088
https://doi.org/10.1007/s11356-020-10275-4

RESEARCH ARTICLE

Study of occupational exposure to brick kiln emissions on heavy metal burden, biochemical profile, cortisol level and reproductive health risks among female workers at Rawat, Pakistan

Mehwish David[1] · Qurat-Ul-Ain[1] · Muhammad Afzal[1] · Muhammad Shoaib[1] · Faiza Aman[1] · Karen J. Cloete[2] · Naheed Turi[1] · Sarwat Jahan[1]

Received: 6 March 2020 / Accepted: 27 July 2020 / Published online: 4 August 2020

Abstract

The present study was planned to understand the heavy metal burden and its possible actions in blood of occupational females working at brick kilns at Rawat, Pakistan. A total of 232 women were included in the study, of which 114 presented control subjects. Apart from collection of demographic data, fertility indicators and body mass index (BMI), blood was collected from subjects that was later used for the determination of heavy metal concentrations using atomic absorption spectroscopy and haematological profile. Blood was centrifuged and plasma was obtained and stored at − 20° to study biochemical variables (sodium dismutase, peroxidase, reactive oxygen species, thiobarbituric acid reactive species, protein estimation), lipid profile and cortisol concentrations among the two groups. Analysis of heavy metal in blood showed elevated levels of cadmium (3.09 ± 0.01 μg/dl), chromium (4.20 ± 0.02 μg/dl) and nickel (5.59 ± 0.03 μg/dl) in worker's group as compared with control. Increased platelet count; decreased antioxidant enzyme and increased oxidants level; increased total cholesterol, low-density lipoprotein (LDL) and triglyceride (TG); decreased total protein and high-density lipoprotein (HDL); and increased cortisol levels were evident among workers as compared with the control group. The study concluded that occupational workers experience increased heavy metals burden in blood and, therefore, pose a risk to human health by causing reduction in antioxidant enzymes concentration and increase in stress conditions.

Keywords Brick kilns · Heavy metals · BMI · Antioxidants · Lipid profile · Cortisol · Public health

Responsible editor: Lotfi Aleya

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11356-020-10275-4) contains supplementary material, which is available to authorized users.

✉ Sarwat Jahan
drsarwatjahan@gmail.com

Mehwish David
mehwish28david@gmail.com

Qurat-Ul-Ain
ainy_choudhary@yahoo.com

Muhammad Afzal
mafzal@bs.qau.edu.pk

Muhammad Shoaib
mshoaib@bs.qau.edu.pk

Faiza Aman
niazi_faiza123@yahoo.com

Karen J. Cloete
kaboutercloete@gmail.com; kcloete@tlabs.ac.za

Naheed Turi
nancymirza90@gmail.com

[1] Reproductive Physiology laboratory, Department of Animal Sciences, Quaid-i-Azam University Islamabad, Islamabad 45320, Pakistan

[2] iThemba Laboratory for Accelerator Based Sciences, National Research Foundation, PO Box 722, Somerset West 7129, South Africa

ARCHIVES OF ENVIRONMENTAL & OCCUPATIONAL HEALTH
https://doi.org/10.1080/19338244.2020.1854645

Evaluation of environmental effects of heavy metals on biochemical profile and oxidative stress among children at brick kiln sites

Mehwish David, Naheed Turi, Qurat-ul Ain, Humaira Rahman, and Sarwat Jahan

Reproductive Physiology Laboratory, Department of Animal Sciences, Quaid i Azam University, Islamabad, Pakistan

ABSTRACT

The present study was designed to study the health risks among children living at brick kiln industries. A survey was conducted, questionnaires were filled out, and demographic data was collected from Punjab, Pakistan. Heavy metals burden and BMI were calculated, hematological and enzyme analysis, comet assay and hormonal ELISA were performed. The results showed decreased BMI, RBC count and hematocrit in the exposed group. Nickel, cadmium, zinc and chromium concentrations in whole blood were detected among exposed children. Antioxidant enzymes and growth hormone concentration decreased, while reactive oxygen species and cortisol level increased in the exposed group. The comet assay findings showed decreased percentage DNA in the head and increased in the tail region among exposed group. It was concluded that children living at brick kiln sites experienced decreased BMI, altered antioxidant enzymes status and hormone levels and cellular DNA damage that pose a major threat on child health.

ARTICLE HISTORY
Received 20 January 2020
Accepted 16 November 2020

KEYWORDS
Brick kiln; children; heavy metals; hormone analysis; stress

Introduction

A large number of gaseous toxic substances and by-products are formed during the baking of bricks at kilns.[1] These hazardous gases as well as heavy metals become part of the environment, thus, affecting all life forms including humans.[2] Heavy metals are released through natural means or by human activities.[2] Brick kiln emissions comprised of particulate air pollutants, heavy metals, and gaseous pollutants such as sulfur and carbon dioxides, nitrogen oxides, carbon monoxide, hydrogen fluoride, and volatile organic compounds.[3] Animals, plants and human are exposed to these emissions both directly and indirectly.[3] Some heavy metals play crucial role in regulating physiological processes of animals.[2] However, due to their oxidation-reduction and chemical coordination potential, they bypass the control mechanisms as well, such as nutrient transport, homeostasis compartmentalization and binding ability cells.[4,5]

Some of the heavy metals released from brick kiln industries have adverse effects on human health.[4,5] They induce toxicity by getting themselves attached with the protein sites that are not compatible for them. They disrupt the original metal from their natural binding position, thereby destroying the cells.[6,7] Chromium (Cr), zinc (Zn), nickel (Ni), and cadmium (Cd) are known to impart adverse actions among humans and other living organisms.[8–10] The degree of toxicity of different metals depends on dose, duration of exposure, route of administration and other physiological factors, such as nutrition.[11] Among brick kiln workers, children living/working at brick kiln sites are at increased risk of exposure to environmental pollutants.[3] Different cities of Punjab province have a large number of operational brick kilns, especially District Rawalpindi, where an estimated 450 brick kilns are located. In Rawat, these brick kilns and road transport smoke pose a huge burden of air pollution on human health and atmosphere.[12]

Few heavy metals act as endocrine disrupting chemicals (EDCs). Various studies have shown that heavy metals affect children growth by disturbing hypothalamic pituitary somatotropic axis.[13] Jahan et al.[9] reported that exposure to some heavy metals (Cd, Cr, and Ni) causes homeostatic imbalances among male brick kiln workers via decreasing antioxidant concentration and reproductive enzymes levels (testosterone),[9] however, no study has yet been conducted to explore the heavy metal burden in blood and its effects on children's health. Industries are

CONTACT Sarwat Jahan drsarwatjahan@gmail.com Reproductive Physiology Laboratory, Department of Animal Sciences, Quaid-i-Azam University, Islamabad 44000, Pakistan

scientific reports

Check for updates

OPEN

Biochemical and reproductive biomarker analysis to study the consequences of heavy metal burden on health profile of male brick kiln workers

Mehwish David[1], Sarwat Jahan[1], Javaid Hussain[2], Humaira Rehman[3], Karen J. Cloete[4,5], Tayyaba Afsar[6], Ali Almajwal[6], Nawaf W. Alruwaili[6] & Suhail Razak[6]✉

The present study aims to assess the effect of a heavy metal burden on general health, biochemical parameters, an antioxidant enzyme, and reproductive hormone parameters in adult male brick kiln workers from Pakistan. The study participants ($n = 546$) provided demographic data including general health as well as body mass index. Blood was collected to quantitatively assess hematological, biochemical, and reproductive hormone parameters as well as heavy metal concentrations using both atomic absorption spectroscopy (AAS) and particle-induced X-ray emission (PIXE). The data showed that 10% of the brick kiln workers were underweight and 10% obese ($P = 0.059$), with workers also reporting multiple health issues. Heavy metal concentrations utilizing AAS revealed significantly ($p = 0.000$) higher levels of cadmium, chromium, and nickel, while PIXE detected more than permissible levels of Si, P, S, Cl, K, Ca, Zn, Ti ($p = 0.052$), Mn ($p = 0.017$), Fe ($p = 0.055$), Co ($p = 0.011$), Ni ($p = 0.045$), and Cu ($p = 0.003$), in the blood of kiln workers. Moreover, a significant increase in platelet count ($P = 0.010$), a decrease in sodium dismutase levels ($p = 0.006$), a major increase in reactive oxygen species ($p = 0.001$), and a reduction in protein content ($p = 0.013$) were evident. A significant increase in cortisol levels ($p = 0.000$) among the workers group was also observed. The concentration of LH and FSH increased significantly ($p = 0.000$), while that of testosterone decreased ($p = 0.000$) in the worker group compared with controls. A significant inverse relationship was found between cortisol, LH ($r = -0.380$), and FSH ($r = -0.946$), while a positive correlation between cortisol and testosterone was also evident ($r = 0.164$). The study concludes that increased heavy metal burden in the blood of brick kiln workers exposes them to the development of general and reproductive health problems due to compromised antioxidant enzyme levels, increased oxidative stress conditions, and a disturbing reproductive axis.

Pakistan is the third largest brick producing country in South Asia, with more than 45 billion bricks being produced per year by approximately 1.8 million brick kiln workers[1,2]. A recent study reported that åve million people are known to be associated with the brick industry[3]. The Punjab province of Pakistan contains the largest number (10,347) of active brick kilns that are operated by approximately 249 682 brick kiln workers[4]. Despite the large numbers of families associated with this profession, it remains a highly unstructured and undocumented area in terms of public and reproductive health risks[5].

[1]Reproductive Physiology Laboratory, Department of Zoology, Quaid-I-Azam University, Islamabad, Pakistan. [2]National Centre for Physics, Quaid-I-Azam University Campus, Islamabad 44000, Pakistan. [3]Biomedical Center, Uppsala University, Husargatan 3, 752 37 Uppsala, Sweden. [4]College of Graduate Studies, UNESCO-UNISA Africa Chair in Nanosciences-Nanotechnology, University of South Africa, Muckleneuk Ridge, PO Box 392, Pretoria, South Africa. [5]Nanoscience's African Network (NANOAFNET), iThemba LABS-National Research Foundation, 1 Old Faure RoadWestern Cape Province, PO Box 722, Somerset West 7129, South Africa. [6]Department of Community Health Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia. ✉email: smarazi@ksu.edu.sa