Evaluation of the Association Between Reproductive Hazards of Vinyl Chloride and KRAS Gene Mutations in Adult Male Factory Workers

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WAQAR AHMAD

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ISLAMABAD

2018

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

IN

ANIMAL SCIENCES

(REPRODUCTIVE PHYSIOLOGY)

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“In the Name of ALLAH, the most Beneficent, the most Merciful”

Dedicated to My Most caring Parents, My Loving Brothers, Sister and My most Generous Supervisor Who Supported Me a lot in every aspect to procced forward in mine academic life and For everything they provided me.

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort and the material contained in this thesis is original. I have not previously presented any part of this work elsewhere for any other degree.

WAQAR AHMAD

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List of Abbreviations

Abbreviations	Full name
VC	Vinyl chloride
PVC	Polyvinyl chloride
EDC	Endocrine disrupting chemical
HPG axis	Hypothalamic-anterior pituitary gonadal axis
RAS	Rat sarcoma
KRAS	Kristen RAS
CYP2E1	Cytochrome P450 2E1
CEO	Chloroethylene oxide
CAA	Chloroacetaldehyde
GnRH	Gonadotropin releasing hormone
LH	Luteinizing hormone
FSH	Folliclestimulating hormone
T	Testosterone
E_2	Estradiol
PG	Progesterone
WHO	World Health organization

Abbreviations	Full name
DEHP	Diester is di-(2-ethylhexyl) phthalate
MEHP	Mono-2-ethylhexyl phthalate
BPA	Bisphenol A
ERs	Estrogen receptors
µM	Micro-molar
GTPases	Guanosine triphosphatases
GAPs	GTPase activating proteins
GEFs	Guanine nucleotide exchange factors
MAP	Mitogen-activated protein
HCCs	Hepatocellular carcinomas
LAS	Liver angiosarcomas
TGCTs	Testicular germ cell tumors
Rpm	Revolution per minute
Ml	Millilitre
µl	Microliter
PCI	Phenol chloroform iso-amylalcohol

Abbreviations	Full name
SDS	Sodium dodecyl sulphate
MgCl2	Magnesium chloride
Nacl	Sodium chloride
EDTA	Ethylene diamine tetra-acetic acid
Gm	Grams
TE buffer	Tris-EDTA buffer
TBE buffer	Tris-Borate electrophoresis buffer
EtBr	Ethidium bromide
X	Times
V	Voltage
A	Ampere
PCR	Polymerase chain reaction
bp	Base pair
C	Centigrade
SSCP	Single Stranded Confirmation Polymorphism
APS	Ammonium persulphate

Abbreviations	Full name
mM	Millimolar
NaOH	Sodium hydroxide
EIA	Enzyme immunoassay
ANOVA	Analysis of variance
ELISA	Enzyme linked immunosorbent assay
ng	nano-gram
SEM	Standard error mean
DNA	Deoxy ribonucleic acid
NRs	Nuclear receptors
hER	Human estrogen receptor
hAR	Human androgen receptors

ACKNOWLEDGEMENTS

*All praises to **Almighty Allah**, Who puts the sun's seal on the tablets of the flowing waters and throws clouds to the skies, Who distills the waters of the clouds over the seas to conceive the pearl in the womb of the oyster, Who creates fire in every stone, colour in the fire, radiance in the colour, Who gives voices to the dust, word to the voices, and life to the world, Who created us as a Muslim and blessed us with knowledge to differentiate between right and wrong. All prays to Him as He blessed us with the **Holy Prophet, Hazrat Muhammad** (SAW) for whom the whole universe is created and whoenabled us to worship only one God. He (SAW) brought us out of darkness and enlightened the way of heaven.*

*It is a matter of great pleasure to express my sincere regards to my honorable Supervisor and head of department, Professor **Dr. Sarwat Jahan**, Department of Animal Sciences 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.*

*I would like to thank all of mine lab seniors **Asad Aryan, Qurat-ul-ain, Humaira Rehman, Hizbullah Wazir and Mehwish Devid** for their sincere effort for me. I would also like to express my gratitude to mine senior **Ghazala Shaheen** for every kind of guide and help. For her kind and generous help, I would like to acknowledge my junior **Saman Ijaz** and have special thanks for all of mine batch fellows **Wardha Khan, Amna Sardar** and **Aneela** for their support, encouragement and cooperation.*

Waqar Ahmad

Abstract

Vinyl chloride (VC) is a monomer of polyvinyl chloride (PVC) which is among one of the most common synthetic polymers used in manufacturing of different plastic products across the world. Like other plasticizers, it also acts as an endocrine disrupting chemical (EDC) and can disrupt normal functioning of hypothalamic-anterior pituitary gonadal axis (HPG axis). It is also a renowned carcinogen and may be the possible cause of mutations and (KRAS) gene is one of the most frequently mutated gene due to VC exposure. Current experiment was performed to evaluate the association between reproductive hazards of VC as EDC and mutations of KRAS gene. Adult male factory workers (n=125) were enrolled in the study and were organized into five groups; exposed groups (n=95) and control group (n=30). Individuals of the first group (n=36) have an exposure between 1-5 years. Individuals with an exposure of 6-10 years were grouped into the second group (n=32). Third group (n=17) and fourth group (n=10) members have an exposure between 11-15 and 16-20 years respectively. Individuals with no exposure were classified into the control group (n=30). Blood samples were obtained from participants for mutational analysis and plasma was separated and stored for hormonal analysis. Mutational analysis was performed separately for exon 1 and exon 2 of KRAS gene. Results of present study revealed a significant decrease of testosterone concentration in first and second groups having an exposure of 1-5 years and 6-10 years respectively but a non-significant decrease was noticed in third and fourth groups having an exposure of 11-15 and 16-20 years respectively. KRAS mutations were also found in members of all the VC exposed groups. Eighteen samples were found mutated of exon 1 out of 95 VC exposed and 14 samples of exon 2 out of 95 samples were mutated irrespective of testosterone levels. In conclusion, our findings suggest that VC exposure reduces testosterone concentration in adult male workers but prolong exposure to VC has non-significant affect on testosterone concentration. Current findings also demonstrated that VC induces KRAS mutations regardless of the testosterone levels in adult male workers. Therefore, present findings did not show any association between testosterone concentration and mutations of KRAS gene.

Introduction

During the previous century, robust development in technology and industrialization has taken place. Manufacturing of huge quantities of synthetic biomedical and industrial chemicals and, undesirable contaminants cause devastating consequences to ecosystem and enact negative health effects to humans and wildlife. According to recent studies, almost 40% human deaths (62 million/year) are ascribed to exposure of chemical pollutants (Pimentel *et al.,* 2007). During past 60 years, production of synthetic chemical substances exceeded 140,000 and about 2,000 per year fresh chemicals are synthesized (Judson *et al.,* 2009). These chemicals are universal and blow-out in soil, water, food and air. Plastic is one of the chemicals humans have been using for making various products. Different chemicals are used in manufacturing the plastics and vinyl chloride (VC) is one of them.

Vinyl Chloride (VC)

VC is a monomer of PVC which is third most widely used synthetic plastic polymer across the world after polyethylene and polypropylene (Allsopp & Vianello, 2000). Vinyl chloride is synthesized on a large scale_ about 13 billion kilograms are formed annually and nearly 31.1 million tons were produced in 2000 (Weissermel & Arpe, 2003). It is used in the manufacturing of plastic products like water pipes, water tanks, shopping bags and plastic coverings of wires etc. VC is absorbed via respiration in workers and is metabolized in the liver. It is a familiar human carcinogen and cytochrome P450 2E1 (CYP2E1) in the liver metabolizes the VC to the reactive intermediates chloroethylene oxide (CEO) and chloroacetaldehyde (CAA). These intermediates can bind to cellular macromolecules causing DNA damage, including mutations of specific cancer-related genes such as RAS and tumor suppressor gene *p*16 (Schindler *et al.,* 2007).

Plasticizers as endocrine disrupting chemicals (EDCs)

Reproductive hormone levels of the body are regulated by HPG axis; the hypothalamus-anterior pituitary-gonadal axis (Yeung *et al.,* 2011). Hypothalamus contains gonadotropin releasing hormone(GnRH) neurons. These GnRH neurons produce and secrete gonadotropin releasing hormone (GnRH). GnRH excites anterior pituitary which synthesizes and secretes luteinizing hormone (LH) and folliclestimulating hormone (FSH) to the gonads (Cheng *et al.,*

2010). The gonads which are testes in males and ovaries in females produce and release the hormones, called sex hormones, in the circulation. Testes produce testosterone (T) in males and ovaries produce estradiol (E_2) and progesterone (PG) in females. HPG axis is under the control of negative feed back mechanism. This negative feed back mechanism is regulated by sex hormone concenterations in blood which are testosterone in males (Tilbrook & Clarke, 2001) and estradiol (E_2) (Chimento *et al.*, 2014) and progesterone (PG) in females and by responsiveness of pituitary to GnRH.

HPG axis can be disrupted through a number of factors which are called endocrine disrupting chemicals (EDCs) or endocrine disruptors (Diamanti-Kandarakis *et al.*, 2009). Endocrine disrupting chemicals (EDCs) as defined by World Health organization (WHO) are a group of exogenous elements which disrupt the normal functioning of endocrine system and pose adversative health effects in an organism, or its descendants, or (sub) populations (WHO/IPCS, 2002). Among natural sources of EDCs fungi, bacteria, plants and man-synthesized chemicals are most prominent. As hormones require very low concentration for their action, so EDCs also require minute quantities to disrupt endocrine system. Different chemicals used in the manufacture of plastics also called plasticizers are well known EDCs and several other chemicals used in manufacture of our daily life products like cosmetics, construction materials, different paper products are also EDCs (Ribeiro *et al.*, 2017). EDCs may leach into the air, soil and water and exposure to these EDCs via drinking of contaminated water, eating contaminated food, exposure with contaminated air and dermal absorption enhances the chances of developing reproductive hazards and endocrine abnormalities (Calafat and Needham, 2007).

In human males, testosterone is major hormone associated with the growth of male reproductive tissues such as testes and prostate, as well as endorsing secondary sexual characteristics like increased muscle and bone mass, and body hair growth (Mooradian *et al.*, 1987). Testosterone is responsible for the maintenance of spermatogenic process; the process responsible for sperm formation (Cheng *et al.*, 2010). Testosterone also causes the embryonic development of male reproductive system and prevents the germ cell apoptosis in the adulthood (Seachrist *et al.*, 2017). Normal testosterone levels are compulsory for above mentioned functions of normal male reproduction, so any decline in testosterone levels will negatively affect male reproduction. Reproductive toxicity is a risk related with several chemicals, which interfere with normal reproduction; these chemicals are called reprotoxic. It poses devastating

effects on sexual function and fertility in adult males and females, and also causes developmental toxicity in the descendants (United Nations, 2015). In recent years, EDCs have been major concern for their toxic effects on reproduction (Damstra *et al*., 2002) and abnormal testosterone levels is one of the reproductive toxicity, or reproductive hazard caused by these EDCs.

Several past studies have linked plasticizers as EDCs with reduced testosterone levels. Phthalate esters are well known plasticizers and are used on a large scale in the manufacturing of chemicals. These are also used in the synthesis of different products used in daily life including insecticides, food peckaging, for providing flexibility in polyvinyl chloride (PVC) products and cosmetics. All phthalate esters are repoted to be EDCs (Sheikh & Beg, 2017). The most commonly used phthalate diester is di-(2-ethylhexyl) phthalate (DEHP). Studies on animals have proved that DEHP exposure causes a decrease in testosterone concenteration, low sperm count and adverse effect on testicular development (Hallmark *et al*., 2007). Chen *et al.* (2017) evaluated the effect of mono-2-ethylhexyl phthalate (MEHP) on the testosterone levels in Taiwanian population on 786 subjects. Study was conducted on adolescents (aged 12-20) and young adults (20-30). In this study, testosterone levels were signifacantly reduced in both adolescents and adults due to MHEP exposure (Chen *et al*., 2017). Taken together, these findings suggest that phthalates are a well endocrine disrupting chemicals (EDCs) and cause testosterone levels.

Bisphenol A (BPA) is also an everyday plasticizer and is produced on a bulky scale. The production of BPA exceeded 5.4 million tons in the year 2016 (Seachrist *et al.*, 2017). BPA is used in the lining of canned food, in synthesizing plastics, dental sealings and thermal paper receipts. The most common source of exposure to BPA is diet through leaching of BPA from the lining of canned food into the food and beaverages and also through the erosion of BPA packaging material (Vandenberg *et al*., 2007). Other sources of exposure to BPA include dermal absorption and inhalation (Ehrlich *et al*., 2014). BPA like other plasticizers is an EDC (Vandenberg *et al*., 2007). It is an estrogenic compound and performs it's estrogenic function by binding to nuclear estrogen receptors (ERs). Seachrist *et al* ., conducted the experiment for assesment of BPA effects on the development and testosterone levels on TM3 murine leyding cell. TM3 leyding cells were exposed to different concenterations of BPA and testosterone

levels were measured. 1, 10 and 100 µM of BPA reduced the testosterone levels by 22%, 28% and 39% respectively (Cristine *et al*., 2018).

Levels *et al*. (2016) evaluated the effect of BPA exposure on male and female children and adolescents. Male chilren were aged between 6-11 years and female children were also between 6-11 years. Male adolescents were between 12-19 years and female adolescents were also between 12-19 years. Levels of BPA were negatively associated with testosterone levels in male adolescents. In female adolescents, high BPA levels were related with high serum testosterone levels. No relation was observed between BPA levels and children (male and female) (Levels *et al*., 2016). Taken together these findings also suggested that there is reverse relation between BPA and total testosterone levels.

Vinyl chloride (VC) may cause abnormal testosterone levels

Vinyl chloride like other plasticizers is also an EDC. It may disturb the HPG axis and may hinder with the normal functioning of endocrine system. It may cause abnormal levels of estrogen and testosterone. As in the males, testosterone plays a key role in process of sperm formation and other male characters as mentioned above, so focus of the current study was to govern the affect of VC on testosterone levels in males.

Rat sarcoma (RAS)

RAS were discovered for the first time as oncogenes in Kirsten and Harvey strains of retroviruses (Kirsten & Mayer, 1969). RAS superfamily proteins are a part of the small G protein class and include more than 150 guanosine triphosphate hydrolases(GTPases) (Colicelli, 2004). GTPases, along with accompanying effectors regulators, take part as chief controller elements of signal transduction pathways which are involved with every part in cell biology. Majority of these GTPases, are included in RAS oncoprotein superfamily (Bourne *et al.*, 1991). This tremendously conserved protein family plays a key part in signal transduction from membrane proteins within the cell (Jonckheere *et al*., 2017).

RAS Biochemistry and Function

A common mechanism of action is shared by all RAS proteins (Vetter & Wittinghofer, 2001). In the GTP-bound state, all RAS proteins exhibit a high affinity binding surface for downstream effector proteins (Marais *et al.*, 1997). However, the structural changes in RAS proteins are

only for a brief period; disengagement of GTP causes reorientation of binding surfaces for downstream effector proteins. As a result, effector proteins are released. In RAS protein activation the rate limiting step is interchange of bound GDP for GTP (Van Dyke *et al*., 1977).GEFs, guanine nucleotide exchange factors, or guanine nucleotide dissociation stimulators or GDSs cause the release of bound GDP (Fig. 1). So they result in RAS activation by loading of GTP for GDP (Chardin *et al.*, 1993). Ras proteins have inherent GTPase activity but this GTPase activity is low (Neal *et al*., 1988). This tends to extend signal transduction. GTPase activating proteins (GAPs) significantly enhance GTP hydrolysis (Turner *et al*., 2001).

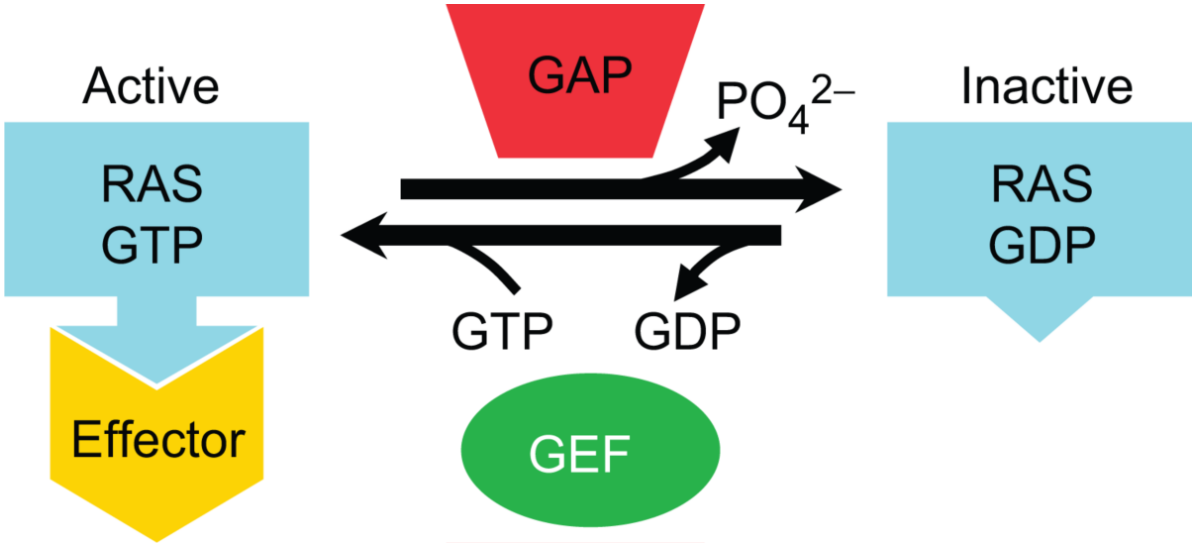

Fig 1.1: RAS proteins cycle between GTP- and GDP-bound forms (Colicelli, 2004).

RAS Protein Functions

RAS is involved in signal transduction within the cell. Therefore, when Ras is activated by incoming signals, it will switch on other proteins. These activated proteins turn on genes involved in cell differentiation, cell growth, and survival. RAS transduces a signal through different effectors. The protein kinases RAF1 (also called c-Raf), ARAF (also termed A-Raf) and BRAF (also termed BRaf) are different effectors for RAS (Van Aelst *et al*., 1993). Activated RAS binds to RAF and kinase activity of RAF is activated. This initiates MEK-ERK

MAP (mitogen-activated protein) kinase cascade, which regulates transcription and various cell functions (Soediono, 1989).

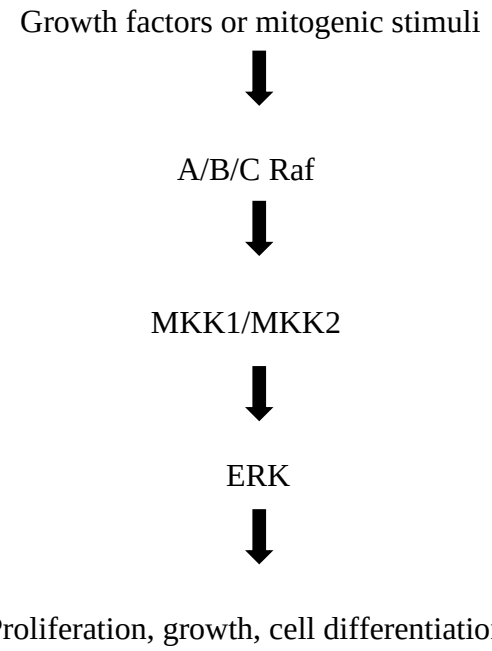

Fig 1.2: Generalized Pathway of RAS genes.

As current experiment was done on humans, so MAP kinase cascades in mammals including humans is debated here. The best-characterized MAPK system of mammals is ERK1/2 pathway. The Raf proteins (A-Raf, B-Raf or c-Raf), are the key mediators of response to growth factors (FGF, PDGF, EGF, etc.). All of the above enzymes phosphorylate and hence activate the MKK1 and/or MKK2kinases, that are highly specific activators for ERK1 and ERK2. The latter phosphorylates a number of substrates important for cell cycle progression, cell proliferation, differentiation and cell division.

The RAS superfamily proteins are categorized into RAS, RAB, ARF, RHO families, and closely associated Gα family. Members of each of these family are classified into evolutionarily preserved branches. This classification reflect structural, functional, and biochemical preservation (Colicelli, 2004). Since our gene of interest KRAS falls within RAS family and RAS oncoprotein branch, so only RAS family and RAS oncoprotein branc is discussed here.

RAS Protein Subfamily (35 members)

RAS oncoprotein branch (HRAS, KRAS, and NRAS)

RAS subfamily contains 35 members and is divided into several branches, but all oncoproteins are included in RAS oncoprotein branch. KRAS, NRAS and HRAS (K, N, HRAS) proteins are included in this branch. Mutations in these proteins effectively transform cells in vivo and in vitro, and these mutations are frequent in a greater variety of human cancers, or tumors. Ras gene mutations are found in a variety of tumors, although the incidence varies greatly. The highest incidences are found in the colon (50%) (Grady & Markowitz, 2002), adenocarcinomas of the pancreas (90%) (Eser *et al*., 2014), in thyroid tumors (50%); and the lung (30%); and in myeloid leukemia (30%) (Arbeitsmedizin & Hannover, 2001). Mutations in codon 12, 13, or 61 are the most common mutations in all of these genes and convert these genes into active oncogenes (Weihrauch *et al*., 2001); (Jackson *et al*., 2017). In some tumors, RAS genes are overexpressed; for example, in breast cancer. Some mutations lead to deletion of negative regulators (for instance, in neurological tumors NF1, a GAP for RAS) and still other mutations lead to overexpression of positive regulators (for example in renal cancer cells SOS1, a GEF for RAS) (Colicelli, 2004). Overall, all of these mutations play an important role in cell transformation by RAS.

KRAS as oncogene

KRAS, our gene of interest as already mentioned above is the member of RAS oncoprotein branch of RAS subfamily. KRAS gene is located on short arm of chromosome 12. KRAS is involved in cell differentiation, cell proliferation, survival, growth, apoptosis and cell migration and the major pathways involved are RAS-RAF-MEK-ERK-MAP cascade and PI3K (Nan *et al.*, 2015*).* Both of these pathways have already been described. As KRAS is involved in cell differentiation and growth, so mutations in KRAS, specially activating mutations cause tumor formation or cancers in different body organs.

Most common mutations are point mutations in codon 12 and 13 of exon 1 and codon 61 of exon 2 of KRAS (Nxan *et al*., 2015). Approximately 90% of the activating mutations are found in codons 12 (GGT) and 13 (GGC) of exon 1 and nearly 5% in codon 61 (CAA) located in exon 2 (Palmirotta *et al*., 2011). Codon 12 normally encodes glycine, codon 13 also encodes

glycine and codon 61 encodes glutamate. G 12 or codon 12 mutations block the binding site for GAP so that bound GTP can not be hydrolysed and hence KRAS remains in the activated form (Eser *et al.,* 2014). G 13 or codon 13 mutations leads to faster GTP/GDP cycle. This causes impairment of sustaining of activation and hence GTP can not remain bound to KRAS. G 61 mutations block the intrinsic GTPase activity of KRAS and so impair GTP hydrolysis and lead to KRAS in permanently activated form even in the absence of external factors (Jonckheere *et al*., 2017).

KRAS mutations are found in 27% of all cancer types. KRAS is mutated in 15-20% of lung cancer or tumors, 98 % of pancreatic adenocarcinomas and 40% of colon adenomas (Eser *et al.,* 2014). These very high rates of K-ras mutations make it the single most common mutated human oncoprotein.

VC and KRAS mutations

A causal relationship between occupational exposure to vinyl chloride and hepatocellular carcinomas (HCCs) is already established through a qnumber of studies. In one study performed by Arbeitsmedizin and Hannover (2001), 5 out of 12 (42%) HCC patients had a mutation of KRAS gene. 4 had a mutation of codon 12 and 1 had a mutation of codon 13. Results of this experiment are presented in the table below.

Table 1.1: Mutations of codon 12 and 13 of exon 1 of KRAS (Arbeitsmedizin and Hannover (2001)

Name of codon	Mutation	Substitution
12	GGT (wild type)	Glycine
12	GGT ⟶ GAT	Aspartate
12	GGT ⟶ GTT	Valine
12	GGT ⟶ TGT	Cystine
12	GGT ⟶ GAT	Aspartate
13	GGC (wild type)	Glycine
13	GGC ⟶ GAT	Aspartate

Both of codons 12 and 13 are of exon 1 in this case. The codon 12 is GGT and encodes for glycine in wild type gene while codon 13 is GGC and also encodes for glycine. The most common mutations are substitution mutations substituting aspartate for glycine or valine or cystine. Aspartate is the most common substitution for glycine at both of the codons 12 and 13 (Arbeitsmedizin & Hannover, 2001).

In another experiment, KRAS mutations were found in 6 out of 18 VC associated HCCs(Weihrauch *et al*., 2001). All of the mutations were found in codon 12 and 13 of exon 1. 4 mutations were found in codon 12 and 2 were found in codon 13. Here, too most common mutations were substitution mutations. At codon 12, 2 out of 4 mutations substituited aspartate for glycine. Other 2 mutations at codon 12 substituited valine and cystine for glycine. Out of 2 mutations at codon 13, 1 mutation substituited cystine for glycine and second mutation substituited aspartate for glycine. Overall, aspartate was the most common substitution for glycine at both codons. The results of this experiment are summarized in the table below.

Table 1.2: Mutations of codon 12 and 13 of exon 1 of KRAS (Weihrauch *et al.*, 2001).

Name of codon	Mutation	Substitution
12	GGT (wild type)	Glycine
12	GGT ⟶ GAT	Aspartate
12	GGT ⟶ GTT	Cystine
12	GGT ⟶ TGT	Valine
12	GGT ⟶ GAT	Aspartate
13	GGC (wild type)	Glycine
13	GGC ⟶ TGC	Cystine
13	GGC ⟶ GAT	Aspartate

Liver angiosarcomas (LAS) are also liver tumors and were first proven liver tumors found in VC workers. Weihrauch *et a*l (2001), examined 12 patients with LAS and observed a very high prevelance of KRAS mutations. 83% cases had KRAS mutations at codon 13 replacing glycine for aspartate (Weihrauch *et al*., 2001). KRAS is also mutated in colon cancer. Palmirotta et al (2011), conducted a study on a colon cancer patient (Palmirotta et al., 2011). In this study, point mutations in exon 1 and exon 2 were found. All mutations were substitution mutations. GGT to TGT substitution (Glycine to Cysteine) at codon 12, and a GAC to AAC substitution (Aspartic Acid to Asparagine) at codon 57 were found in this study (Palmirotta *et al.*, 2011).

Colorectal cancer is another cancer in which KRAS gene is most frequently mutated. In a study coducted on 136 colorectal patients, 53 were identified having KRAS mutations and 2 were identified having double mutation in codon 12 and 13 and 1 had multiple mutations in codon 12. In another study performed on Mexican population, 101 colorectal cancer patient were examined. 2 patients were found of having a double mutation in codon 12 and 1 having a concurrent mutation of both codons 12 and 13 (Palmirotta *et al*., 2011). In a current study performed in pancreas tumor biopsies gained from 222 patients, Guo *et al*. recognized a new KRAS mutation at codon 76 of exon 1, simultaneously with a codon 12 of same exon (Palmirotta et al., 2011). Kimura *et al*. (2015) performed mutational screening on 236

gastrointestinal human tumors and four colorectal cancer and one gastric cancer cases with double K-ras codon 12 and 13 mutations were reported in this study (Kimura *et al*., 2015).

Testicular cell cancers are another tumors involving KRAS mutations. Testicular cancers are a very relative rare type of tumors in men and accounts for 1% of all tumors in men. But at the same time, testicular cell cancer is the most common malignancy in the men aged between 15 to 40 years. 95% of all testicular cell tumors are testicular germ cell tumors (TGCTs). TGCTs are further divided into two types: Seminomas and non-seminomas. Seminomas occur primarily within the testes and non-seminomas occur in extragonadal sites and include several subtypes of tumors like embryonal cell carcinoma, choriocarcinoma and yolk sac tumors. Hacioglu *et al* (2017), examined 24 pure seminoma patients and 19 non-seminoma patients. 4 out of 24 pure seminoma and 3 out of 19 non-seminoma patients had KRAS mutations. These mutations were found in different codons of different exons. . The most common KRAS mutation was in the codon 146 of the exon 4 (in 3 cases). In addition, two patients had GGT>AGT mutation in the codon 12 of the exon 2, one patient had GGC>GAC mutation in the codon 13 of the exon 2, and another had CAA>CTA mutation in the codon 61 of the exon 3. In another study (Sommerer *et al.*, n.d.), 30 seminoma and 32 non-seminoma patients were examined. 2 out of 30 seminoma and 3 out of non-seminoma patients had KRAS mutations. All the patients had a mutation of codon 12 of exon 1 but none had a mutation of codon 13 or 61 in this experiment.

VC may cause KRAS mutations

All of the studies performed above are on verified tumors due to vinyl chloride exposure. No one perfomed mutation analysis of KRAS gene on VC workers who are non tumorous. As majority of mutations of KRAS gene are in codons 12 and 13 of exon 1 and codon 61 of exon 2, prime focus of present study was to assess the mutation status of exon 1 and exon 2 in VC workers who are working in VC factories upto 20 years but did not develop any tumors.

Aims and Objectives

EDCs may interrupt with normal functioning of HPG axis and cause abnormal levels of sex hormones or reproductive hormones (Wen *et al*., 2017). Different chemicals act as EDCs and plasticizers are also included in them. Phthalates are the plasticizers and reduced testosterone

levels in animals (Hallmark *et al*., 2007) and in humans (Chen *et al*., 2017). BPA another plasticizer reduced testosterone levels in leyding cells (Cristine *et al*., 2018) and human male adolescents (Levels *et al*., 2016). However, very small data regarding the affect of VC, a common plasticizer, on testosterone levels is present. Similarly, VC is also a familiar mutagen, causes mutaions in different genes and KRAS is most commonly mutated gene due to VC. Arbeitsmedizin and Hannover (2001) found 42% KRAS mutations in hepatocellular carcinomas patients due to VC exposure. 83% mutations were discovered in liver angiosarcoma (LAS) patients (Weihrauch *et al*., 2001) due to VC exposure and KRAS mutations were also present in VC-associated colon cancer patients. All of the studies performed above documented KRAS mutations in confirmed tumors due to VC exposure. To date, no study was performed to find mutations in VC workers who were non-tumorous. First objective of the current study was to find effect of VC exposure on testosterone concentration in human adult male workers. Another objective was to find KRAS mutations in non-tumorous VC workers. Lastly, major objective of the present study was to find association between KRAS mutations and testosterone levels in VC exposed male workers.

Materials and Methods

The current study was conducted at the Animal Sciences Department of Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, with the permission of Ethical Committee for research on humans.

Collection of samples

The different areas of Pakistan were visited; Rawalpindi and Lahore for searching the plastic industries. In Rawalpindi, Golra Mor area was explored to find plastic industries there in which vinyl chloride was being used as the main ingredient. Industries were in the form of small units in which plastic pipes, shopping bags and water tanks were being synthesized. Performa was prepared for seeking consent for taking blood from factory workers. In the performa questions about the name of worker, age, experience of working with vinyl chloride, smoking status and infectious disease record were recorded. In Lahore, Shahdrah region was explored for finding plastic industries. Factories in Lahore manufactured water pipes and plastic bags for shopping. Informed consent was also gained from factory workers by filling the same performa. The workers were having an exposure of upto 20 years with VC. Blood was collected from the adult male VC workers. Blood was also taken from healthy control adult males. The ages of the VC workers were between 19 to 48 years. All other risk factors for developing KRAS mutations like drugs, metabolic disorders or autoimmune chronic hepatitis, alcohol abuse and smoking were excluded through perfoma filling. Control samples were also taken from the individuals of all ages included in the VC workers. Data was organized on the basis of exposure to VC into the following categories.

Control (C): Having no exposure to VC

Group 1 (G1): Having exposure between 1-5 years

Group 2 (G2): Having exposure between 6-10 years

Group 3 (G3): Having exposure between 11-15 years

Group 4 (G4): Having exposure between 16-20 years

Collection of blood

After having written information, blood samples were collected from venous blood vessel in a 5ml syringe by a professional laboratory technician. 2.5ml of the blood was then drawn into the 5ml heparinized tube and 2.5ml was left into the syringe for serum separation. The blood

in the heparinized tubes was then stored at -4˚C for DNA extraction. Blood in the syringe was taken into the 3ml tube. Blood was centrifuged at 13000 revolution per minute (rpm) for 20 minutes to separate plasma and was transferred to the eppendrof tubes.

DNA Extraction

DNA extraction was performed in two days. Steps are as following.

Day 1

1- 0.5 ml of blood and 0.5 ml of solution A was taken in 1.5 ml of eppendrof tube.
2- Blood and solution A was mixed by inverting tubes 4-6 times and tubes were put at room temperature for 20-40 minutes.
3- Then mixture was centrifuged at 13000 rpm for 3-4 minutes.
4- Nuclear pellet was resuspended in 0.5 ml of solution A after discarding supernatant.
5- When pellet was clear, then discarded supernatant and resuspended nuclear pellet in solution B (400µl), 20 % SDS (12 µl) and proteinase kinase (2-4 µl).
6- Overnight incubation was done at 37˚C for or 65˚C for 3 hours.

Day 2

7- 0.5 ml of fresh mixture of PCI was added to tubes and was mixed. Tubes were then centrifuged for 10 minutes at 13000 rpm.
8- The aqueous phase (upper layer) was collected in a new tube and 500 µl of solution C was added in tubes and centrifuged at 13000 rpm for 10 minutes.
9- Aqueous phase was placed in new tube and was precipitated by adding Sodium Acetate (3M) (55µl) and Isopropanol (500 µl) and inverted tubes several times for precipitating the DNA.
10- Centrifugation was done for 10 minutes to settle the DNA and discarded supernatant.
11- To the tubes 0.5 ml of 70% ethanol was added and tubes were then centrifuged for 5 minutes to settle the DNA and supernatant was discarded.
12- Ethanol was evaporated by inverting tubes on tissue paper.
13- DNA was dissolved in 60µl of TE buffer and was kept at 37˚C for overnight and then stored in freezer at -20.

Recopies of Solutions

Solution A for 250 ml

Sucrose 27.36 gm

Tris 0.303 gm

MgCl2 0.254 gm

Then autoclaved the mixture. After autoclave 2.5 ml tritron X-100 was added.

Solution B

Tris 0.182 gm

NaCl 3.52 gm

EDTA 0.11 gm

All the reagents were dissolved in 200 ml of distilled autoclaved water and were mixed well before use.

Solution C

Iso-amyl alcohol 2 ml

Chloroform 48 ml

The two reagents mentioned above were taken in a 50 ml falcon tube and were mixed well.

PCI (Phenol, Chloroform, Iso-amy alcohol)

Phenol 25 ml

Chloroform 24 ml

Iso-amy alcohol 1 ml

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

20% Sodium dodisyl 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 0.08 gm

EDTA 0.014 gm

Above mentioned chemicals were dissolved in in 50 ml of autoclaved water, then raised the volume to 70ml and autoclaved the mixture.

10x Tris Boric EDTA buffer (TBE Buffer)

Tris 109 gm

Boric acid 55 gm

EDTA 9.3 gm

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, 0.1 gm 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 and dissolving in 100 ml of 1X TBE (Tris-Borate electrophoresis buffer) buffer and then heating the mixture. Heating was performed by heating the mixture for 2 minutes in the oven. After heating 5 µl of ethidium bromide (EtBr) was added to gel solution. Gel solution was poured in the gel plate having dual comb 16 teethed casters. Then the solution was allowed to solidify at room temperature. When the gel was solidified, then 2 µl diluted DNA was mixed with 2 µl of 10X loading dye and was loaded in the wells. Control samples were also run in some wells. Gel was run at 90V and 500A for 40 minutes. UV trans-illuminator (Bio Rad, UK) was then used for visualizing gel.

Polymerase Chain Reaction (PCR)

Details of the PCR are as following.

Primer Designing

We designed the primers for exon 1 and exon 2 of KRAS gene through a number of previously published papers (Sommerer *et a*l., n.d.), (Hacioglu *et al*., n.d), (Weihrauch *et al.*, 2001). Primers were checked by using BLAST for specific amplification. These primers were used for amplification of exon 1 and exon 2 of KRAS gene. Details of the primers are given in table 2.1.

Table 2.1: Primers of exon 1 and exon 2 of KRAS gene.

Number of exon	**Length (bp)**	**Primer sequence (5-3; a, forward; b, reverse)**
Exon 1	164	f-GGCCTGCTGAAAATGACTGAA r-GGTCCTGCACCAGTAATATGC
Exon 2	133	f-CAGGATTCCTACAGGAAGCAAGTAG r-CACAAAGAAAGCCCTCCCCA

Optimization of PCR products

Both of primers for exon 1 and exon 2 were optimized at the same conditions. PCR profile after optimization for both exons consisted of an initial melting temperature of 95˚C for 5 minutes, 94˚C for 45 sec, annealing temperature of 57˚C for 45 sec, 34 cycles of 72˚C for 1 minute and the last extension step at 72˚C for 10 minutes and then at 4˚C for infinity. Composition of PCR reaction mixture is presented in the table 2.2.

Table 2.2: Composition of PCR reaction.

Reagents	**Volume/reaction**
PCR master mix	10µl
Forward primers	0.5µl
Reverse primers	0.5µl
DNA	2µl
PCR water	7µl

PCR gel electrophoresis

1.5% gel for PCR amplified product, was used. Gel was made by mixing the 1.5gm of agarose in 100ml of 1XTBE buffer and then heating the mixture. After heating, 5µl of ethidium bromide was added and the mixture was poured into the gel plate having 16 teeth combs for solidifying the gel. After the gel was solidified at the room temperature, combs were removed carefully and gel was placed into the gel tank also filled with 1X TBE buffer. Gel was loaded with 5µl of 100bp ladder in the first well and 5µl of amplified product in both rows. Gel was run at 120V and 500A for 50 minutes. After the gel was run, gel was pictured by UV trans-illuminator (Bio Rad, UK).

Single Stranded Confirmation Polymorphism (SSCP)

SSCP is a cheap and highly sensitive method for detection of genetic variations.

Polyacrylamide gel Preparation

8% of non-denaturing gel for SSCP was made by the use of acrylamide and bisacrylamide. Glass plates were arranged with 0.4-mm spacers and held together. Chemicals for preparing the gel are as follows.

- 13.5 ml = 30 % acrylamide Solution
- 5 ml = 10X Tris base EDTA
- 350µl = 10% Ammonium persulphate (APS)
- 20µl = TEMED
- Water = total volume made to 50 ml

Gel solution was dispensed between the glass plates and shark tooth combs were implanted for making wells. The gel was then permitted to solidify.

Preparation of samples and denaturation

After confirmation of amplification by PCR, 5µl amplified product was taken into the new PCR tube and was mixed with 5µl of denaturant (formamide/ 10mM NaOH). Then SSCP samples were first heated to 95ºC for 8 min for denaturation of DNA and single stranded configuration was created. SSCP samples were transferred to the ice for sustaining of single stranded confirmation. 5µl gel loading dye was added to each tube at the end.

Vertical gel electrophoresis

After solidification of gel, plates were arranged into vertical gel apparatus (GibcoBRL, Life Biotechnologies, UK). after the removal of shark tooth comb, samples with loading dye added were loaded into wells. Gel was run at 120volts for 1hr and 30 min in pre-cooled 1XTBE.

Gel staining and visualization

Staining of gel was done in ethidium bromide tank containing 200ml of running buffer and 1: 10,000 dilution ethidium bromide for 4-10 min. After staining was done, gel was visualized by using UV transilluminator (Bio Rad, UK).

Determination of serum testosterone (T)

The concentrations of testosterone were measured by using EIA kits. The procedures and principles of the of the assay are given below

Enzyme Immunoassay (EIA)

EIA tests kit (Biocheck, Inc, USA) for testosterone was used for testosterone concentration determination. The assay was performed in accordance with protocol provided in kit.

Assay Procedure

1- For determining testosterone concentration, desired number of coated wells were secured in the holder.
2- 10µl of standards, specimens, and controls were dispensed into the appropriate wells.
3- 100µl of testosterone-HRP conjugate reagent was dispensed into the each well.
4- To each well, 50µl of rabbit anti-Testosterone reagent was then dispensed and was thoroughly mixed for 30 seconds.
5- The microtiter well plate was then incubated at 37˚C for 90 minutes.
6- The incubation mixture was then removed by flicking plate contents into a waste container. The microtiter wells were then washed 5 times with deionized or distilled water.
7- Into the each well, 100 µL TMB reagent was then dispensed and was mixed gently for 5 seconds.
8- Plate was then incubated for 20 minutes at room temperature.
9- Reaction was then paused by dispensing 100 µL of Stop Solution into the each well.
10- The micro well contents were then mixed for 30 seconds. It was made sure that all the blue colour was turned to yellow colour fully.

11- The absorbance was read at 450 nm with a microtiter well reader within 15 minutes.
12- Results were then described in ng/ml.

Statistical analysis

The data was tabulated in the form of mean, standard deviation and standard error. Analysis of data was performed with one-way ANOVA, carried by a computer software Graph Pad Prism (version 5).

Results

Current study consisted of a total of 125 male subjects. Subjects were organized into five groups with control group (n=30), group 1 or G1 (n=36), group 2 or G2 (n=32), group 3 or G3 (n=17) and group 4 or G4 (n=10) with the average age (years) of 26.7±1.16, 25.5±1.47, 28.1±1.15, 30.2±0.75, 36±0.93 respectively (Table 3.1, Fig 3.1). Controls were unexposed to VC, G1 males were having an exposure between 1 to 5 years with an average exposure of 2.2 years, G2 having an exposure between 6 to 10 years with an average exposure of 8.5 years, G3 having an exposure between 11 to 15 years with an average exposure of 14.2 years and G4 having an exposure between 16 to 20 years with an average exposure of 18.5 years. The mean time of exposure to VC was 12 hours per day in all exposed groups. None of them were having any infectious disease e.g hepatitis and no one was cigarette smoker among them. All other risk factors for tumor were excluded through performa filling.

HORMONAL ANALYSIS

Testosterone Levels

For assessment of testosterone concentration, ELISA was performed for 21 control samples, 16 samples of G1, 14 samples from G2, 10 samples from G3 and 8 samples from G4. Mean plasma testosterone (ng/ml) levels were evaluated to be significantly reduced ($p < 0.001$) in G1 and G2 as compared to control. Testosterone levels were lower in G3 and G4 but the change was not found to be significant. Results of ELISA are summarized in the table 3.2 (Fig 3.2).

Table 3.1: Mean ± SEM of age of control group, G1, G2, G3, G4 and average exposure to VC.

Groups	Number	Age (years)	Exposure (years)	Mean exposure (years)
Control	30	26.7±1.16	No exposure	No exposure
G1	36	25.5±1.47	1-5	2.2
G2	32	28.1±1.15	6-10	8.5
G3	17	30.2±0.75	11-15	14.2
G4	10	36±0.93	16-20	18.5

Values expressed as Mean ± SEM.

Table 3.2: Mean ± SEM Plasma Testosterone concentrations in control group, group 1 (G1), group 2 (G2), group 3 (G3) and group 4 (G4)

Reproductive hormone	Control (n=21)	G1 (n=16)	G2 (n=14)	G3 (n=10)	G4 (n=08)
Testosterone (ng/mL)	5.59 ± 0.39	4.10 ±0.12***	4.00 ± 0.12***	4.84 ± 0.32	5.30 ±0.33

Values expressed as Mean ± SEM;

*, **, *** indicate significant difference at probability value $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to the control (ANOVA followed by Dunnet's multiple comparison test).

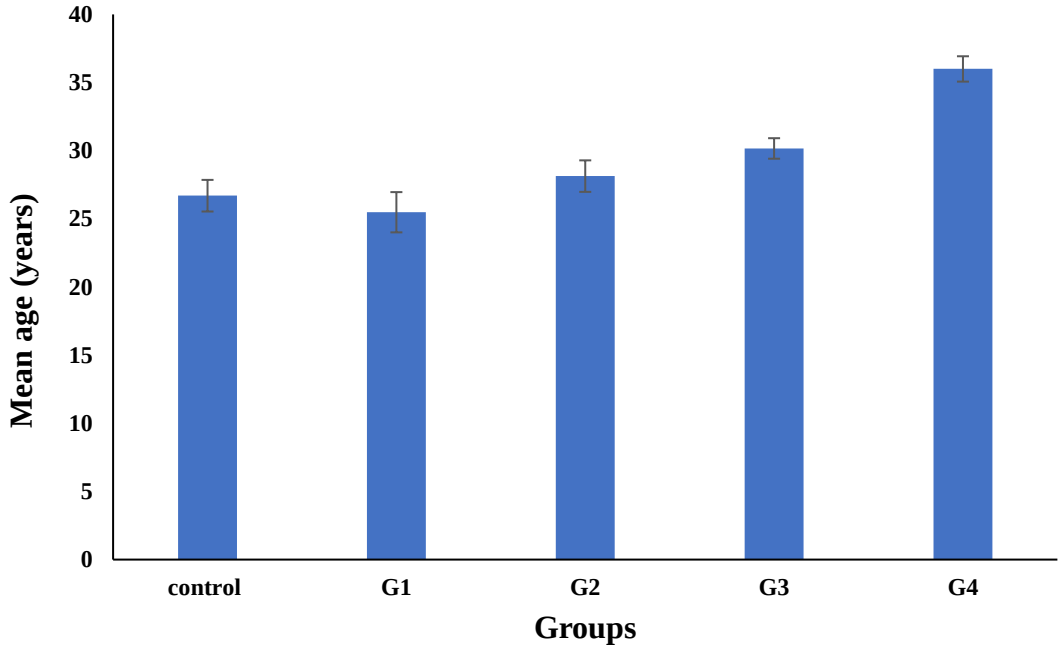

Fig 3.1: Mean± SEM Age (years) of control, G1, G2, G3 and G4.

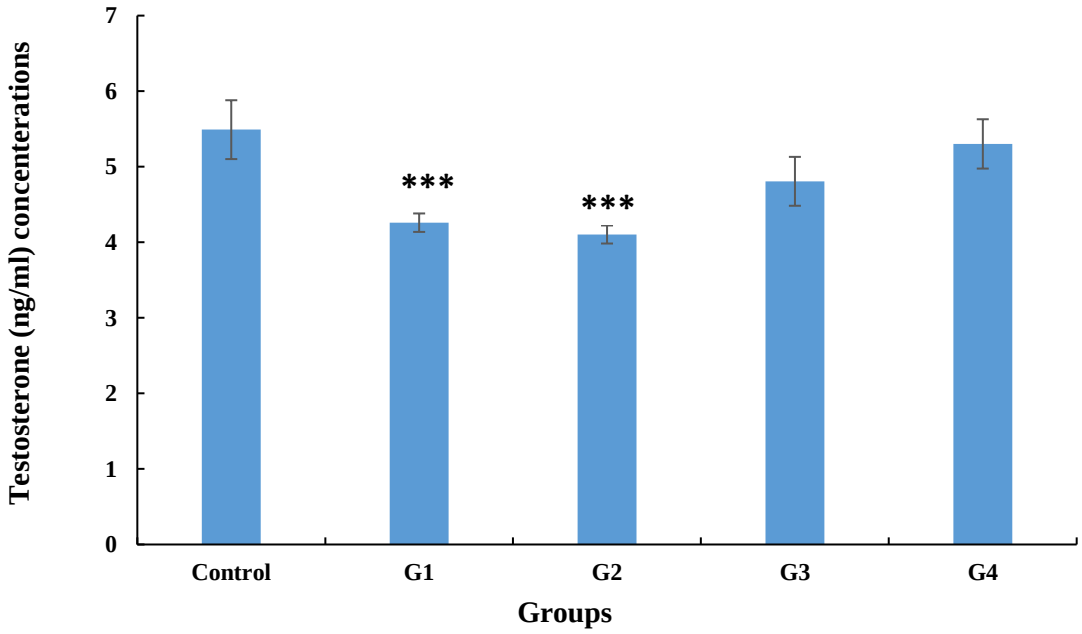

Fig 3.2: Mean ±SEM Testosterone concentration in control, G1, G2, G3 and G4.

MOLECULAR ANALYSIS

DNA extraction and amplification

DNA extraction was performed according to protocol already mentioned. 1% gel with EtBr staining was used for DNA visualization. Electro photomicrographs of extracted DNA samples of controls and exposed are given in figure 3.3 and fig 3.4 respectively. DNA samples were then employed for amplification of exon 1 and exon 2 of KRAS gene. 100 base pairs DNA ladder was used to evade chance of non-specific amplification, to verify exact size of the amplified products. Amplified products of exon 1 and exon 2 of KRAS gene were visualized on 1.5% agarose gel electrophoresis stained with EtBr. Electro photomicrographs of amplified products of exon 1 and exon 2 are given in fig 3.5 and fig 3.6 respectively.

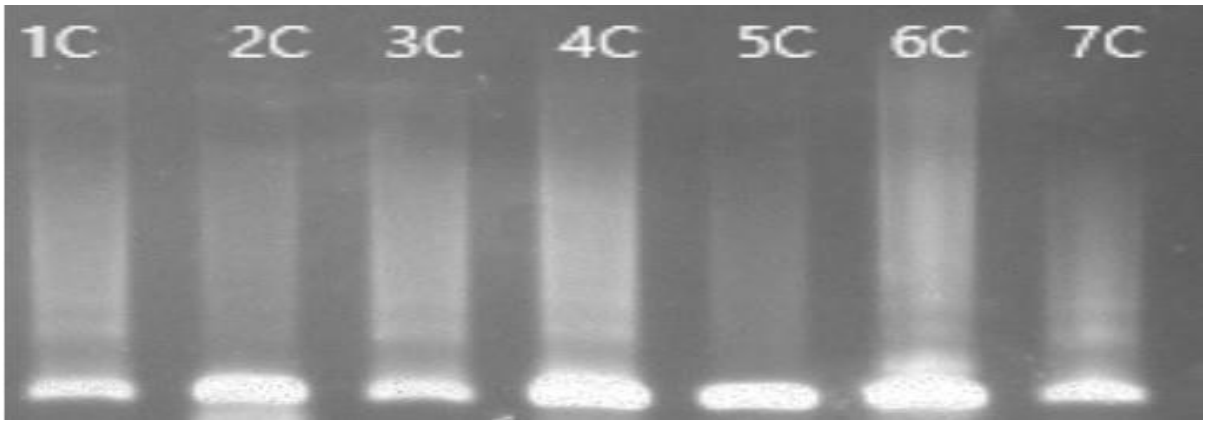

Fig 3.3: Electro photomicrographs of extracted DNA samples, where C stands for control subjects.

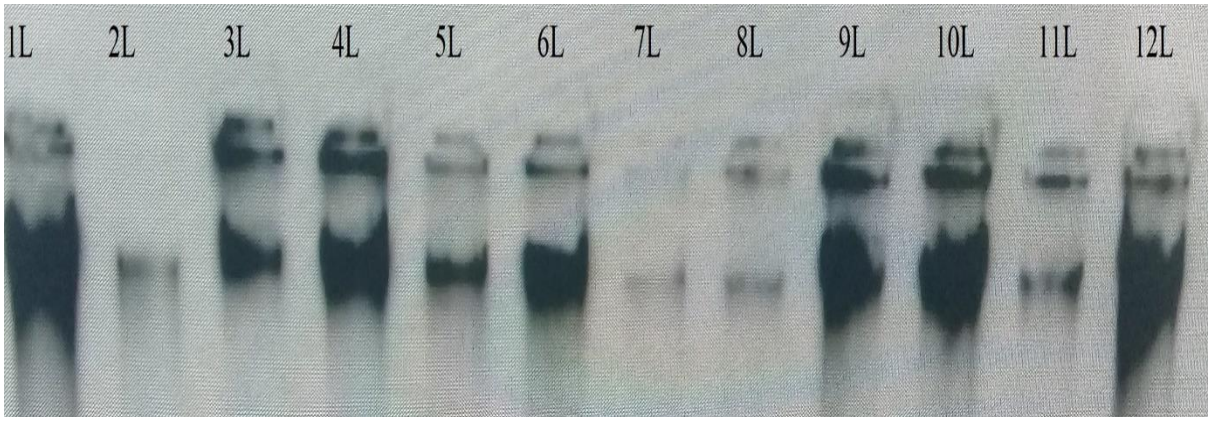

Fig 3.4: Electro photomicrographs of extracted DNA samples of VC exposed subjects, where L stands for VC exposed.

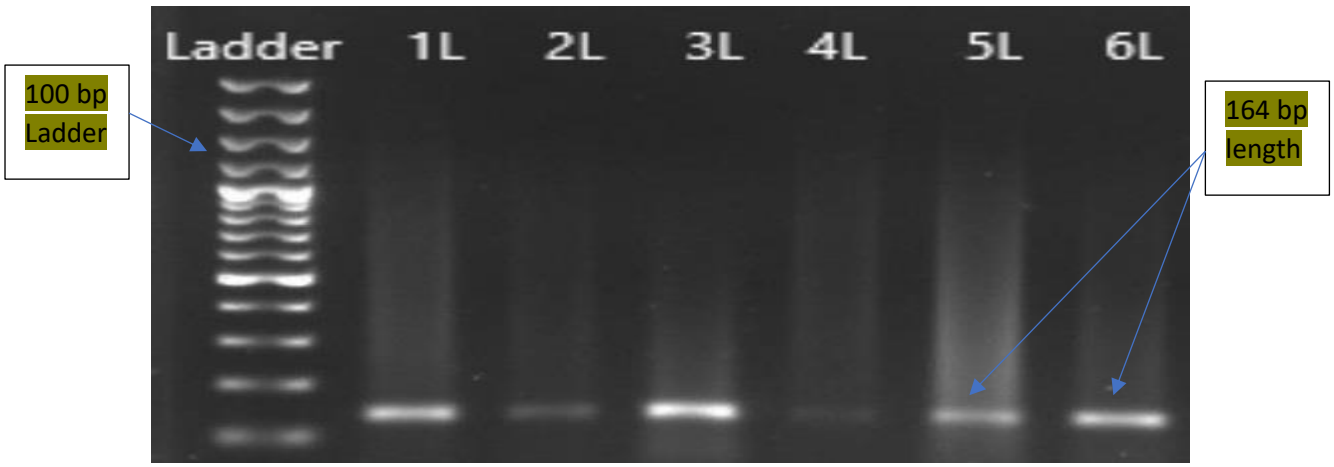

Fig 3.5: Electro photomicrographs of amplified DNA showing the amplified products of exon 1 of KRAS gene in accordance with 100 base pair DNA ladder.

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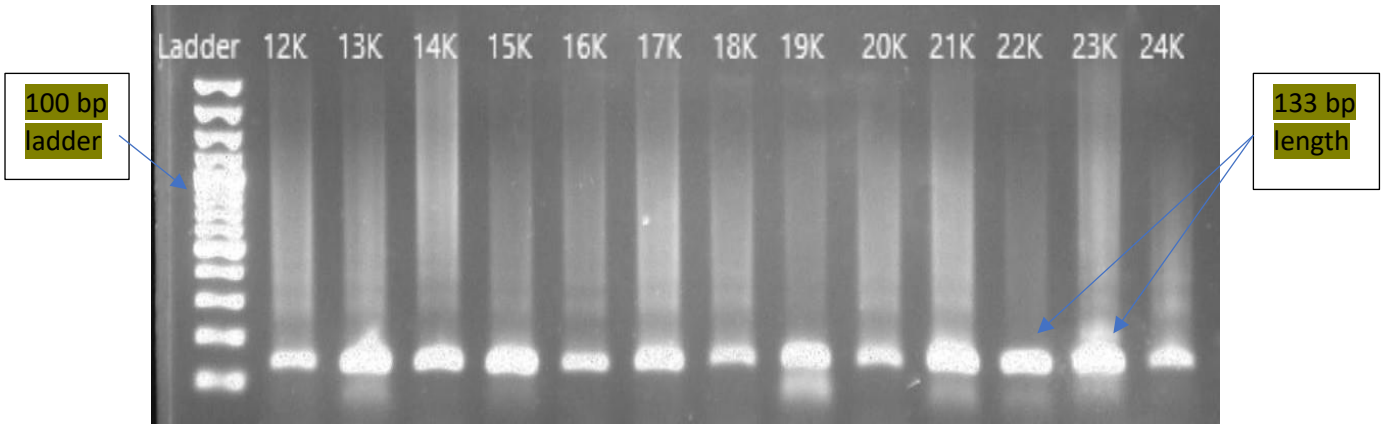

Fig 3.6: Electro photomicrographs of amplified DNA showing the amplified products of exon 2 of KRAS gene in accordance with 100 base pair DNA ladder

Mutation detection of exons of KRAS gene

SSCP was used for detection of mutations in exon 1 and exon 2 of KRAS gene as already discussed in chapter 2. SSCP is a cheap and sensitive method for determination of genetic mutations and more precise procedure for distinguishing heterozygous and homozygous mutations. Mutation identification via SSCP depends on conformational changes and mobility alteration of single stranded molecules because of mutations.

SSCP

125 samples were examined for mutation detection. 30 comprised control group and 95 comprised exposed group. All samples were separately analysed for exon 1 and exon 2 of KRAS gene by SSCP. SSCP samples exhibiting an electrophoretic mobility shifts were recognized as positive for KRAS mutations. 18 samples of VC exposed out of 95 of exon 1 (Fig 3.7 A, B) and 14 samples out of 95 of exon 2 (Table 3.3, Fig 3.8 A, B) were examined to be showing mobility shifts. No mobility shift was observed for remaining samples. Control samples were used as standard to compare the mobility shift with exposed (Fig 3.7, 3.8).

Table 3.3 Number of mutated samples of KRAS.

Number of exon	Number of mutated samples
Exon 1	18/95
Exon 2	14/95

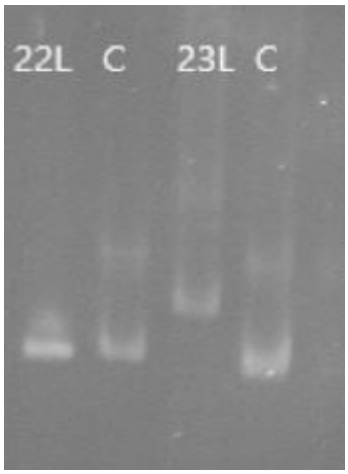

Fig 3.7(A)

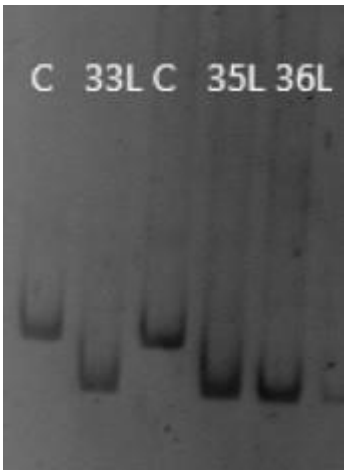

Fig 3.7(B)

Fig 3.7: Electropherograms of mobility shifts of exon 1 of KRAS gene, where C stands for control samples and L stands for exposed samples.

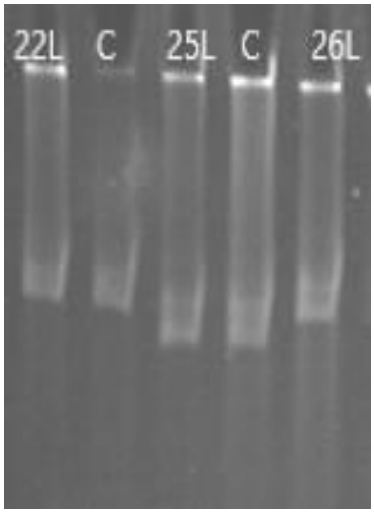

Fig 3.8 (A)

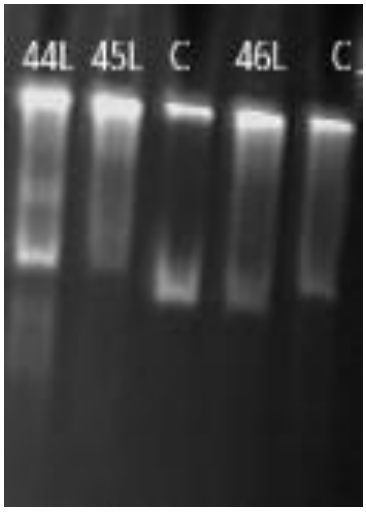

Fig 3.8 (B)

Fig 3.8: Electropherograms of mobility shifts of exon 2 of KRAS gene, where C stands for control samples and L stands for exposed samples.

Association between KRAS mutations and testosterone levels

Results of ELISA and SSCP were analysed to find association between KRAS mutations and testosterone levels. According to current study, 7 samples of G1 (exposure between 1-5 years) were mutated, 5 samples of G2 (exposure between 6-10 years), 9 samples were mutated in G3 (exposure between 11-15 years) and 11 samples of G4 (exposure between 16-20 years) were mutated. As mentioned above, testosterone levels were significantly reduced in G1 and G2 and non-significant decrease was observed in G3 and G4. KRAS mutations were present in all groups regardless of the testosterone levels. If testosterone levels were significantly reduced in all the groups, then we could relate KRAS mutations with testosterone levels. But as testosterone levels were almost normal in G3 and G4 and greater number of KRAS mutations occurred in these groups so we could speculate that VC exposure might cause KRAS mutations irrespective of the testosterone levels in adult male workers. Hence, no association was found between KRAS mutations and testosterone levels in human adult males (Table 3.4).

Table 3.4: Number of mutated samples in G1, G2, G3 and G4.

Groups	Number of mutated samples
G1	7
G2	5
G3	9
G4	11

Discussion

Of the hundreds of chemicals present in the environment, those supposed of exerting hazardous effects on the health of living organisms by disturbing the endocrine systems, pronounced as endocrine-disrupting chemicals (EDCs), have attained much attention during previous two decades. These EDCs interfere with normal working of HPG axis and result in irregular levels of sex hormones or reproductive hormones (Wen *et al*., 2017). This extremely heterogenous group of EDCs comprises of plasticizers and plastics, pharmaceutical products, herbicides, pesticides and numerous organic pollutants.

Testosterone is among different hormones effected by EDCs which is key reproductive hormone in males involved with development of reproductive organs, stimulating growth of secondary sex characters and maintaining spermatogenic process. As normal testosterone levels are necessary for above mentioned functions of male reproduction. Any decline in testosterone levels will negatively affect male reproduction.

VC is a monomer of PVC and is ranked as third most widely used synthetic plastic polymer all over the world (Allsopp & Vianello, 2000). Different plastic items like water tanks, plastic pipes, shopping bags etc are made of PVC of which VC is the major part. Reproductive hazards of various plasticizers through distressing HPG axis resulting in abnormal testosterone levels have been established through a number of studies (Chen *et al*., 2017; Seachrist *et al.*, 2017; Cristine *et al*., 2018; Levels *et al*., 2016). VC is also a well- known human carcinogen and is metabolized by the liver (El Ghissassi *et al*., 1998). KRAS, our gene of interest, is involved in cell differentiation, cell proliferation, survival, growth, apoptosis and cell migration. Several studies have established a fundamental relationship between VC exposure and KRAS mutations (Weihrauch *et al*., 2001; Nan *et al*., 2015; Palmirotta *et al.*, 2011) which make it one of the most commonly muatated gene in all cancers and accounts for 27% of all cancers (Eser *et al.,* 2014).

Previous literature demonstrarted that plasticizers are EDCs and so irregulate HPG axis by disturbing the normal testosterone levels. The direct interaction of these EDCs chemicals with nuclear receptors (NRs), is a renowned mechanism of endocrine disruption. NRs are members of the steroid receptor superfamily, a large family of ligand-dependent transcriptional factors (Germain *et al.,* 2006). EDCs pose deleterious effects on sexual development and reproduction caused by interference with steroid signalling via human estrogen (hER) and androgen (hAR) receptors (Satoh *et al.,* 2004). Phthalate esters are plasticizers and repoted to be EDCs (Sheikh

& Beg, 2017). Borch *et al* (2006) showed that di-(2-ethylhexyl) phthalate (DEHP) exposure is associated with reduced testosterone concentration in animals (Borch *et al*., 2006). In another human study performed by Ferguson *et al* (2014), DHEP exposure was associated with reduced testosterone levels in children (Ferguson *et al.*, 2014). Chen *et al.* (2017) evaluated the effect of mono-2-ethylhexyl phthalate (MEHP) on the testosterone levels in Taiwanian population. In that study, testosterone levels were signifacantly reduced in both adolescents and adults male and female due to MHEP exposure (Chen *et al*., 2017). Bisphenol A (BPA) is widely used in many plastic products is also an EDC. BPA-containing product can errode the chemical into food, water, and ecosystems. BPA binds with estrogen receptor or androgen receptor and has both estrogenic and anti-androgenic effects (Lee *et al*., 2003). Tohei *et al.* (2001) demonstrated that BPA exposure decreases testosterone levels in the serum or testis (Tohei *et al*., 2001). Levels *et al*. (2016) showed that levels of testosterone were lower in male adolescents and were high in female adolescents due to BPA exposure (Levels *et al*., 2016). A nonsignificant association between BPA concentrations and lower tesosterone levels among 8–14 year old boys (n=113) was also reported by Ferguson *et al.* (2014) (Ferguson *et al*., 2014). Scinicariello and Buser (2016) found significant relationship between urinary BPA concentrations and lower serum testosterone levels among male adolescents around 15 years of age (n = 161) (Scinicariello & Buser, 2016).

Our results also demonstrated a decrease in tesosterone levels of adult males due to VC exposure. In males having eposure of 1-10 years, highly significant decline was observed. Decrease was found also in males having exposure between 11-15 years but decrease was not significant. A very little change was observed in males who have an exposure between 16-20 years. These random findings may partially be due to variations in the characteristics and sizes of the study populations mentioned above. For example, the participants in the present study were all adult males and population size of present study was also small. Moreover, VC exposure levels are also different among present study and above-mentioned studies.

Highly significant decrease in VC workers having an exposure between 1-10 years suggests that VC may obstruct testicular functions in adult males via changes in the expression of steroidogenic enzymes, which then disturb steroid synthesis (T production) and circulating steroid levels as was observed in the case of BPA exposure in pubertal and adult rodents (Akingbemi *et al*., 2004; Nanjappa *et al*., 2012). Also VC might have antagonistic activity at the androgen receptor (AR) like BPA to modify the expression of steroidogenic enzymes, and to interfere with thyroid, PPAR-γ, and glucocorticoid signaling pathways (Mustieles *et*

al.,2015). With the passage of time, AR receptors might have lost sensitivity to VC and receptor desensitization might have occurred due to which non-significant decrease was detected in males having long term exposure to VC.

Similarly, numerous past studies also demonstrated KRAS mutations due to VC exposure in different tumors. Weihrauch *et al*. (2001) performed study on 16 hepatocellular carcinomas (HCCs) due to VC exposure and spotted KRAS mutations in 8 patients and all of the mutations were in exon 1 (Weihrauch *et al*., 2001). Arbeitsmedizin and Hannover (2001) detected mutations in codon 12 and 13 of exon 1 of KRAS gene in 5 out of 12 (42%) HCC patients (Arbeitsmedizin and Hannover ,2001). Marion *et al.* (1991) found mutations of KRAS gene in human Angiosarcoma of the Liver ASL associated with VC exposure. G>A changes were detected at codon 13 of exon 1 in 5/6 tumor samples examined (Marion *et al.*,1991). Guido *et al*. (2016) described an exceptional case of consecutive incidences of hepatocellular carcinomas and angiosarcoma of the liver, in a vinyl chloride exposed worker without cirrhosis and any known risk factor for chronic liver disease. KRAS mutation of codon 12 of exon 1 was reported in this study (Guido et al., 2016).

About 90% of the activating KRAS mutations are found in exon 1 and nearly 5% in exon 2 (Palmirotta *et al*., 2011). Exon 1 mutations block the binding site for GAP (GTPase activating protein) and so bound GTP can not be hydrolysed and hence KRAS persists in the activated form and hence becomes mutant (Eser *et al.,* 2014). Exon 2 mutations block the inherent GTPase activity of KRAS and so impair GTP hydrolysis and cause KRAS in always activated form even in the absence of external factors (Jonckheere *et al*., 2017).

In the current study, mutations in exon 1 and 2 of KRAS were found in VC workers who were non-tumorous as were found in previous studies. As, all the risk factors for developing KRAS mutations were ruled out through performa filling, hence it was confirmed that VC exposure was responsible for KRAS mutations. As VC is metabolized in liver hence reactive intermediates of VC metabolism, chloroethylene oxide (CEO) and chloroacetaldehyde (CAA), might have mutated both exons of KRAS as described by (Schindler *et al.,* 2007). Mutations in exons 1 and 2 might have blocked binding sites for GAPs as was demostrated by Eser *et al.* (2014) or might have inhibited intrinsic GTPase capacity of KRAS as Jonckheere *et al*. (2017) elobrated. These might have caused KRAS in a permanently activated form but exact mechanism of KRAS mutations remains unrevealed in the present study.

All previous literature reported the affect of plasticizers on testosterone levels as all plasticizers are EDCs but data regarding association of KRAS mutation with the release of testosterone are

still scant no eveidance is yet to be present. This association was confirmed by present study results which demonstrated a significant decrease in testosterone concenterations in adult male factory workers having an exposure of 1-10 years and a non-significant decline was obsereved in males having prolonged exposure of 11-20 years to VC. Current study also proved mutagenic affect of VC in adult males and demostrated exon 1 and exon 2 KRAS gene mutations. To conclude, present results suggested that there is no association between testosterone levels and KRAS mutations in VC exposed males workers.

Conclusion

Results of the current study demonstrated that vinyl chloride (VC) reduce testosterone levels in adult male workers but very long exposure to VC has non-significant affect on testosterone levels. Moreover, VC also cause KRAS mutations in non-tumorous male workers. However, no association was found between KRAS mutations and testosterone levels in adult male workers. As, data about affect of VC exposure on testosterone levels in adult males is infrequent, further studies are required to clarify this task. Also, more detailed studies are required to confirm the role of VC exposure in causing KRAS mutations in non-tumorous males.

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