# POTENTIAL OF KISS-1 PEPTIDE IN MITIGATING SODIUM ARSENITE-INDUCED TESTICULAR TOXICITY IN ADULT MICE AND ITS COMPARISON WITH N-ACETYL CYSTEINE


**A Thesis submitted in Partial Fulfilment of The Requirements for The Degree of Doctor of Philosophy**

**BY**

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*Dedicated*

*To*

*My beloved parents*

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*Iffat fatima*

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| CAT | Catalase |
| DMEM | Dulbecco's modified eagle's medium |
| ELISA | Enzyme linked immunosorbent assay |
| FSH | Follicle stimulating hormone |
| GSH | Reduced glutathione |
| H & E | Hemoatoxylin and eosin |
| KP-10 | Kisspeptin-10 |
| LH | Luteinizing hormone |
| $NaAsO_2$ | Sodium arsenite |
| PBS | Phosphate buffer saline |
| POD | Peroxidase |
| ROS | Reactive oxygen species |
| SOD | Superoxide dismutase |
| TBARs | Thiobarbituric acid reactive substances |
| WHO | World Health Organization |

# Abstract

Contamination of soil and water reservoirs with arsenic has severely affected human health. Arsenic which is a naturally found metalloid has been enlisted among such contaminants that impose toxic effects on animal and human health upon exposure to above the safe levels (<10 ppb) recommended by World Health Organization. Besides other pathophysiological changes, trivalent form of arsenic is specifically found to cause andrological dysfunctions in several animal species. Arsenic-induced reproductive toxicity has been previously evaluated through spermatological, biochemical, hormonal, histological and molecular parameters. Redox imbalance leading to oxidative damage resulting in apoptosis is now considered the main underlying mechanism mediating the deleterious effects of arsenic.

Considering the rapid increase in the prevalence of andrological disorders and exposure to various pollutants like arsenic, potential therapeutics have also been investigated. These include several naturally found compounds like vitamins and other plant based chemicals etc. N-acteyl cysteine (NAC) is one such antioxidant that has well-established role as an antidote against several oxidizing agents including the trivalent form of arsenic as sodium arsenite ($NaAsO_2$).

Recent clinical research on finding the endogenous regulators of reproductive functions which could be used for treatment of several fertility disorders with minimum un-desired effects have highlighted the kisspeptin which is a neuropeptide initially discovered as “metastatin”. Considering its therapeutic potential in regulating hormonal dysfunctions and recently reported anti-oxidative properties against oxidizing stressors, present study was designed to comprehensively evaluate and compare the mitigating role of Kiss-1 peptide (KP-10) along with NAC (well-known antioxidant) against $NaAsO_2$-induced testicular toxicity at seminal, biochemical, hormonal, histological and genetic level through a series of *in vivo* and *in vitro* experiments on adult albino mice.

*In vivo* study comprised of following experiments. An initial pilot study on adult male mice evaluated the efficacy of KP-10 (50 nmol) intraperitoneal dosing regimens (continuous and intermittent) upon exposure to low (4 ppm) and high (10 ppm) doses of $NaAsO_2$ in drinking water for the whole length of mice spermatogenic cycle (35

days). Later, taking into account the findings of first experiment, similar experiment was conducted to compare the efficacies of both KP-10 (50 nmol) and NAC (for 5 alternative days) against arsenic toxicity in adult mice. However, only once a week intermittent dosing of KP-10 was selected for this experiment to avoid the extraneous stress caused by overhanding.

Outcomes of these *in vivo* experimentations revealed that KP-10 has the efficacy comparable to NAC in mitigating the toxic effects of $NaAsO_2$ on male reproductive system. Additionally, when provided in combination both therapeutics produce synergistic effect which is depicted by all investigated parameters. Such as, spermatological parameters including the sperm count, motility and viability were significantly improved upon supplementation both KP-10 and NAC along with $NaAsO_2$ exposure at both 4 and 10 ppm doses, when compared to $NaAsO_2$ alone treatment group. Whereas, testicular oxidative stress level was also decreased as depicted by significantly decreased ROS and TBARS levels, while elevating the activities of antioxidant enzymes including SOD, POD, CAT and non enzymetic reduced glutathione GSH. Moreover, testicular lactate dehydrogenase LDH levels were also found to be significantly decreased, when compared to $NaAsO_2$ alone treatment group thus indicating prevention of tissue damage. Circulating testosterone levels were also increased to near control level (tap water provided) in supplemented groups as compared to $NaAsO_2$ alone treatment groups, thus indicating the protection against arsenic-induced endocrine disruption. Histological evaluation also supported the protective effects of the respective therapeutics as germinal layers in seminiferous tubules showed normal arrangement and replenished sperm cells which were prominently lost upon $NaAsO_2$ alone exposure at both 4 ppm and 10 ppm doses. Therefore, current substantial *in vivo* study provides evidence of spermatological, biochemical, hormonal and histoprotective effects of KP-10 against $NaAsO_2$-induced testicular damage for the first time. It also suggests the potential of Kiss-1 peptide to be used in combination with other known therapeutics such as NAC for treatment of andrological disorders.

To further verify these *in vivo* findings and evaluate the direct effect of KP-10 treatment, organ culture technique was employed in the current study. It involved 2 and

24 h incubation of testicular fragments with culture media containing respective doses of KP-10 (1 μM), $NaAsO_2$ (100 μM) and NAC (1 μM) according to study design. Whereas, treatment free media served as control for these *in vitro* experiments. Evaluation of biochemical, hormonal and histological parameters also revealed the protective effects of KP-10 and NAC supplementation when compared to $NaAsO_2$ alone treated cultures following both 2 and 24 h incubation. It was found that both therapeutics equally prevented the testicular damage by significantly decreasing the ROS, TBARS and LDH levels while increasing the activities of antioxidant enzymes including SOD, POD, CAT and non enzymetic GSH. Additionally, histoprotective effects of these therapeutics were demonstrated by significant recovery in germ cells within seminiferous tubules which were depleted in $NaAsO_2$ alone incubated cultures. Intra-testicular testosterone levels also demonstrated the significant increase upon KP-10 and NAC supplementation in a time dependent manner which was reduced upon $NaAsO_2$ alone incubation for both 2 and 24 h. These observations also suggest the direct effect of KP-10 on testicular steroidogenesis which is compromised due to arsenic toxicity. It is concluded therefore that KP-10 protects against oxidizing and endocrine disrupting effect of $NaAsO_2$ by involving indirect and direct mediatory pathways.

Considering the outcomes of *in vivo* and *in vitro* evaluation of biochemical, hormonal and histological parameters which suggest the anti-apoptotic and steroidogenic effect of KP-10, expression levels of mRNAs for genes involved in apoptosis and steroidogenesis were further evaluated by real time PCR. The target genes included Caspase 3, StAR and Cyp11a1. The results demonstrated the significantly increased expression of Caspase 3 upon $NaAsO_2$ alone exposure in tissue samples from both *in vivo* and *in vitro* experiments in a dose and time dependent manner respectively, when compared to respective controls. Whereas, both KP-10 and NAC supplementation significantly decreased the mRNA expression of Caspase 3 as compared to $NaAsO_2$ alone treatment. Moreover, expression of mRNAs for both StAR and Cyp11a1 were also found to be significantly elevated upon supplementation with KP-10 and NAC when compared to $NaAsO_2$ alone treated groups thus indicating the steroidogenic effect of these therapeutics. Additionally, synergistic effect of both KP-10 and NAC was observed when given in combination in both experimental regimens. Therefore, these

findings suggest the protective effect of both KP-10 and NAC against $NaAsO_2$-induced testicular damage at genetic level. Current study is novel in the aspect that for the first time it evaluates Kiss-1 peptide both alone and in combination with a well-known therapeutic agent NAC through spermatological, biochemical, hormonal, histological and genetic parameters in both *in vivo* and *in vitro* conditions. The outcomes from both regimens conclude that as a potent endocrine regulator KP-10 effectively mitigates the endocrine disruption caused by arsenic. Moreover, it is also suggested that it can counteract the oxidizing effect of trivalent arsenite by promoting testicular oxidant-antioxidant balance thus preventing cellular apoptosis which is depicted at both biochemical and genetic level. Additionally, it promotes steroidogenesis by involving either indirect or direct intra- testicular signalling pathways. Furthermore, synergistic effect of both KP-10 and NAC strongly suggest that combination of these therapeutics can be used as a potential infertility treatment.

# CHAPTER # 1
# GENERAL INTRODUCTION

# GENERAL INTRODUCTION

## 1.1 Male factor infertility

Reproduction is an essential physiological function for the survival of any living species. Besides, continued supply of dairy products and meat from production animals also owe to reproductive system (Foster, 2017). As regards human populations, most of the reproductive disorders do not have underlying life threatening reasons as compared to other diseases like cancer and diabetes etc. It is even common among healthy individuals to delay the child-bearing until late 30s. Consequently, field of reproductive physiology is somehow undermined as pharmaceutical industries also avoid taking risk of exposing unborn child to extrinsic chemicals. That is why most of the recent fertility drugs have been still formulated from previously discovered substances and there is deficit of novel therapeutic agents (Ivell and Anand-Ivell, 2021).

Inability to produce an offspring after practicing regular unprotected intercourse for one year is defined as infertility. According to the latest WHO's statistical evaluation, infertility affects approximately 50-80 million human population worldwide. About half of the reported cases are contributed by female factors and 20-30 percent caused by male factors. However, worldwide prevalence of this issue is not rightfully reflected due to certain limiting factors like inappropriate statistical methods and cultural constraints etc. Underlying mechanisms and causes of male factor infertility have still not been well understood which makes it a challenging research problem (Babakhanzadeh et al., 2020).

### 1.1.1 Male gonads

Reproductive system is comprised of primary and secondary sex organs. Gonads are the primary organs which are involved in synthesis of gametes and hormones while accessory glands and ducts are considered as secondary organs playing their role in completing the stages of gamete production (Ampatzidis et al., 2021). Testes are the male gonads which are placed in scrotum and enclosed by a capsule of tunica albuginea. Each testis is morphologically and functionally separated into two parts including tubular and intertubular portions. Former is comprised of seminiferous tubules and intercellular spaces while latter is responsible for blood supply and eliciting the immune responses (Li et al., 2016). Seminiferous tubules are functional units of testes and comprise 60 to 80% volume of a testis (Bhushan et al., 2016). Testicular testosterone is

produced by the Leydig cells which synthesize testosterone, an androgen under the action of LH. In addition, intercellular components are comprised of immune cells, nerve cells, blood and lymphatic vessels and fibroblasts etc. (Ye at al., 2017). Seminiferous tubules are surrounded by epithelial tissue which is composed of Sertoli cells and spermatogenic cells. Sertoli cells are responsible for nourishment of sperm through different stages of spermatogenesis. Additionally, Sertoli cells are also involved in regulation of luminal release of sperm and removal of additional cytoplasm and degraded germ cells (Loveland et al., 2015).

### 1.1.2 Spermatogenesis

This is the most crucial stage of gamete formation within seminiferous tubules, slightest deviation of which can lead to male infertility. Male gametes are developed in the epithelial tissue of seminiferous tubules from diploid spermatogonia and released into the tubular lumen as haploid germ cells (Xu et al., 2016). Spermatogenic cycles are regulated at both endocrine and paracrine stages. Testosterone and follicle stimulating hormone (FSH) are essential for successful completion of spermatogenesis (Dimitriadis et al., 2015). There are four general phases of spermatogenesis which include a) mitotic division (spermatogoniogenesis) b) meiotic division of spermatocytes into spermatids c) formation of elongated sperm from round spermatids (spermiogenesis) d) sperm release into tubular lumen (spermatogenesis) (Yao et al., 2015).

#### 1.1.2.1 Neurological regulation

Hypothalamus is responsible for initiation of spermatogenesis through hormonal regulation. It stimulates the anterior pituitary to release luteinizing hormone (LH) and follicle stimulating hormone (FSH) by releasing gonadotropin releasing hormone (GnRH). LH stimulates the Leydig cells to produce testosterone while Sertoli cells are triggered by FSH and assist the developmental stages of spermatogenesis (Sharma and Agarwal, 2011).

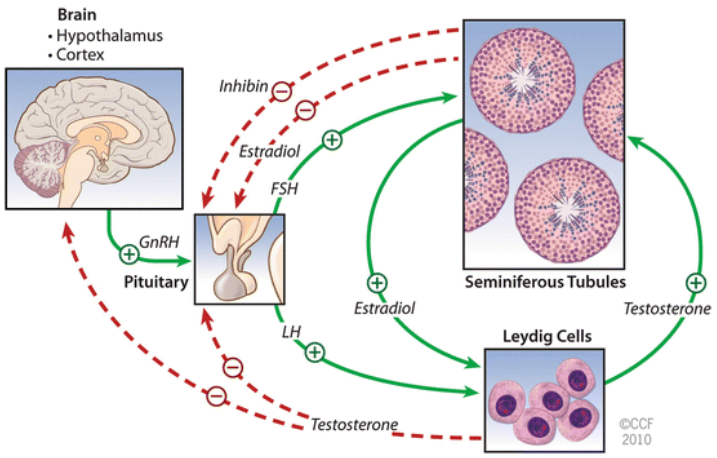


Fig. 1.1 Hypothalamus pituitary gonadal axis and feedback system (Sharma and Agarwal, 2011)

### 1.1.3 Male factor infertility

Semen analyses is the routine practice to diagnose fertility issues of masculine origin. The parameters which are observed for any abnormality include sperm count, morphology and motility (De Kretser and Baker, 1999). There are several contributing factors to male infertility. The most important of which include hormonal, physical, genetic and environmental issues (Krausz and Riera-Escamilla, 2018; Lee and Ramasamy, 2018; Lotti and Maggi, 2018).


Fig. 1.2 Main causes of male infertility (Wasef et al., 2011)

### 1.1.3.1 Endocrine disruption and infertility

Chemical substances which exert either agonistic or antagonistic endocrine effects in animals and humans are defined as endocrine disruptors. These substances either disrupt the normal biosynthesis of endogenous hormones or affect their activities (Georgescu et al., 2011). Endocrine disrupting chemicals (EDCs) found in the environment have long been known to target the reproductive system resulting in reduced reproductive performance (Gore et al., 2015).

There exist several kinds of endocrine disruptors in the environment which are synthetic chemicals that include pesticides and microplastics etc. affecting various physiological systems even with exposure to very low concentrations (La Merrill et al., 2020). Recent recognition of some pharmaceutical agents as EDCs has further complicated this issue (Ivell and Anand-Ivell, 2021). Previously, feminization of male fish within natural water systems due to exposure to a non-degradable contraceptive component, the ethinyl estradiol had been reported (Joblings et al., 2006).

Besides these synthetic chemicals, some heavy metals are also known to cause endocrine disruption in both animals and humans. Among these, metals like arsenic (As), lead (Pb) and mercury (Hg) etc. are found to specifically target reproductive systems by altering steroidogenesis. Considering their activities, these metals are categorized as endocrine-disrupting metals. There is an ongoing research on mode of action of these metals which is providing further evidence in this regard (Georgescu et al., 2011).

## 1.2 Metal-induced reproductive toxicity

### 1.2.1 Heavy metals

There is no consensus on chemical definition of heavy metals. However, due to their high densities i.e. > 5 g/cm$^3$, these have acquired the name of 'heavy metals'. These are placed in groups 3-16 within periodic table. Their occurrence in the natural environment has long been dated since birth of the planet earth (Jarup, 2003). Considering their usefulness, they have become widely dispersed during burst of industrialization and anthropogenic manipulation of the environment. Physical, chemical and radioactive properties (uranium) of these metals make them ideal for industrial use. Trace amounts of heavy metals including zinc (Zn), iron (Fe), manganese

(Mn) and copper (Cu) are required in several biological processes. However, in higher concentrations, they become extremely toxic due to their high reactivity. Among these heavy metals, five have been comprehensively investigated for their impact on reproductive performance. These include arsenic (As), cadmium (Cd), lead (Pb), mercury (Hg) and uranium (U) (Dyer, 2007).

### 1.2.2 Arsenic

As a naturally occurring metalloid, As is ubiquitously distributed in earth crust and ground water. Lower concentrations of As are also found in the atmosphere and living organisms especially crustaceans (Carline et al., 2016). It exists in three major forms i.e. organic, inorganic and arsine gas. Its three valence states are namely arsenic element (0), pentavalent arsenate ($As^{+5}$) and trivalent arsenite ($As^{+3}$). Both organic (monomethyl arsenic) and inorganic trivalent (arsenite) compounds of arsenic are known to exert more toxic effects as compared to their pentavalent counterparts. Additionally, organic compounds are considered less toxic than inorganic arsenic compounds (Olsen and Mørland, 2004).

Arsenic is released in the environment via both natural and anthropogenic activites which include volcanic eruption and mining processes respectively. In addition, smelting of different ores is also found to produce arsenic as a byproduct (Carline et al., 2016). Various synthetic compounds were previously manufactured using arsenic metalloid. These include herbicides, preservatives and defoliants used in alcohol industry etc. (Huang et al., 2012). Gallium arsenide is an essential compound which is still used in the manufacture of several electronic devices including semiconductors, diodes and photoelectric chemical cells etc. (Hong et al., 2014).

#### 1.2.2.1 Epidemiology

Current increase of arsenic in drinking waters up to toxic levels has become a cause of health concern worldwide. The contamination rates however vary according to geographical distribution. The most affected countries are India and Bangladesh with approximately 27 million people being exposed to toxic levels of arsenic i.e. 50 ppb via drinking water. Meanwhile, World Health Organization (WHO) recommends that concentration < 10 ppb is safe. Worldwide, private wells have also been contaminated with As including the United states (US) (Yunus et al., 2016). Other countries like Pakistan, Iran, Hungary, Serbia and Spain are facing similar situation (Hettick et al.,

2015). Anthropogenic production and release of As containing chemicals in the environment is another means of exposure. Occupational exposure involves production of chemicals including herbicides and pesticides and wood preservatives and the procedures like mining and smelting, manufacturing of glass and semiconductors etc. (Huang et al., 2012; Hong et al., 2014). Although As toxicity is dominantly regulated by geographical distribution however, it has also been reported that toxic effects of As are influenced by the gender of the exposed person, in some conditions males are more vulnerable to arsenic-induced toxic effects as compared to females (Ferrario et al., 2016).

#### 1.2.2.2 Arsenic Metabolism

Inorganic As is the most widely distributed form of As in the environment. Its absorption takes place through the gastrointestinal (GI) tract and is biotransformed in liver and other peripheral tissues. Its biotransformation in mammals and rodents involves alternating events of reduction and oxidation (Vahter 2002; Carter et al., 2003). During this process, inorganic pentavalent As is ultimately converted into dimethylarsinous acid and trimethylarsine oxide. The sequential forms include trivalent arsenite, monomethylarsonic acid, monomethylarsonous acid, and dimethylarsinic acid. Enzymes called reductases and non-enzymatic glutathione in addition to electron donors facilitate the reduction reactions. Whereas, methyltransferases and methyl donating S-adenosylmethionine catalyze the oxidative methylation. Arsenic methylation is more efficiently performed in rats and mice as compared to humans (Lu et al., 2003).

#### 1.2.2.3 Pathophysiology

Drinking water is the primary source of human exposure to As while dermal contact and inhalation are considered secondary routes. It is a well-known carcinogen which promotes tumor formation in lungs, liver, kidney and bladder. Its exposure is also associated with the development and progression of vascular disease. Accumulation of approximately 70-90% of inorganic As in different organs including liver, kidney, lungs, bladder, muscles and nerves have been found after absorption through the GI tract. It is excreted from the body through urinary tract (Palma-Lara et al., 2020).

Toxic effects of As are associated with its metabolism however; the underlying mechanisms are still not completely understood. Considering the valence state, two

different mechanisms have been proposed for trivalent arsenite and pentavalent arsenate. Due to similarity in structure and chemical properties, pentavalent arsenate is being considered replacement for phosphate in several metabolic reactions. It has been found through *in vitro* investigations that arsenate interferes with carbohydrate metabolism by forming glucose 6 arsenate which acts as a substitute substrate for glucose 6 phosphate dehydrogenase enzyme thus affecting glycolysis. Ultimately, both aerobic and anaerobic pathways are compromised leading to depletion of ATP. Arsenite is considered more toxic as it affects multiple proteins and enzymes by reacting with their thiol and sulfhydryl groups. Pyruvate dehydrogenase is such an enzyme which is activated in the presence of dithiol. Interference by arsenite leads to impaired cellular respiration and ATP depletion. Additionally, mechanisms mediating carcinogenic effects of As include altered DNA repair, oxidative damage leading to genotoxicity (Hughes, 2002). Toxic effects of arsine gas are mediated through its hemolytic activity. It is proposed to be directly related to increased oxidative stress and decreased activities of antioxidant enzymes (Pakulska and Czerczak, 2006).

#### 1.2.2.4 Pro-oxidant shifting oxidant-antioxidant balance

Oxidative stress is considered to be the essential part of mechanisms underlying inorganic arsenic toxicity (Gao et al., 2013). Exposure to sodium arsenite shifts the oxidant-antioxidant balance by stimulating production of reactive oxygen species (ROS) (Samuel et al., 2005). As regards body's antioxidants, most important of these are non-enzymatic glutathione (GSH) (scavenger of free radicals), and enzymes including glutathione peroxidase, catalase and superoxide dismutase (White et al., 2003). If these antioxidant enzymes fail to offset excess free radicals then lipid peroxidation sets in (Mena et al., 2009). Oxidative damage is evaluated by measurement of malondialdehyde (MDA) which is produced by peroxidation of polyunsaturated fatty acids (Zhao et al., 2017).

Arsenic-induced oxidative stress primarily originates within mitochondria which alters the ultrastructure of mitochondria leading to deterioration of membrane potential (Olsen et al., 2013). This altered membrane potential promotes production of reactive oxygen species which gradually compromises the cellular defense against oxidative stress by decreasing the GSH molecules (Forman, 2016).

### 1.2.2.5 Arsenic as an endocrine disruptor

Endocrine disruption mediated by As exposure has been found in research animals. Such as, consumption of drinking water containing arsenite at dose of 5 mg/kg of body weight for four weeks resulted in pathological changes in adult rats. These included decreased gonadotrophins which caused lowering of testicular androgen levels and a degeneration of germ cells. All these changes mimic the effects of estrogen agonists thus suggesting the estrogenic activity of As (Jana et al., 2006). Likewise, male mice intravenously injected with sodium arsenite at 0.5 mg/kg once a week for twenty weeks, showed hyperplasia of interstitial cells of testes and degeneration of seminiferous tubules. Female mice also exhibited pathological changes in uterus including cyctic hyperplasia. All these changes depicting estrogen like activity of arsenic (Waalkes et al., 2000).

Arsenic is known to disrupt the activities of glucocorticoid hormones in various biological reactions by binding to glucocorticoid receptors. Unlike organochlorides, instead of activation, it inhibits the activation of genes regulated by glucocorticoid receptors. Estrogen receptors are inhibited in a similar manner by As. Such as, it is found to suppress the transcription of 17β-estradiol-inducible vitellogenin gene in liver of chicken embryos which is normally activated by estrogen receptor dependent manner. Moreover, breast cancer MCF-7 cells in humans also demonstrated significant inhibition of estradiol receptor regulated effects even at 2-225 ppb concentration which are considered non cytotoxic (Davey et al., 2007).

Arsenite binds the estrogen receptor at zinc finger region (Kitchin and Wallacw, 2005). It alters gene expression in rats and human cells by binding to estrogen receptor at low micromolar concentrations while, at > 100 μM concentrations, it exerts its toxic effects by non-specifically damaging DNA or proteins by increasing oxidative stress level (Valko et al., 2005). It is suggested that As may exhibit estrogenic activity however, there might be some indirect effects due to the fact that it also interferes with metabolism by decreasing insulin levels which could compromise the responses of reproductive tissues (Dyer, 2007).

### 1.2.2.5 Arsenic-induced Andrological dysfunction

Exposure to trivalent form of As through drinking water (Chinoy et al., 2004) and intraperitoneal administration (Sarkar et al., 2003) are known to exert toxic effects on

male reproductive system. It disrupts the normal spermatogenic pathway by interfering with enzymes involved in gamete formation (Pant et al., 2001; Pant et al., 2004). As an endocrine disruptor, it also decreases levels of pituitary gonadotrophins and testicular testosterone (Chinoy et al., 2004). These observations suggest both indirect and direct effect of As on male gonads (Sarkar et al. 2003).

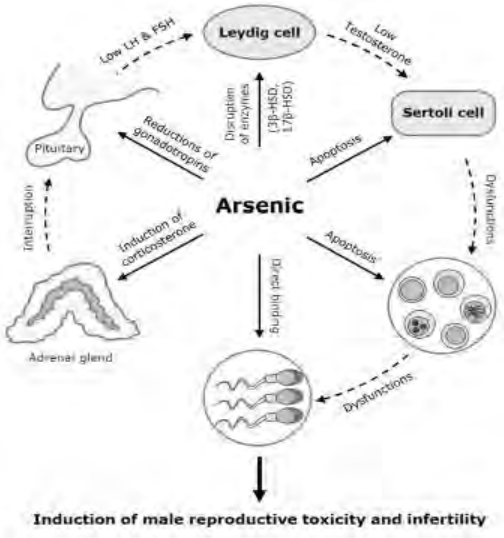


Fig. 1.3 Arseinc-induced reproductive toxicity (Kim and Kim, 2015)

Recent investigations on organ cultures of testes and epididymides of mice have demonstrated the toxic effects of sodium arsenite ($NaAsO_2$) at higher doses i.e. (50 and 100 µM). These include increased oxidative stress depicted by higher levels of reactive oxygen species and reduced antioxidant enzymes, decreased testicular testosterone concentration and sperm DNA damage (Anwar and Qureshi, 2019). Similar pathological changes have also been observed in mice exposed to $NaAsO_2$ (4 ppm and 10 ppm doses) through drinking water for 35 days (Fatima and Qureshi, 2022). Additionally, arsenic was found to induce apoptosis leading to germ cell depletion in a dose dependent manner in testes of post-natal day 5 CD1 (Anwar et al., 2020). Previously, $NaAsO_2$ (533.90µmole/L for 35 days) exposure via drinking water has been found to reduce the activity of 17β-hydroxysteroid dehydrogenase (HSD) enzyme which is essential for testosterone production. In contrast, lactate dehydrogenase (LDH) and and γ -glutamyltranspeptidase (γ GT) levels were increased indicating the elevated level of oxidative stress within mice testes (Meroni et al., 2000). Mice treated with trivalent arsenic have also shown altered sperm parameters including reduced sperm count, motility and viability (Reddy et al., 2011; Fatima and Qureshi, 2022). Arsenic-

induced abnormalities in sperm might be due to its binding to thiol groups which are found in chromatins of sperm nuclei and flagella (Uckun et al., 2002). Along with direct effect of As on sperm, its indirect effect through endocrine disruption has also been reported as late stages of spermatogenesis are sensitive to testosterone. Arsenic is known to decrease, LH, FSH and testosterone levels thus triggering the degeneration of spermatids (Sarkar et al., 2003).

### 1.3 Therapeutic approaches

Considering the increase in case of male factor infertility, identification of prognostic biomarkers and then finding the most suitable therapeutic solution has become a challenge research in the field of andrology. In this regard, finding the regulatory genes of infertility using animal models is a novel approach which can be directed in two ways. These include candidate genic approach and whole genome approach. The former involves identification of genes leading to fertility issues and predicting their function in humans while later approach employs the use of advanced techniques like whole genome sequencing and single nucleotide polymorphism (SNP) microarray for gene identification (Carrell and Aston, 2011; Aston, 2014).

Increased levels of oxidative stress are associated with reproductive dysfunction in males (Agarwal et al., 2014). Arsenic being an environmental pollutant is known to increase the ROS within testes (Doshi et al., 2012) which in turn exert deleterious effects on male reproductive system (Kim and Kim, 2015). These include testicular dysfunction, poor semen quality and altered levels of gondaotrophins and androgens (Zubair et al., 2017).

These findings provide evidence that oxidative stress plays leading role in mediating the toxic effect of arsenic on testes (Jomova et al., 2011). Since, As disrupts the homeostatic balance between oxidative species and antioxidant defences thus there is need to strengthen the antioxidant mechanisms of the body. In this context, natural antioxidants have been found to be effective against As-induced testicular toxicity (Mehrzadi et al., 2018).

#### 1.3.1 Natural antioxidants

There is an ongoing research on finding the potent antioxidants from natural sources to attenuate the testicular toxicity caused by As exposure. Several compounds have been

enlisted in the category of natural antioxidants. These include ellagic acid (Mehrzadi et al., 2018), lutein (Li et al., 2016), arjunolic acid (Manna et al., 2008), quercetin (Jahan et al., 2015) and *N*-acetylcysteine (Reddy et al., 2011). Additionally, vitamin C (Im Chang et al., 2007) and vitamin E (Zubair et al., 2017) have also been found to specifically protect against As-induced testicular damage.

Besides these naturally occurring antioxidants provided through extrinsic sources, potent countering agents of intrinsic origin have also been recently investigated. These include endogenous neuropeptides like melatonin (Reiter et al., 2016) and kisspeptin (Aydin et al., 2010; Fatima and Qureshi, 2022).

#### 1.3.1.1 Ellagic acid

Ellagic acid (EA) is a naturally occurring poly phenol in nuts and fruits and is well-known for its antioxidant properties. Its anti-inflammatory effect in various diseases of heart, liver, kidney and nerves has been well documented (Sarkaki et al., 2016; Mehrzadi et al., 2018). It is known to alleviate oxidative stress by scavenging ROS including hydrogen peroxides, peroxynitrites, hydroxyls and hydroxyl radicals. It also activates the physiological defense against oxidative stress by increasing the activities of antioxidant enzymes (Çeribaşı et al., 2012). It is considered a potent therapeutic agent against diseases involving mitochondrial dysfunctions due to its capability to sustain complex functions of mitochondria (Keshtzar et al., 2015). The protective effect of EA against As-induced testicular damage has been investigated in adult rats. The findings revealed improvement of histological and biochemical parameters and elevated serum and tissue levels of testosterone and antioxidant enzymes (Mehrzadi et al., 2018).

#### 1.3.1.2 Quercetin

Quercetin is another strong acting, polyphenol antioxidant which is commonly obtained from various plant species (Kahraman and Inal, 2002; Ikizler et al., 2007). It can also be chemically synthesized and incorporated in human diet as an essential supplement. Owing to its antioxidant properties, it has been investigated for its protective role in several diseases and toxicities. As regards the As-induced testicular toxicity, quercetin has been found to reduce the testicular oxidative stress level in $NaAsO_2$ treated rats. It also protects the tubular structure and inhibits significant decrease in activities of antioxidant thus preventing apoptosis (Baltaci et al., 2016).

#### 1.3.1.3 Lutein

Lutein (LU) is also another plant based xanthophyll, which is abundantly found in green leafy vegetables including kale, spinach, lettuce, celery, squash and peas etc. (Chang et al., 2013). It is considered beneficial for eye health. Its presence has been observed in macula which is retinal region regulating central vision. It is hypothesized that LU protects the eyes from adverse effects of high energy photons found in blue light and oxidative stress. Several studies have reported the association between intake of LU with eye pigmentation (Krinsky et al., 2003). Lutein has been found to counteract the arsenic-induced reproductive toxicity in mice by reducing oxidative damage. It is indicated by increased activities of antioxidant enzymes and improved sperm count and testicular somatic index. Additionally, LU treatment also increased the mRNA and protein expression of genes involved in mediating protective effects of LU (Li et al., 2016).

#### 1.3.1.4 Vitamin C

L-Ascorbic acid or vitamin C is naturally occurring vitamin in citrus fruit and green leafy vegetables (Abdullah et al., 2022). It cannot be biosynthesized and is only available through diet and supplements. It is absorbed through intestinal route and is distributed throughout the body (Castro et al., 2008). It plays regulatory role in several physiological functions including collagen biosynthesis and free radical scavenging etc. (Castro et al., 2008; Abdullah et al., 2022). It plays its antioxidant role by scavenging ROS and reactive nitrogen species (RNS). It is also capable of regenerating a few antioxidants like GSH, vitamin E and urate etc. (Carr and Frei, 1999). It has been found to protect male reproductive functions by promoting androgen synthesis and protecting spermatogenesis (Fernandes et al., 2011; Siervo et al., 2015).

Protective effects of vitamin C have been reported against arsenic-induced testicular toxicity in mice. It was observed that vitamin C treatment recovers the steroidogenic activities of 3β-hydroxysteroid dehydrogenase (HSD) and 17β-HSD enzymes within testes. Additionally, arsenic mediated oxidative stress was also reduced by increased levels of antioxidant GSH (Im Chang et al., 2007).

#### 1.3.1.5 Vitamin E

Vitamin E is a naturally occurring lipid soluble vitamin (Salama et al., 2015) mainly obtained from nuts, olives, avocados wheat and soy beans etc. Its eight isoforms exist in nature i.e. alpha, beta, gamma, and delta-tocopherol and alpha, beta, gamma, and delta-tocotrienol (Medina and Gupta, 2022). It is well recognized for its antioxidant properties within the biological systems (Colombo, 2010). It protects the cellular membranes from free radical-mediated oxidative damage (Salama et al., 2015). Its deficiency is associated with andrological dysfunctions including reduced synthesis of testosterone (Erdemli et al., 2019) and degeneration of testes (Lomnitski et al., 1991).

Its protective role has been investigated against various environmental pollutants. (Momeni et al., 2012; Mehranjani et al., 2009). It has been found to attenuate testicular damage caused by chronic exposure to $NaAsO_2$ in teddy goat bucks. The findings reveal significant recovery of tubular structure and spermatogenesis (Zubair et al., 2017).

#### 1.3.1.6 Melatonin

It is a neurohormone released from the pineal gland following a circadian rhythm. Its role as a strong antioxidant has long been well established. Generally, it is involved in regulation of various physiological functions including nerve protection (Zararsiz et al., 2007) gonadotrophin regulation (Kus et al., 2000) and maintaining the endocrine rhythms (Forsling et al., 1993).

#### 1.3.1.7 *N*-acetyl cysteine

*N*-acetylcysteine (NAC) is a plant based (naturally found in onions) glutathione precursor. Its use as an antidote drug against toxicity has been routinely practiced since 1960s. It is also used to make cosmetics and several nutraceuticals (Šalamon et al., 2019). Its use as an antioxidant is well established in animal research and clinical investigation. It is being investigated for its antioxidant potential against oxidative stress induced by several stressors (Pedre et al., 2021).

Three different assumptions have been made to explain its beneficial biological effects. Firstly, it may reduce the disulfide bonds found in either intracellular or extracellular space. Secondly, it acts as a scavenger of oxidants like hydrogen peroxide $H_2O_2$ etc. through its sulfhydryl group. Thirdly, it may boost the GSH levels by providing its precursor amino acid the cysteine.

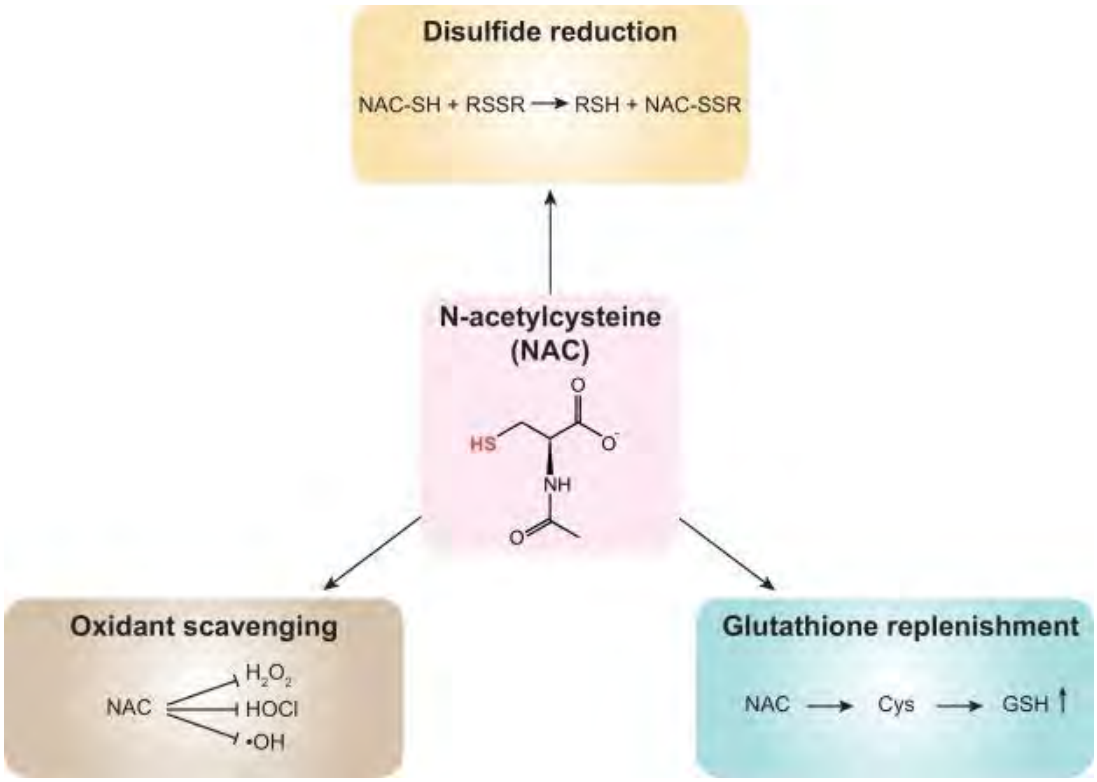


Fig. 1. 4 Proposed narratives for biological effects of NAC (Pedre et al., 2021)

#### 1.3.1.4.1 Attenuating role against arsenic toxicity

In the context of arsenite toxicity, protective effect of NAC has been found in cells taken from anterior pituitary glands of rats which involve decreased expression of oxidative stress biomarker genes including HMOX1 and MT1 (Ronchetti et al., 2016). Likewise, protective effects of NAC against $NaAsO_2$-induced reproductive toxicity have been documented in male mice exposed for 35 days through drinking water. It was observed that NAC improves the sperm parameters, activities of testicular enzymes and circulating testosterone level in mice provided with drinking water containing $NaAsO_2$ (4ppm). It is also found to decrease the lipid peroxidation and increase the activity of antioxidant enzymes in testes (Reddy et al., 2011).

Like most of other antioxidants, it had also been investigated for its therapeutic potential against As-induced testicular damage in rats. It was revealed that melatonin ameliorated the deleterious effects of As on testicular tissue by reducing the germ cell apoptosis, lipid peroxidation and structural deterioration (Uygur et al., 2016).

## 1.4 Kisspeptin

### 1.4.1 Discovery of kisspeptin

Lee et al. (1996) first discovered kisspeptin as a novel metastasis suppressor in melanoma cell lines. It belongs to a peptide family encoded by KISS1/kiss1 gene and derived from the cleavage of a common prepro-kisspeptin precursor. All of the cleavage products share similar structure and are categorized as 'RF amide peptides family'. The name is given due to presence of Arg-Phe-NH2 motif which is characteristic to neuroactive peptides (Clements et al., 2001). The most abundantly circulating kisspeptin in the human body is kisspeptin-54 which is further cleaved to smaller peptides having 14, 13 and 10 amino acids (Kotani et al., 2001).

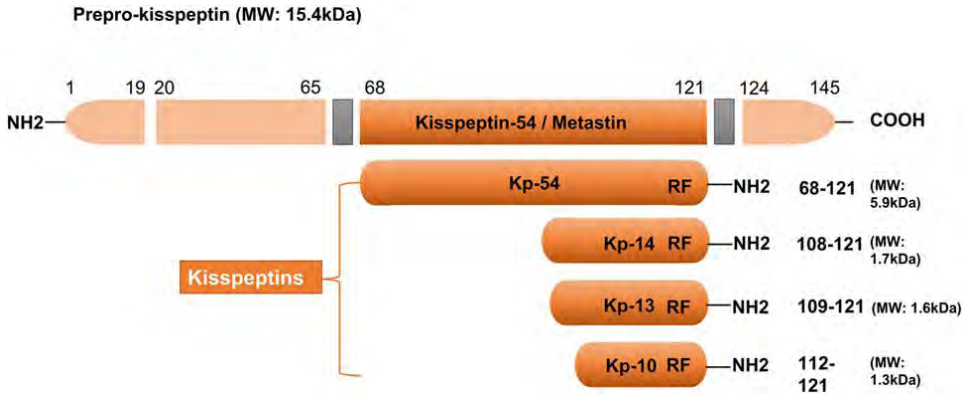


Fig. 1.5 Products of Kiss1 gene (Hu et al., 2018)

### 1.4.2 Kisspeptin receptor

Kisspeptin receptor originally named as GPR54 was discovered four years later KP (Gottsch et al., 2009) discovery. It resembles the galanin receptor and belongs to the rhodopsin family of G-protein coupled receptors (Clements et al., 2001; Kotani et al., 2001; Ohtaki et al., 2001). Kisspeptin ligand binds to its receptor and a cascade of events is initiated by secondary messenger recruitment which results in activation of protein kinase C via release of intracellular calcium (Muir et al., 2001; Liu et al., 2008; Constantin et al., 2009). Calcium ions are released in two phases including former rapid phase and later slower. Second phase is crucial in preventing receptor desensitization as it involves receptor internalization and recycling (Min et al., 2014).

### 1.4.3 Distribution of kisspeptin and its receptor

Kisspeptin encoding genes were first found to be expressed in the placenta (Ohtaki et al., 2001; Muir et al., 2001) and later in other organs including ovary, testes, pituitary gland and spinal cord (Muir et al., 2001; Gaytan et al., 2009). In rodents, kisspeptin and its receptor are centrally expressed in the hypothalamus at two neuronal sites, the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) (Gottsch et al., 2004). While it is primarily expressed within the infundibular nucleus in humans and primates, is equivalent to ARC of other mammalian species (Rometo et al., 2007).

### 1.4.4 Sexual dimorphism in neuronal distribution of kisspeptin

In rodents, sexual dimorphism exists in the form of higher population of kisspeptin neurons at the AVPV nuclei in females as compared to males (Clarkson and Herbison, 2006; Kauffman et al., 2007). Likewise, in humans, possibility of such differential expression in the rostral periventricular area of the third ventricle (RP3V) and infundibulum has also been observed (Hrabovszky et al., 2010; Hrabovszky et al., 2011). In addition, the regulatory role of estradiol in female mice has also been noticed during development leading to increased kissppeptin expression in RP3V region (Clarkson et al., 2009; Bakker et al., 2010).

### 1.4.5 Regulatory role of Kisspeptin in activation of HPG axis

A variety of species exhibit close proximity of gonadotrophin releasing hormone (GnRH) and kisspeptin neurons in the hypothalamic region (Rance et al., 1993; Clarkson and Herbison, 2006) along with the expression of kisspeptin receptor on GnRH neurons (d'Anglemont de Tassigny et al., 2008; Herbison et al., 2010). Both *in vivo* and *in vitro* investigations have revealed that GnRH is released under the stimulation by kisspeptin neurons (Irwig et al., 2004; Liu et al., 2008; Novaira et al., 2009; Padda et al., 2021) a phenomenon which can be inhibited by GnRH antagonistic drugs (Shahab et al., 2005).

Animal and human studies have reported that both central and peripheral administration of kisspeptin is found to elevate the circulating LH levels (Gottsch et al., 2004; Thompson et al., 2004; Dhillo et al., 2005; Leonardi et al., 2022). In the preoptic region, kisspeptin neurons make synapses with both cell bodies of GnRH neurons and median

eminence in proximity with GnRH nerve endings (Gottsch et al., 2004, Clarkson and Herbison, 2006; Uenoyama et al., 2011).

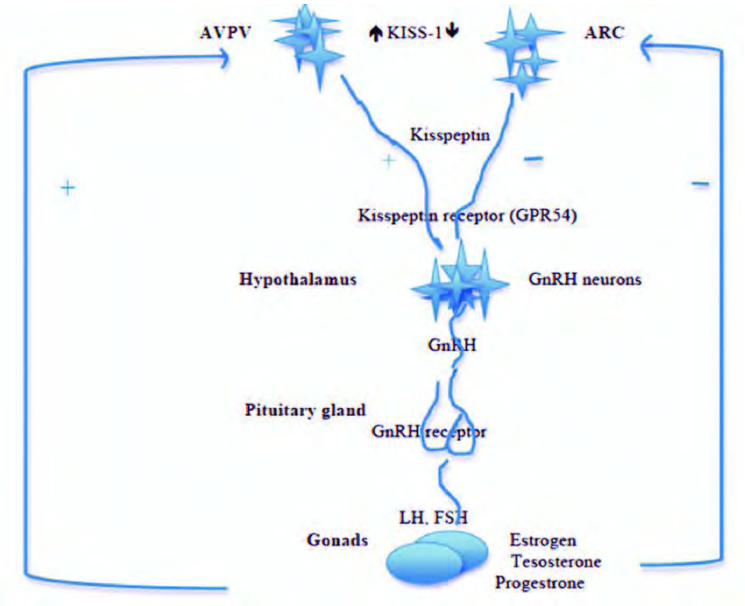


Fig. 1. 6 Kisspeptin signaling in HPG axis (Babiker and Al shaikh, 2016)

Considering this neuronal distribution, it is suggested that hypothalamic GnRH neurons are stimulated by kisspeptin leading to release of GnRH into hypothalamic-pituitary portal circulation. This ultimately stimulates the anterior pituitary to release goadotrophins (Messager et al., 2005; Yeo et al., 2018). It is further evident from the observation that GnRH release is inhibited in the ovariectomized pubertal monkeys which is restored after estradiol replacement. This indicates significance of estradiol for kisspeptin-induced stimulation of GnRH neurons (Guerriero et al., 2012).

### 1.4.6 Kisspeptin and puberty onset

Various mutations in GPR54 have been previously discovered in patients with congenital hypogonadotropic hypogonadism (CHH) (de Roux et al., 2003; Seminara et al., 2003). These findings have also directed the research towards finding more mutations in kisspeptin receptor of humans (Semple et al., 2005; Tenenbaum-Rakover et al., 2007; Breuer et al., 2012). The genetic basis of CHH phenotype suggests the primary role of kisspeptin in puberty onset (Chan et al., 2011). Mechanisms underlying kisspeptin-regulated sexual maturation have been comprehensively investigated by using knockout mouse model. As, it has been found that the hypogonadotropic hypogonadism (HH) is displayed in mice devoid of kisspeptin receptor gene. These

animals showed reduced circulating LH and FSH levels, smaller male gonads and delayed opening of vagina in females. However, their condition was corrected by treatment with exogenous GnRH (Seminara et al., 2003; Uenoyama et al., 2018).

### 1.4.7 Gonadal expression and actions of kisspeptins

Expression of Kiss1, GPR54 and kisspeptin in mammalian ovary had been known before 2010 however, its localized role in gonads has just been recently reported (Ruohonen et al., 2020). It has been concluded from these finding that ovarian kisspeptins are involved in controlling puberty (Ricu et al., 2012) ovarian aging (Hu et al., 2018) and modulate granulosa lutein cells in humans (Owens et al., 2018). Furthermore, significance of peripherally expressed kisspeptin is confirmed by pharmacological investigations using kisspeptin antagonists which blocked action of kisspeptin in the rat ovary which compromised ovarian maturation (Ricu et al., 2012). While, kisspeptin injected directly to ovaries restored the ovarian maturation (Fernandois et al., 2017). Recent evidence by Gpr54 ablation in mice oocytes resulting in premature failure of ovulation also support the localized action of kisspeptins (Ruohonen et al., 2022).

As regards the male gonads, kisspeptin is only expressed in testicular Leydig cells in mice. Its level in Leydig cells is regulated by pituitary LH which is for Leydig cell functions (Salehi et al., 2015). In vivo study on monkeys has revealed supporting role of kisspeptin to LH agonist (human chorionic gonadotropin hCG) in testosterone secretion (Irfan et al., 2014). *In vitro* experiments revealed that application of kisspeptin antagonists inhibits the hCG-induced secretion of testosterone from isolated goat leydig cells (Samir et al., 2018). Recently, cell line studies have also suggested the steroidogenic role of kisspeptin in leydig cells (Hsu et al., 2020).

In contrast, kisspeptin administration has been found to cause no significant change in basal testosterone levels in monkeys (Tariq and Shahab, 2017). Additionally, it has been found that Gpr54 is expressed in seminiferous tubules of mice (on spermatids possibly) (Chiang et al., 2021) and Sertoli cells in primates (Irfan et al., 2016). Considering these controversial findings, localized effect of kisspeptin on testicular functions still remains inconclusive (Sharma et al., 2020).

### 1.4.8 Kisspeptin and stress

It is a well-known fact that reproductive functions are inhibited by stress-induced suppression of GnRH release. The mechanisms involved however still remain unclear but role of corticotrophin releasing hormone/factor (CRH/CRF) which is a hypothalamic neuropeptide has been proposed (Cates et al., 2004; Li et al., 2006). It is observed that centrally injected CRF reduces the expression of kisspeptin and its receptor in the ARC in mice. Similar results have been found in response to various exogenous stressors including, lipopolysaccharide (LPS), insulin induced hypoglycemia and physical restraint (Kinsey-Jones et al., 2009). These findings suggest the contribution of kisspeptin to compromised reproductive function in response to stress (He et al., 2003).

### 1.4.9 Impact of endocrine disruptors (EDCs) on Kiss1 neurons

It has been recently documented that EDCs specifically target the Kiss1 neurons and interfere with the onset of puberty and other reproductive functions. One such chemical, is bisphenol A (BPA) which has been found to lessen the Kiss1 expression in ARC while elevate in the AVPV region leading to early onset of puberty in mice which were exposed to low doses of BPA during gestational period (Ruiz-Pino et al., 2019). In addition, a developmental study on female rats exposed to a mixture of EDCs have revealed the delayed puberty onset caused by disrupted expression of key genes including Kiss 1 (Lopez-Rodriguez et al., 2021).

## 1.5 Therapeutic application of kisspeptin in reproduction

Recent advances in the understanding of mechanisms underlying kisspeptin signaling in the reproductive system have suggested its therapeutic potential in various fertility related disorders. Kisspeptin is known to control reproductive functions through central regulation however numerous animal studies have demonstrated that its peripheral administration may also exert stimulatory effect on GnRH neurons (Thompson et al., 2004, Matsui et al., 2004). Furthermore, no adverse effects of kisspeptin administration have been reported by human research (Dhillo et al., 2007; Chan et al., 2011; George et al., 2011).

These findings suggest the possibility to use kisspeptin for treatment of several endocrine disorders by manipulating the HPG axis. Effects of exogenous kisspeptin on

LH secretion have been investigated in humans (Clarke et al., 2015). It was found that kisspeptin increases circulating gonadotrophin and testosterone levels in healthy males administered intravenous kisspeptin doses (Dhillo et al., 2005). Likewise, subcutaneous administration of kisspeptin was also found to increase the LH levels in plasma of healthy pre-menopausal healthy females (Dhillo et al., 2007). Women with hypothalamic amenorrhoea also got temporary relief (for two weeks) following subcutaneous administration of kisspeptin twice a day (Jayasena et al., 2009). Treatment of these women with kisspeptin twice a week however sustained the LH secretion for more than a month (Jayasena et al., 2010).

Effects of exogenous kisspeptin on endogenous GnRH pulse have also been investigated. It has been noticed that peripheral administration of single bolus dose starts an immediate response in GnRH neurons by causing LH pulse which is not affected by previously generated endogenous LH pulse. Moreover, kisspeptin is found to reset the GnRH pulse generator by delaying the generation of endogenous pulse (Chan et al., 2011). Similarly, continuous infusion with the bolus dose of kisspeptin-10 increases the pulse amplitude and frequency of LH in healthy humans (George et al., 2011).

### 1.5.1 Both a neurohormone and potential antioxidant

A relationship between oxidative stress and gonadotropins has already been demonstrated by previous studies (Appasamy et al., 2008; Terasaka et al., 2017). For instance, production of ROS leads to hypothalamic dysfunction via generation of oxidative stress and subsequent lowering of LH and FSH (Darbandi et al., 2018). Although its role as a binding bridge between sex steroid levels and GnRH has been extensively suggested but literature relating kisspeptin with infertility is scant (Trevisan et al., 2018).

Keeping in view the endogenous origin of kisspeptin as a neurohormone and that no adverse effects have been reported in experimental animals or even human trials, its therapeutic application has been recently suggested (Qureshi and Fatima, 2020; Abbara et al., 2020). Besides, antioxidant potential of KP-10 has been found in male rats treated with methotrexate drug. It was found that kisspeptin effectively prevented the toxic effects of methotrexate treatment by increasing antioxidant enzyme activites and decreasing lipid peroxidation in testicular tissue. Additionally, sperm parameters

including sperm count, motility and viability were also improved in kisspeptin treated rats (Güvenç and Aksakal, 2018). Previously, both direct and indirect effects of exogenous kisspeptin have been reported on oxidant-antioxidant system of rat liver. Kisspeptin alone treatment resulted in increased activities of antioxidant enzymes when compared with its treatment with a GnRH agonist gosereline suggesting its prominent indirect effects (Aydin et al., 2010). Interestingly, another neurohormone, melatonin has also been reported for its antioxidant potential. It was found to suppress production of free radicals and activate cellular antioxidant enzymes (Maitra and Hasan, 2016; Hacışevki and Baba, 2018).

### 1.5.2 Kisspeptin and arsenic-induced testicular toxicity

Research is in its infancy as regards the mechanisms through which kisspeptin may exert its therapeutic effects against several types of endocrine disruptors including the heavy metals. To fill this research gap, we have recently performed our pilot study (Fatima and Qureshi, 2022) based on the hypothesis; whether kisspeptin10 is potent enough to attenuate sodium arsenite (trivalent form of arsenic) -induced testicular damage in adult mice?

Sodium arsenite has been comprehensively investigated for its toxic effects on male reproductive system. It was found to increase oxidative stress by promoting ROS production and lipid peroxidation and decreasing antioxidant enzyme activities in mice testes. (Reddy et al., 2011; Anwar and Qureshi; 2019). Moreover, *in vitro* investigations on mice testes have revealed the induction of apoptosis and germ cell loss followed by exposure to high doses of sodium arsenite (Anwar et al., 2020).

Our recent *in vivo* study has revealed the kisspeptin-induced attenuation of biochemical, spermatological, histological and hormonal parameters in mice provided with sodium arsenite in drinking water at doses of 4 ppm and 10 ppm for 35 days. These include improved sperm count, motility and viability, recovery of tubular structure within testes, sustained circulating testosterone level and decreased oxidative stress (Fatima and Qureshi, 2022).

These are initial outcomes which need comprehensive evaluation to find the central and peripheral pathways through which kisspeptin may have prevented arsenic toxicity. A platform has been provided to further investigate the direct effect of kisspeptin in testicular cultures through *in vitro* experiments. There is still need to evaluate the

molecular markers of oxidative stress and steroidogenic genes involved in kisspeptin mediated recovery of testicular tissue. Lastly, comparative evaluation with a well-established therapeutic NAC at spermatological, biochemical, hormonal, histological and genetic level will also verify the efficacy of Kiss-1 peptide as a potential therapeutic to be used either alone or in combination with other therapeutics for treatment of arsenic-induced reproductive toxicity.

**Aim**

Current study aims to comprehensively evaluate the attenuating role of Kiss1-peptide (KP-10) against sodium arsenite-induced reproductive toxicity in adult male mice and its comparison with *N*-Acetyl cysteine which is a potent antioxidant.

**Objectives**

- Comparative assessment of spermatological, biochemical, hormonal and histoprotective effects of KP-10 and N-acetyl cysteine against sodium arsenite-induced testicular toxicity in adult mice
- Comparative assessment of biochemical, hormonal and histoprotective effects of KP-10 and N-acetyl cysteine against sodium arsenite-induced toxicity in testicular cultures of adult mice
- Molecular assessment (through apoptosis and steroidogenesis related genes) of mitigating role of KP-10 and N-acetyl cysteine against sodium arsenite-induced testicular damage through *in vivo* and *in vitro* experiments

**Abstract**

Current increase in environmental arsenic levels due to anthropogenic activities has resulted in higher human exposure worldwide. Arsenic as a strong pro-oxidant metalloid is known to cause various types of toxicities. Toxic effects of arsenic on male reproductive system have been known for decades and several naturally found compounds have been investigated for their healing properties. Kisspeptin being an endogenous neuropeptide and an essential player in regulation of hypothalamo-pituitary gonadal axis is considered a potential therapeutic agent against arsenic-induced testicular damage. Its capability to reduce oxidative stress has also been reported previously. Therefore, present study was designed to investigate the protective ameliorative effect of intraperitoneally administered kisspeptin-10 (50 nmol/day) against reproductive toxicity in adult male mice challenged with 35 days of exposure to sodium arsenite in drinking water. Mice were assigned to tap water control, sodium arsenite alone (4 ppm and 10 ppm), kisspeptin alone (intermittent and continuous) and combined (sodium arsenite + kisspeptin-10 intermittent & continuous) treatment groups. Results revealed protective effect of both intermittent and continuous kisspeptin doses on reproductive organs against sodium arsenite induced toxicity. This was indicated by an increase ($p<0.001$) in the activity of antioxidant enzymes and a decrease ($p<0.001$) in the levels of oxidative stress biomarkers. Concomitant significant increase was noticeable in the relative organs weight ($p<0.01$), serum testosterone, seminal fructose ($p<0.001$) and significant improvement ($p<0.001$) in sperm parameters. A significant downregulation of lactate dehydrogenase concentration demonstrated further the protective effect of kisspeptin against tissue damage. Histologically, both treatment regimens of kisspeptin combined with sodium arsenite exposure, prevented massive germ cell loss and tissue damage, a condition prominent in sodium arsenite alone treated mice. The study demonstrated for the first time kisspeptin's potential to mitigate the biochemical and histo-toxic effects of arsenic on male reproductive system.

## 2.1 Introduction

Chronic exposure to heavy metals may indeed be one of the underlying causes of rapidly increasing male infertility (Ma et al., 2019). Heavy metals are those environmental contaminants which are known to cause reproductive dysfunction in males. A negative correlation has been found between progressive motility of mature sperm and levels of cadmium (Cd) and arsenic (As) in humans (Li et al., 2018; He et al., 2020). Oxidative damage caused by the generation of free radicals (reactive oxygen or nitrogen species, ROS/RNS) is considered to be the underlying cause of heavy metal poisoning (Souza et al., 2016). In this context, the presence of high content of unsaturated fatty acids in the cell membranes of spermatozoa make them more susceptible to oxidative damage (Tremellen, 2008), a major contributing factor toward male factor infertility (Bisht et al., 2017).

Being well-known toxic metalloid and having ubiquitous distribution, arsenic is considered a serious health hazard. Exposure to arsenic has been linked with diseases like cancer, diseases of the gastrointestinal tract (GIT), diabetes and neurological disorders. Inorganic arsenic is also known to cause impairment of the andrological functions via lowering the sperm count and decreasing the weights of gonadal and accessory sex glands (Kim and Kim, 2015). Trivalent arsenic is considered to be more toxic than its pentavalent counterparts. This is so because it readily blocks several enzymatic pathways (Brachowsky et al., 1999). Deleterious effects of sodium arsenite ($NaAsO_2$) on testicular and epididymal organ cultures in mice have been recently documented. These include, increased ROS levels, decreased antioxidant enzyme levels, decreased blood testosterone concentration and excessive sperm DNA damage, particularly at slightly high concentrations (50 and 100 µM) (Anwar and Qureshi, 2019). In addition, dose-dependent induction of apoptosis and germ cell loss were also reported in post-natal day 5 CD1 mice testes exposed to arsenic (Anwar et al., 2020).

To counter the arsenic-induced oxidative stress which is the major cause of testicular damage (Kim et al., 2011), a biochemical agent(s) with strong antioxidant properties is(are) required as a candidate countering agent(s). Kisspeptin (KP-10), which is an endogenous reproduction related small neuropeptide, appears to bear such potential. It was initially isolated from melanoma cell lines and was characterized as a 54 amino acid protein encoded by an oncosuppressor gene "KISS1" (Lee et al. 1996). Later, it was established that kisspeptide binds as a ligand to a G protein coupled receptor called

GPR54 (Ohtaki et al., 2001). KISS1 gene products include several isoforms comprising 54, 14, 13 and 10 amino acids. All of these isoforms are in general termed kisspeptins (KPs). The decapeptide KP-10 is considered to be the main peptide since this sequence of amino acids which is common to all isoforms, is the sequence required for complete stimulation of GPR54 (Pasquier et al., 2014). KP-10 is although mainly expressed in the hypothalamic area of the central nervous system (CNS) but its expression has also been reported in several peripheral tissues including the ovaries, testes, liver, pancreas etc. (Richard et al., 2008). KP-10 has long been considered a puberty onset protein (Tena-Sempere, 2006). It stimulates the release of gonadotropin releasing hormone (GnRH) from the hypothalamus which then regulates the release of anterior pituitary gonadotropins, the luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Mikkelsen and Simonneaux, 2009).

A relationship between oxidative stress and gonadotropins has already been demonstrated by previous studies (Appasamy et al., 2007; Terasaka et al., 2017). For instance, production of ROS leads to hypothalamic dysfunction via generation of oxidative stress and subsequent lowering of LH and FSH (Darbandi et al., 2018). Although its role as a binding bridge between sex steroid levels and GnRH has been extensively suggested but literature relating KPs with infertility is still deficient (Trevisan et al., 2018). Considering that KP-10 is an endogenous hormone and safe to use in experimental animals or even humans, its use as a potential therapeutic agent has also been suggested (Qureshi and Fatima, 2020; Abbara et al., 2020). Besides, antioxidant potential of KP-10 has also been reported in a study which demonstrated that it can protect testicular damage in male rats caused by methotrexate-induced oxidative stress (Güvenç and Aksakal, 2018). Antioxidant activities, including suppression of ROS/RNS, stimulation of antioxidant enzymes and suppression of proxidant enzymes of another neuropeptide hormone namely, melatonin (Maitra and Hasan, 2016; Hacışevki and Baba, 2018) are already on record. KP-10 is a reproduction related neuropeptide hormone but similar protective potential of KP-10 against oxidative stress has not been studied. This study explored the potential protective effect of KP-10 against $NaAsO_2$ -induced spermatotoxicity and testicular damage in adult male laboratory mice.

## 2.2 Materials and methods

### 2.2.1 Ethics statement

All experiments were approved by the "Bioethical Committee of Quaid-i-Azam University" on care and use of Animals for Scientific Research (BEC-FBS-QAU/2021-308). Animal handling also strictly conformed with Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

### 2.2.2 Chemicals and reagents

KP-10 (KiSS-1; 112-121; H-YNWNSFGLRF-$NH_2$) was obtained as lyophilized powder from Calbiochem (La Jolla, CA, USA). Sodium pentobarbital (Sigma, Germany) and Heparin (Kota Bharu, Kelantan, Malaysia) were purchased locally. Inorganic sodium arsenite ($NaAsO_2$) was purchased from Sigma-Aldrich (Germany).

### 2.2.3 Animals

Male Swiss albino mice (35-50 days old) were procured from the National Institute of Health (NIH), Islamabad and maintained in the Animal House Facility of Quaid-i-Azam University, Islamabad. These were housed in transparent polycarbonate small rodent cages (6 mice per cage) and maintained under standard laboratory conditions (temperature 25±4 °C, photoperiod of 12:12 Light: Dark hours and 40% humidity). Mice were acclimatized for one week. They were given free access to standard rodent chow and fresh drinking water *ad libitum.*

### 2.2.4 Dose selection and preparation

Doses of both inorganic arsenic as $NaAsO_2$ and KP-10 were selected as per previous investigations (Ayturk et al., 2017; Guvenc et al., 2018). For $NaAsO_2$, 100 ppm stock solution was prepared in distilled water. This was further diluted with drinking tap water to obtain the required concentrations of low (4 ppm) and high doses (10 ppm). For KP-10, 1 mg lyophilized powder was used to make stock solution in normal saline, and final injectable dose of 50 nmol/day was worked out in distilled water. $NaAsO_2$ was given orally in drinking water while, KP-10 was administered as intraperitoneal (i.p) injections. Doses were prepared fresh before use.

### 2.2.5 Experimental design

Mice (n=54) were selected randomly and allocated to nine groups; each contained six animals. Taking into consideration the length of the spermatogenic cycle, mice were exposed to respective doses of $NaAsO_2$ and KP-10 for 35 days (Reddy et al., 2011). Two regimens of KP-10 doses were devised; intermittent treatment (once a week) and continuous (daily dose for 35 days). Experimental groups were: control having free access to drinking tap water without any treatment, KP alone (intermittent) + drinking tap water; KP alone (continuous) + drinking tap water; $NaAsO_2$ alone 4 ppm in drinking water, $NaAsO_2$ alone 10 ppm in drinking water; $NaAsO_2$ 4 ppm orally in drinking water + KP intermittent; $NaAsO_2$ 4 ppm + KP continuous; $NaAsO_2$ 10 ppm + KP intermittent, $NaAsO_2$ 10 ppm +KP continuous.

### 2.2.6 Necropsy

Toward the end of 35 days of experimental period, mice were fasted overnight, weighed next morning and sacrificed with an overdose of sodium pentobaribitone (80 mg/kg b.w). Animals were quickly dissected and venous blood was collected through the heart, centrifuged at 12204 $g$ for 5 min, serum was collected and stored at –20 °C for the determination of testosterone concentration. Testes, epididymides and seminal vesicles were collected, rinsed with saline. One of the testes was fixed in 10% buffered formalin for histology, while the other testis was stored at –20°C to prepare homogenate for the determination of biochemical parameters. Epididymides and seminal vesicles were collected to determine the extent of sperm damage and seminal fructose concentration respectively. Relative organ weights were calculated using the following formula;

$$Weight\ of\ the\ organ\ (g) \div Weight\ of\ the\ animal\ body\ (g) \times 100$$

### 2.2.7 Sperm analyses

Sperm count, motility and viability were determined by diluting the epididymal fluid with normal saline according to the method of Belsy et al. (1980) using the improved Neubauer's hemacytometer. Total motile and non-motile sperm were taken into account with motility expressed as percentage of total motile sperm of the total sperm count. Whereas, sperm viability was determined with 1% trypan blue solution as described by Talbot and Chacon (1981). Unstained spermatozoa were considered viable and viability was expressed as percentage of unstained spermatozoa of the total count.

### 2.2.8 Tissue histology

Fixed testicular tissues were processed for standard histology. Briefly, testicular tissues were dehydrated in ascending ethanolic grades, cleared in xylene and embedded in molten paraffin wax prewarmed to 56°C. Thick sections (5µm thickness) were cut on a rotary microtome (Shanden, Finesse, Italy). Sections were then dewaxed and rehydrated with descending grades of alcohol. These were stained with conventional Hematoxylin and Eosin (H & E) stains, washed in tap water, dehydrated again and cover slipped with DPX (BDH, Germany) mountant medium. Sections were photographed on a light microscope (OLYMPUS CX41, Japan) with an attached camera (Canon, Japan) at 40x magnification. Photo plates were prepared using the Adobe Photoshop (ver. 7. 0, Microsoft, Inc. USA).

### 2.2.9 Morphometry

Seminiferous tubular sections (n=30) from each group were used for quantification of morphometric parameters. Sections were randomly selected with preference given to circular symmetry. Parameters including tubular diameter, lumen diameter and germinal epithelium height were measured at 40x magnification using the software Image J, software (USA). Image J was first calibrated to accept the distance of 50 µm (scale bar). Then by using appropriate tools, the measures of seminiferous tubules were taken to calculate the average distance (µm) between respective components. Tubular diameter was calculated from average distance between the opposite points at the tubular boundaries. Similarly, the average distance between tubular and luminal boundaries was taken as epithelial height. Whereas, the distance between opposing points at the inner layer of epithelial membrane was considered as luminal diameter.

### 2.2.10 Homogenate preparation

Testicular tissues were homogenized in 1 ml of phosphate buffered saline (PBS) and centrifuged at 12204 *g* for 10 min. Supernatants were collected and stored at –20 °C to conduct later the biochemical assays.

### 2.2.11 Oxidative stress biomarkers, antioxidant enzymes and non-enzymatic GSH

Testicular homogenates were prepared according to the methods as described (Anwar and Qureshi, 2019), for the assessment of reactive oxygen species (ROS) and lipid peroxidation (TBARS), levels of reduced glutathione (GSH) and antioxidant enzymes, the superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT).

### 2.2.12 Lactate dehydrogenase (LDH)

Testicular LDH levels were determined from stored supernatants using a commercial LDH kit (AMP, diagnostics, Austria) and following the protocol given therein by the manufacturer.

### 2.2.12 Seminal fructose

For seminal vesicle homogenates, tissue was mixed with 1 ml distilled water and centrifuged at 12204 *g* for 10 min. Supernatants were then collected and stored at –20 °C for determination of fructose concentration (Ramzan et al., 2013). Seminal vesicle fructose concentration was then determined using a commercial fructose kit (FertiPro NV, Industriepark Noord 32, 8730 Beernem, Belgium). Assay was carried out according to manufacturer's instructions and fructose levels were determined on a spectrophotometer (Agilent 8453, USA). The absorbance of control and samples was measured at 505 nm.

### 2.2.13 Determination of testosterone concentrations

Serum testosterone levels were determined through enzyme linked immunosorbent assay (ELISA) using the commercial kit (Bioactive, Germany). Inter and intra-assay coefficients of variation were respectively 6.4% and 3.8%. The absorbance of control and samples was read at 450 nm.

### 2.2.14 Statistical analysis

Data were analyzed statistically using the software GraphPad Prism (version 5.0; San Diego, California, USA). One-way analysis of variance followed by Tukey's multiple comparison test was applied to compare the difference between the means of control and treatment groups. The level of significance was $p<0.05$, $p<0.01$ & $p<0.001$. Data are expressed as mean ± standard error of mean (SE).

## 2.3 Results

### 2.3.1 Body weight and relative organ weight

No significant alteration occurred in the body weights in any of the experimental groups. Relative organ weights of testes ($p<0.05$), epididymides ($p<0.05$; $p<0.01$) and seminal vesicles ($p<0.01$) were however found significantly lowered in mice exposed to 4 ppm and 10 ppm doses of $NaAsO_2$, while no such change was observed in mice where a treatment of intermittent or continuous KP-10 doses was given along with exposure to $NaAsO_2$ (Table 2.1).

### 2.3.2 Sperm count, motility and viability assay

Epididymal sperm count, progressive sperm motility and percentage of viable sperm decreased significantly ($p<0.001$) upon treatment with 4 ppm and 10 ppm doses of $NaAsO_2$. In contrast, co-administration of KP-10 as intermittent or continuous doses along with respective doses of $NaAsO_2$ prevented the decline in the above parameters (Table 2.2).

### 2.3.3 Biochemical analysis

#### 2.3.3.1 Testicular lactate dehydrogenase (LDH)

Significantly increased ($p<0.001$) LDH levels in mice exposed to $NaAsO_2$ alone (4 ppm and 10 ppm), indicated testicular damage which was restored with KP-10 treatment to nearly control level (Fig. 2.1).

#### 2.3.3.2 Seminal fructose level

Exposure to $NaAsO_2$ (4 ppm and 10 ppm) alone led to significant decline in seminal fructose concentrations ($p<0.001$). This decline was prevented by intermittent or continuous KP-10 treatment in the combined $NaAsO_2$ and KP-10 groups of mice (Fig. 2. 2).

#### 2.3.3.3 Oxidative stress biomarkers and antioxidant enzymes

Testicular homogenates procured from mice exposed to $NaAsO_2$ alone doses, revealed significantly increased levels of ROS and TBARS ($p<0.01$; $p<0.001$) with a concomitant significant decrease in the levels of antioxidant enzymes the SOD, POD and CAT ($p<0.001$). In contrast, KP-10 treatment combined with $NaAsO_2$ doses led to

a significant reduction in the ROS and TBARS levels whilst improving the activity of antioxidant enzymes (Table 2.3).

### 2.3.3.4 Reduced glutathione (GSH)

Testicular GSH levels were found significantly decreased ($p<0.001$) in $NaAsO_2$ (4 ppm and 10 ppm) treatment groups but exposure to $NaAsO_2$ along with KP-10 treatment revealed prevention of GSH decline in the testes (Table 2.3).

### 2.3.3.5 Serum testosterone

Mice exposed to 4 ppm and 10 ppm doses of $NaAsO_2$ demonstrated significant decrease ($p<0.001$) in serum testosterone. Whereas, treatment with intermittent or continuous KP-10 doses along with respective doses of $NaAsO_2$, prevented the decline in serum testosterone concentration with no significant difference from control mice (Fig. 2. 3).

### 2.3.3.6 Histology

Control groups of mice (Fig 2.4) demonstrated well preserved normal histological structure of testes. Treatment with $NaAsO_2$ (4 ppm) (A) resulted in expanded lumen with abnormally arranged cells and prominent germ cell loss. distorted seminiferous tubules, damaged Leydig cells, torn basement membranes (BM) along and a disruption of spermatogonia (SG), Sertoli cells, round spermatids and elongated spermatids (ES). In contrast, both intermittent (B) and continuous (C) administration of KP-10 along with $NaAsO_2$ (4 ppm) treatment, showed improved distribution of germ cells and normal lumen space in the seminiferous tubules (Fig. 2.5). Likewise, treatment with high dose of $NaAsO_2$ (10 ppm) resulted in severe damage to testicular histology. This was indicated by distorted basement membranes and massive loss of germ cell layers and spermatozoa (A). However, where exposure to $NaAsO_2$ (10 ppm) was combined with the intermittent (B) or continuous (C) KP-10 treatment, it led to a restoration of the germinal epithelium, normal lumen, well-formed basement membranes and a marked increase in the germ cell and sperm population across seminiferous tubules (Fig. 2.6).

### 2.3.3.7 Histomorphometry of seminiferous tubules

Tubular diameters of seminiferous tubules demonstrated no significant change across all experimental groups. However, treatment with $NaAsO_2$ (4 ppm & 10 ppm) alone resulted in significantly increased luminal diameter (65.33±1.23 μm and 72.50±1.00

µm respectively from control, $p<0.001$, Table 4). Whereas, KP-10 supplementation along with $NaAsO_2$ exposure, prevented the expansion of luminal diameter within the seminiferous tubules at both intermittent and continuous dose (50.66±1.00 µm, 50.78±2.12 µm; 50.33±1.00 µm, 48.16±1.04 µm respectively at 4 and 10 ppm arsenic doses) (Table 4). Likewise, height of germinal epithelium was significantly decreased ($p<0.001$) due to massive germ cell loss in $NaAsO_2$ alone treated groups (Table 4). While, both intermittent and continuous KP-10 treatment maintained the epithelial height equivalent to control. Moreover, alone KP-10 treatment either intermittent or alone showed no significant change in morphometric parameters as compared to control group (Table 2.4).

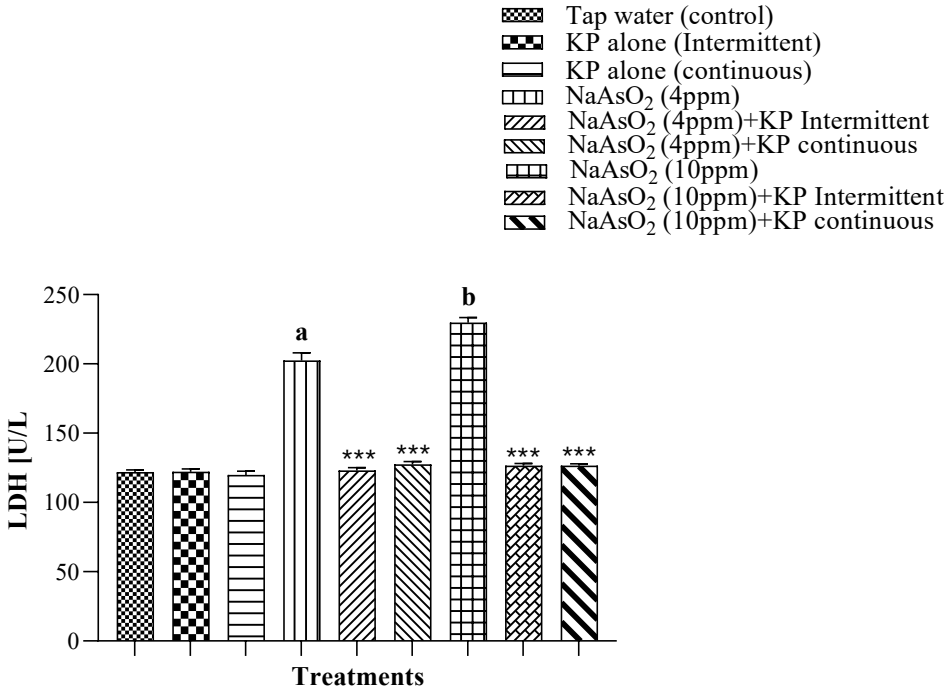


Fig. 2.1 Mean testicular LDH level [U/L] of adult male mice following 35-days exposure to 4 ppm and 10 ppm doses of $NaAsO_2$ and KP-10 (50 nmol/day). letters a & b depict significant difference of 4 ppm & 10 ppm $NaAsO_2$ doses from control groups. *** $p<0.001$ significant difference between KP+ $NaAsO_2$ and $NaAsO_2$ alone treated groups

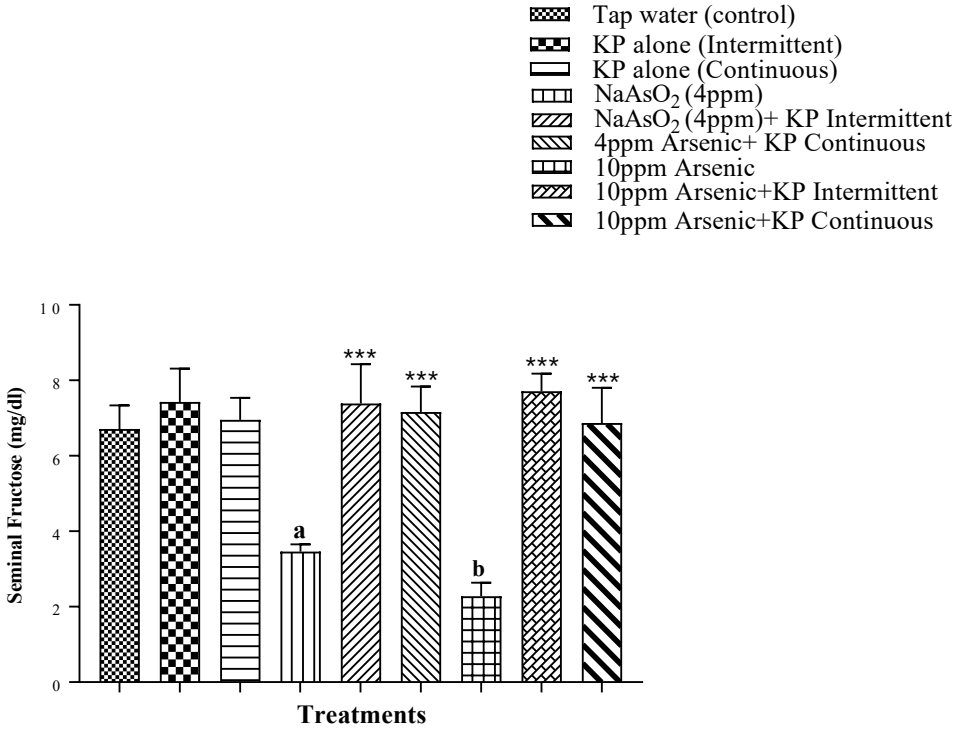


Fig. 2.2 Mean seminal fructose concentration (mg/dl) of adult male mice following 35-days exposure to 4 ppm and 10 ppm doses of $NaAsO_2$ and KP-10 (50 nmol/day). letters a & b depict significant difference of 4 ppm & 10 ppm $NaAsO_2$ doses from control groups. *** $p<0.001$ significant difference between KP+ $NaAsO_2$ and $NaAsO_2$ alone treated groups

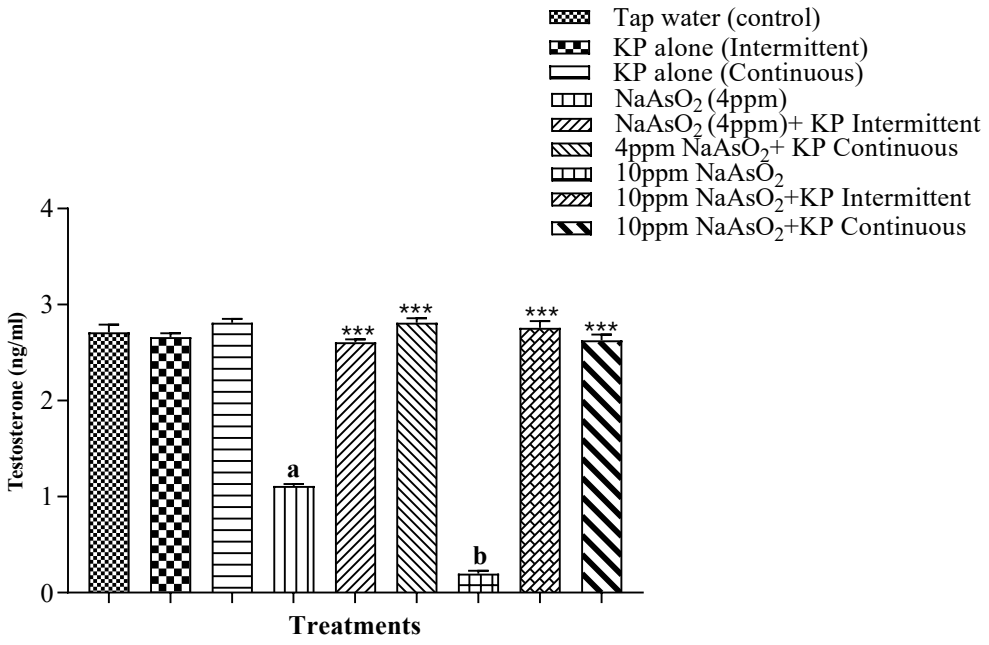


Fig. 2.3 Mean serum testosterone (ng/dl) of adult male mice following 35-days exposure to 4 ppm and 10 ppm doses of $NaAsO_2$ and KP-10 (50 nmol/day). letters a & b depict significant difference of 4 ppm & 10 ppm $NaAsO_2$ doses from control groups. *** $p<0.001$ significant difference between KP+ $NaAsO_2$ and $NaAsO_2$ alone treated groups

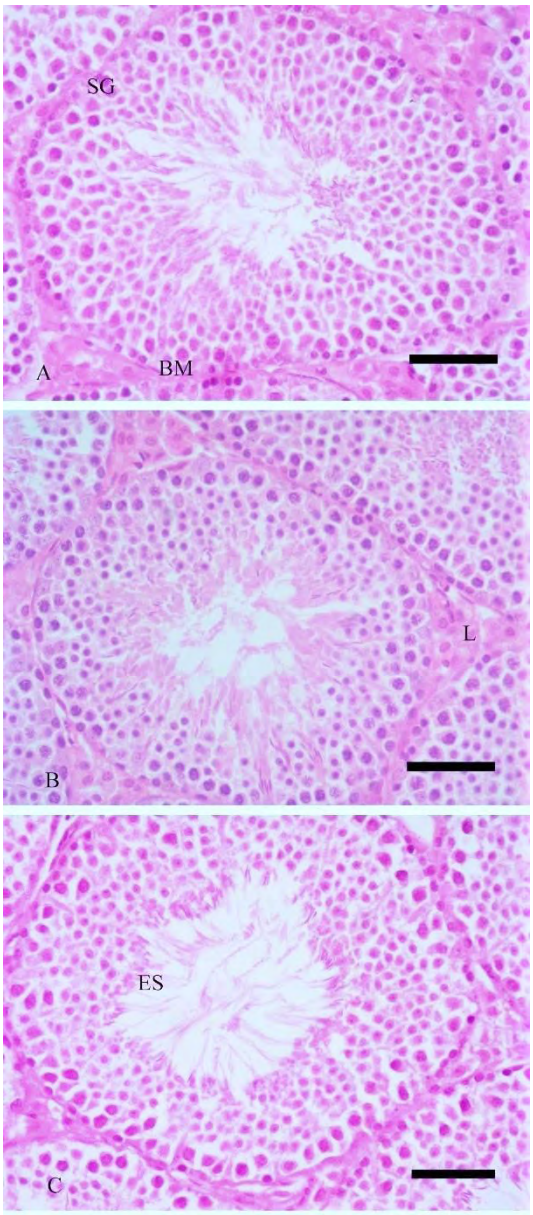


Fig. 2.4 Photomicrograph of testicular sections (5 µm thick) of adult mice upon 35 days exposure to tap water control (A), KP-10 alone; intermittent (B) and continuous (C) (50 nmol/day): normally arranged spermatogonia (SG), elongated spermatids (ES) and basement membrane (BM) Scale bar =50 µm

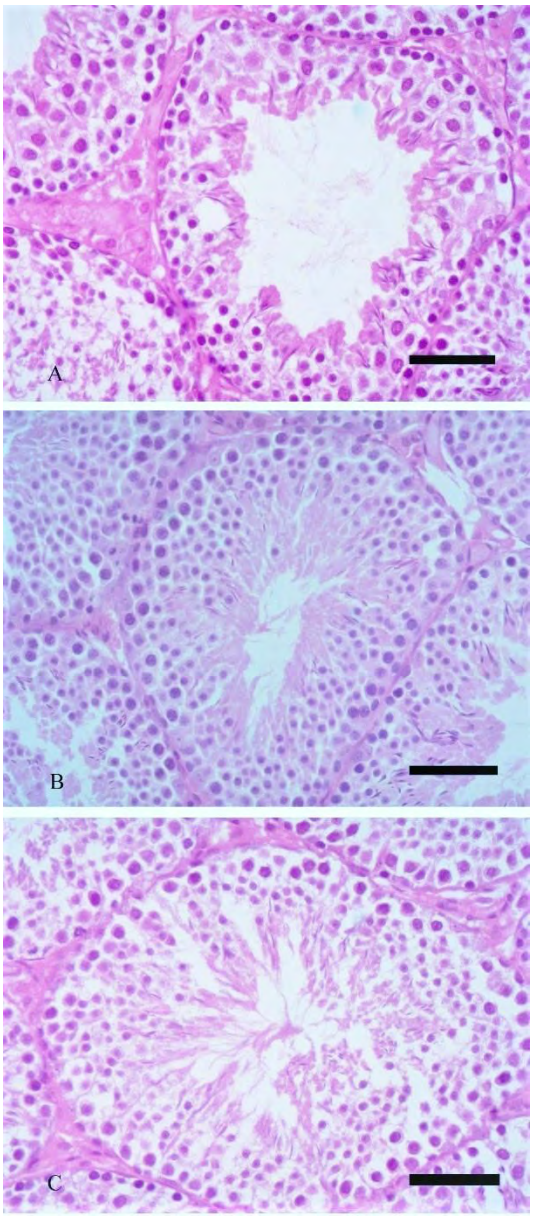


Fig. 2.5 Photomicrograph of testicular sections (5 µm thick) of adult mice upon 35 days exposure to $NaAsO_2$ alone 4 ppm (A): Expanded luminal space and prominent germ cell loss; combined with KP-10 intermittent (B) and KP-10 continuous (C): normal luminal space and improved germ cell distribution Scale bar =50 µm

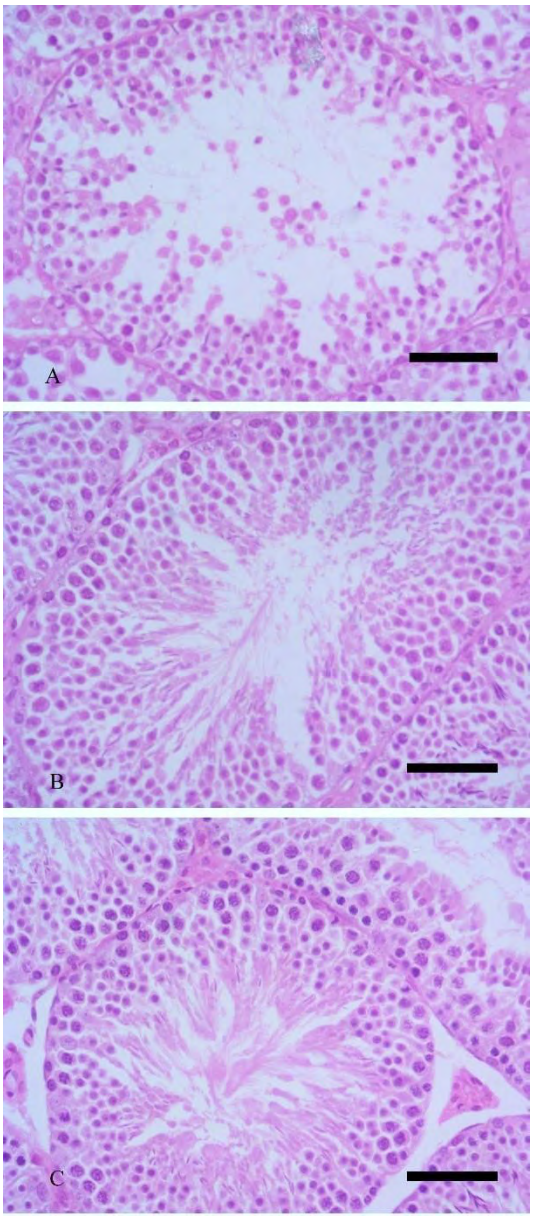


Fig. 2.6 Photomicrograph of testicular sections (5 µm thick) of adult mice upon 35 days exposure to $NaAsO_2$ alone 10 ppm (A): Massive germ cell loss; combined with KP-10 intermittent (B) and KP-10 continuous (C): restored germinal epithelium, normal lumen and basement membranes Scale bar =50 µm

Table 2.1

Body weight and relative organ weights (W/W%) of mice following 35 days exposure to 4 ppm and 10 ppm doses of Sodium Arsenite ($NaAsO_2$) and Kisspeptin-10 (KP-10) (50 nmol/day). Values are Mean ±S.E.

| Treatments | Bodyweight(g) | Testes | Epididymides | Seminal vesicles |
|---|---|---|---|---|
| Tap water(control) | $38.33^{a} \pm 0.81$ | $0.70^{a} \pm 0.02$ | $0.51^{a} \pm 0.01$ | $0.73^{a} \pm 0.02$ |
| KP alone (Intermittent) | $35.00^{a} \pm 0.82$(-8.68) | $0.73^{a} \pm 0.04$(4.28) | $0.49^{a} \pm 0.04$(-3.92) | $0.69^{a} \pm 0.02$(-5.47) |
| KP alone (continuous) | $34.92^{a} \pm 0.55$(8.89) | $0.74^{a} \pm 0.04$(5.71) | $0.46^{a} \pm 0.01$(-9.80) | $0.71^{a} \pm 0.02$(-2.73) |
| $NaAsO_2$ (4ppm) | $37.92^{a} \pm 1.52$(-1.06) | $0.52^{b} \pm 0.03$*(-25.71) | $0.39^{b} \pm 0.02$*(-23.52) | $0.51^{b} \pm 0.03$**(-30.13) |
| $NaAsO_2$ (4ppm)+KP Intermittent | $34.75^{a} \pm 1.08$(-9.33) | $0.62^{a} \pm 0.05$(-11.42) | $0.41^{a} \pm 0.03$(-19.60) | $0.68^{a} \pm 0.04$(-6.84) |
| $NaAsO_2$ (4ppm)+KP continuous | $35.42^{a} \pm 0.88$(-7.59) | $0.64^{a} \pm 0.02$(-8.57) | $0.48^{a} \pm 0.04$(-5.88) | $0.71^{a} \pm 0.03$(-2.73) |
| $NaAsO_2$ (10ppm) | $34.58^{a} \pm 1.26$(-9.78) | $0.50^{c} \pm 0.03$*(-28.57) | $0.29^{c} \pm 0.02$**(-43.13) | $0.47^{c} \pm 0.02$**(-35.61) |
| $NaAsO_2$ (10ppm)+KP Intermittent | $38.50^{a} \pm 1.20$(0.44) | $0.63^{a} \pm 0.03$(-10) | $0.50^{a} \pm 0.02$(-1.96) | $0.67^{a} \pm 0.02$(-8.21) |
| $NaAsO_2$ (10ppm)+KP continuous | $36.75^{a} \pm 1.60$(-4.12) | $0.66^{a} \pm 0.02$(-5.71) | $0.45^{a} \pm 0.04$(-11.76) | $0.70^{a} \pm 0.03$(-4.1) |

Different superscripts in a column show significant difference at *$p < 0.05$ & **$p < 0.01$. values in parentheses represent %change from Tap water control. (n=6/group

Table 2.2

Sperm parameters in mouse testicular homogenate following 35-day exposure to 4 ppm and 10 ppm doses of Sodium Arsenite ($NaAsO_2$) and Kisspeptin-10 (KP-10) (50 nmol/day). Values are Mean ± S.E.

| Treatments | Sperm count ($10^6$ $mL^{-1}$) | Sperm motility% | Sperm viability% |
|---|---|---|---|
| Tap water (control) | $67.66^a \pm 1.14$ | $67.00^a \pm 2.59$ | $64.00^a \pm 1.93$ |
| KP alone (Intermittent) | $71.66^a \pm 1.66$ | $62.66^a \pm 2.37$ | $67.33^a \pm 1.85$ |
| KP alone (continuous) | $74.16^a \pm 2.18$ | $68.66^a \pm 2.04$ | $68.33^a \pm 2.02$ |
| $NaAsO_2$ (4ppm) | $46.54^b \pm 2.39$*** | $43.16^b \pm 2.03$*** | $47.83^b \pm 2.67$*** |
| $NaAsO_2$ (4ppm)+KP Intermittent | $65.33^a \pm 1.68$ | $68.16^a \pm 2.27$ | $68.66^a \pm 2.06$ |
| $NaAsO_2$ (4ppm)+KP continuous | $67.00^a \pm 2.29$ | $67.16^a \pm 2.25$ | $67.00^a \pm 2.46$ |
| $NaAsO_2$ (10ppm) | $31.52^c \pm 1.94$*** | $33.83^c \pm 1.90$*** | $33.33^c \pm 1.94$*** |
| $NaAsO_2$ (10ppm)+KP Intermittent | $67.50^a \pm 2.09$ | $66.00^a \pm 1.36$ | $69.16^a \pm 2.22$ |
| $NaAsO_2$ (10ppm)+KP continuous | $64.83^a \pm 2.85$ | $67.33^a \pm 2.15$ | $68.66^a \pm 2.55$ |

Different superscripts in a column show significant difference at ***$p < 0.001$. (n=6/group)

Table 2.3

Oxidative stress parameters in mouse testicular homogenate following 35-day exposure to 4 ppm and 10 ppm doses of Sodium Arsenite ($NaAsO_2$) and Kisspeptin-10 (KP-10) (50 nmol/day). Values are mean ± S.E.

| Groups | ROS (abs) | TBARS (nM $min^{-1}$ mg $protein^{-1}$) | SOD unit $mg^{-1}$ | POD unit $min^{-1}$ | CAT unit $min^{-1}$ | GSH µM/g |
|---|---|---|---|---|---|---|
| Tap water control | $1.17^{a} \pm 0.03$ | $0.81^{a} \pm 0.04$ | $16.04^{a} \pm 1.28$ | $7.35^{a} \pm 0.75$ | $6.31^{a} \pm 0.33$ | $8.06^{a} \pm 0.57$ |
| KP alone (Intermittent) | $1.15^{a} \pm 0.02$ | $0.79^{a} \pm 0.10$ | $13.28^{a} \pm 1.08$ | $6.41^{a} \pm 0.55$ | $5.10^{a} \pm 0.39$ | $7.95^{a} \pm 0.51$ |
| KP alone (continuous) | $1.15^{a} \pm 0.02$ | $0.89^{a} \pm 0.13$ | $12.68^{a} \pm 1.83$ | $6.41^{a} \pm 0.69$ | $5.54^{a} \pm 0.27$ | $8.76^{a} \pm 0.36$ |
| $NaAsO_2$ (4ppm) | $1.48^{b} \pm 0.04$** | $1.62^{b} \pm 0.06$*** | $5.27^{b} \pm 0.05$* | $2.29^{b} \pm 0.46$*** | $3.68^{b} \pm 0.26$*** | $3.98^{b} \pm 0.52$*** |
| $NaAsO_2$ (4ppm)+KP Intermittent | $1.22^{a} \pm 0.02$ | $0.89^{a} \pm 0.09$ | $12.93^{a} \pm 0.87$ | $6.09^{a} \pm 0.55$ | $5.47^{a} \pm 0.33$ | $7.78^{a} \pm 0.66$ |
| $NaAsO_2$ (4ppm)+ KP continuous | $1.22^{a} \pm 0.03$ | $0.81^{a} \pm 0.07$ | $15.75^{a} \pm 1.27$ | $6.46^{a} \pm 0.53$ | $6.09^{a} \pm 0.29$ | $8.56^{a} \pm 0.25$ |
| $NaAsO_2$ (10ppm) | $1.71^{c} \pm 0.14$*** | $1.93^{c} \pm 0.05$*** | $3.29^{b} \pm 1.07$** | $1.30^{c} \pm 0.21$*** | $0.79^{c} \pm 0.05$*** | $2.19^{c} \pm 0.35$*** |
| $NaAsO_2$ (10ppm)+KP Intermittent | $1.24^{a} \pm 0.03$ | $0.72^{a} \pm 0.11$ | $12.54^{a} \pm 3.63$ | $6.13^{a} \pm 0.47$ | $5.54^{a} \pm 0.33$ | $7.93^{a} \pm 0.39$ |
| $NaAsO_2$ (10ppm)+KP continuous | $1.21^{a} \pm 0.05$ | $0.69^{a} \pm 0.05$ | $12.40^{a} \pm 2.58$ | $6.76^{a} \pm 0.69$ | $5.52^{a} \pm 0.50$ | $6.61^{a} \pm 0.52$ |

Different superscripts in a column show significant difference at *p <0.05, **p < 0.01 & ***p <0.001. (n=6/group)

Table 2.4

Morphometric measurements of seminiferous tubules of mice testes following 35 days exposure to 4 ppm and 10 ppm doses of Sodium Arsenite ($NaAsO_2$) and Kisspeptin-10 (KP-10) (50 nmol/day). Values are Mean ±S.E.

| Treatments | Tubular diameter (µm) | Lumen diameter (µm) | Epithelial height (µm) |
|---|---|---|---|
| Tap water(control) | $201.16^{a}$±3.85 | $48.00^{a}$±1.00 | $82.00^{a}$±1.06 |
| KP alone (Intermittent) | $205.66^{a}$±2.56 | $48.16^{a}$±1.22 | $81.66^{a}$±1.00 |
| KP alone (continuous) | $203.33^{a}$±1.08 | $48.00^{a}$±1.46 | $82.00^{a}$±1.03 |
| $NaAsO_2$ (4ppm) | $206.00^{a}$±2.30 | $65.33^{b}$±1.23*** | $54.33^{b}$±1.40*** |
| $NaAsO_2$ (4ppm)+KP Intermittent | $208.66^{a}$±2.13 | $50.66^{a}$±1.00 | $80.50^{a}$±1.01 |
| NaAsO2 (4ppm)+KP continuous | $204.50^{a}$±2.02 | $50.78^{a}$±2.12 | $80.50^{a}$±1.05 |
| NaAsO2 (10ppm) | $208.16^{a}$±2.25 | $72.50^{c}$±1.00*** | $32.83^{c}$±1.32*** |
| $NaAsO_2$ (10ppm)+KP Intermittent | $206.67^{a}$±2.89 | $50.33^{a}$±1.00 | $81.66^{a}$±1.14 |
| $NaAsO_2$ (10ppm)+KP continuous | $205.66^{a}$±1.56 | $48.16^{a}$±1.04 | $82.00^{a}$±1.25 |

Different superscripts in a column show significant difference at ***$p < 0.001$. (n=30 sections/group)

## 2.4 Discussion

The present study investigated the protective potential of KP-10 (kisspeptin, a hypothalamic reproduction related neuropeptide) against heavy metal induced reproductive toxicity. Laboratory mice were exposed orally to two different doses of $NaAsO_2$ (4 ppm and 10 ppm) in drinking water for 35 days, while separate groups of mice were given equivalent exposure to $NaAsO_2$ but supplemented along with intermittent or continuous KP-10 doses. Results demonstrated lowering of ROS and TBARS, increase in the activity of antioxidant enzymes the SOD, POD and CAT, restoration of circulating testosterone, seminal fructose and testicular LDH levels to within control concentrations upon supplementation with KP-10 in mice exposed to $NaAsO_2$. Concomitant significant improvement occurred in the epididymal sperm count, percentage of motile and viable sperm. Apparently it was the KP-10 which facilitated the recovery of germ cell layers within the seminiferous tubules and a restoration of reproductive dysfunction. The toxic effects of $NaAsO_2$ included; decreased tissue indices, increased levels of oxidative stress biomarkers and lactate dehydrogenase along with decreased activities of antioxidant enzymes, serum testosterone, seminal fructose and massive germ cell loss.

Presently, no significant difference was observed in the body weights of control or treatment groups. A slight decrease was however observed in the body weight of mice upon KP-10 treatment as depicted by percent weight change given in Table 1. This might be due to the reason that KP-10 is found to interfere with food intake and gastrointestinal hormones in mice, thus acting as an appetite suppressor (Dong et al., 2020). However, the observed change was not significant enough to compete with the toxic effects of arsenic exposure that quite possibly led to significantly decreased tissue indices of testes and accessory sex organs.

As regards the $NaAsO_2$ induced reproductive toxicity, outcomes of the current experimentation are in agreement with previous studies which have frequently reported similar observations. For instance, Reddy et al. (2011) have demonstrated similar decrease in testicular weights, sperm parameters, serum testosterone and antioxidant enzyme activities in mice upon exposure to 4 ppm dose of $NaAsO_2$ in drinking water. Likewise, Souza et al. (2016) also noticed decreased reproductive performance in rats chronically exposed to both 0.01 ppm (0.01mg/L) and 10 ppm (10 mg/L) doses of

$NaAsO_2$. Of note, tissue toxicity caused due to the activation of oxidative stress mechanisms has also been comprehensively studied in the pituitary gland of rats during *in vitro* and *in vivo* investigations (Ronchetti et al., 2016).

Besides direct assessment of oxidative stress through testicular oxidant and antioxidant system, we also analyzed tissue damage through the determination of testicular LDH activity. LDH levels are known to elevate in several diseases due to which its activity is used as a biochemical biomarker of disease (Klein et al., 2020). Increased tissue LDH indicates enhanced lipid peroxidation caused by oxidative stress (Jovanovic et al., 2010). Current results also revealed significantly increased LDH levels upon exposure to $NaAsO_2$ alone. However, testicular LDH levels remained significantly lower in mice exposed to $NaAsO_2$ but supplemented with intermittent or continuous KP-10 supplementation. These results suggest that KP-10 mitigated the oxidative damage and preserved cellular integrity by decreasing the lipid peroxidation as indicated by a decrease in the levels of ROS and TBARS (Table 2.3).

As regards the KP-10 alone treatment, no significant change was observed in biochemical, spermatological, hormonal or histological evaluation. Such as, levels of oxidative stress biomarkers and antioxidant enzyme activities remained nearly equal to tap water control group. Similarly, testicular LDH and seminal fructose levels also showed no significant change. Tissue histology and morphometry also revealed no significant change in the arrangement of germ cells within seminiferous tubules, tubular diameter, luminal space or epithelial height. Similarly, serum testosterone levels also showed no significant increase upon treatment with either intermittent or continuous KP-10 alone. Although, KP-10 treatment is well known to stimulate the HPG-axis thereby increasing the circulating LH and testosterone levels in experimental animals (Saito et al., 2012), in the present study KP-10 was continuously administered for 35 days which have somehow desensitized its receptors thus depicting no such increase in circulating testosterone levels.

Keeping in view the past literature on KPs, it is reiterated therefore that KP-10, being an endocrine regulator and an antioxidant both (mentioned elsewhere), can counter the toxic effects of arsenic heavy metal which is a strong pro-oxidant and a known endocrine disruptor. The reason for selecting KP-10 as an intrinsic antioxidant was that it is an endogenous neuropeptide that regulates puberty onset and reproduction, and that

this sequence of amino acids is common to all isoforms and is required for receptor activation (Tena-Sempere, 2006). Presently, KP-10 treatment was found to be very effective as both intermittent and continuous supplementation. KP-10 equally prevented the $NaAsO_2$ induced oxidative damage and also maintained the serum testosterone levels to near normal concentration at 50 nmol dose. Investigators have although tested variable doses (low and high) of kisspeptin to study reproduction in several animal species (Tavor et al., 2006; Ramaswamy et al., 2007). However, it is pertinent to mention that our present study demonstrates that KP-10 is capable of preventing the toxic effects of arsenic on reproduction at 50 nmol dose. Thus unlike $NaAsO_2$ alone treatment, tissue indices of testes, epididymides and seminal vesicles did not show significant decrease upon KP-10 supplementation. Histological examination also revealed well-preserved seminiferous tubules depicting normal germ cell layers. These observations are justifiable since the proper growth and functioning of testes and accessory glands requires continuous stimulation by androgens (Dohle et al., 2003).

In the present study, two experimental regimens, intermittent and continuous, were selected for supplementation with KP-10. The reasons were; firstly, to compare the potential efficacy of these treatments for the first time, secondly, to circumvent the risk that if continuously administered, instead of mitigating the toxic effect of $NaAsO_2$, it might promote testicular degeneration due to desensitization of its receptor, and lastly, to minimize the risk of getting erroneous results due to the stress imposed from continuous handling and dosing for such a long duration. As regards the selection of high dose of KP-10 (50nmol) for current study, Thompson et al. (2006) compared the different isoforms of KP peptide; KP-10, KP-14 and KP-54 at the dose of 50nmol for their efficacy to activate the HPG axis. They found that chronic subcutaneous administration of kisspeptin-54 in adult male rats resulted in decreased LH and testosterone levels which resulted in testicular degeneration. However, significantly elevated LH levels were observed following 1 day treatment with respective dose of KP-54. Although Ramzan and Qureshi, (2011) have previously reported dose-dependent testicular degeneration but this was demonstrated in pre-pubertal rats. Current findings however, did not present any deteriorating effect of KP-10 on adult male mouse reproductive system, instead it occurred to be a protective agent against the toxic effects of $NaAsO_2$. In the present study we also carried out alone KP treatment as intermittent or continuous doses. The results revealed that KP-10 supplementation

maintained ROS, and TBARS to control values, kept antioxidant enzymes, SOD, POD and CAT, LDH, testosterone and seminal fructose to normal levels, Moreover, KP-10 maintained the testicular histology and sperm in healthy and viable state. If the present study is compared with the previous ones, the reason for contrasting results might be that aforementioned studies have specifically reported the dose-dependent damaging effect of KPs in pre-pubertal and adult male rats without any further intervention which could have disrupted the HPG axis. Therefore, it is likely that in the absence of any endocrine disruptor, continuous exposure to KP-10 doses could have desensitized its receptor. Conversely, in the present study, mice were exposed to $NaAsO_2$ that readily affects the HPG axis resulting in downregulation of testosterone secretion. Presumably then, exogenous supplementation with the KP-10 not only restored serum testosterone but also prevented testicular degeneration which would otherwise have been caused by arsenic.

The mechanism through which KP-10 mediates its antioxidant effects necessitates further investigations because literature is still in its infancy as regards the role of KP-10 during chemical or metal induced toxicity. However, existing knowledge related to the regulatory role of KP-10 in puberty onset and reproduction can provide the most logical explanation. As Hamden et al. (2008) has previously reported that sex steroids, specifically estrogen, play an antioxidant role against free-radical damage. Moreover, testosterone is converted into estradiol by the activity of aromatase enzyme thus suggesting its indirect effect as a potential antioxidant (Lephart, 1996). Since, KP-10 is currently found to prevent the significant decline in testosterone concentration which was observed upon $NaAsO_2$ alone exposure. Therefore, it is proposed that the ameliorative effect of KP-10 against arsenic toxicity might have been mediated through the pathway mentioned above. Furthermore, previous literature also supports the current outcomes such as Güvenç and Aksakal (2018) who observed improvement in spermatological parameters upon KP-10 administration in rats treated with methotrexate (an anti-cancer drug). Moreover, oxidative stress was also decreased as indicated by increased activities of antioxidant enzymes. Similarly, Aydin et al. (2010) had also reported the antioxidant potential of KP in rat liver. Considering the outcomes of interventional studies on human subjects, therapeutic potential of KPs in fertility disorders has been suggested (Clarke et al., 2015). These authors presume that deep down mechanism of KP-10 in the restoration of altered reproduction related parameters

at biochemical and cellular level upon exposure to arsenic can be attributed to activation of the free radical scavenging system of the cell.

Localization of KP and its receptor in testes and germ cells is well established (Sharma et al., 2020). In addition, KP-10 has been shown to increase the intracellular calcium concentration in spermatozoa (Hsu et al., 2014). Knowingly, calcium is essential in regulating the motility and capacitation of spermatozoa (Costello et al., 2009). Considering these studies, current findings also suggest the direct effect of KP-10 through sperm activating proteins. Further *in-vivo* and *in-vitro* studies are definitively required to explore the protective effect of KP-10 on spermatozoa challenged with heavy metal exposure. In the present case sperm count, motility, and viability was significantly improved upon KP-10 supplementation. For instance, sperm motility elevated on average to 67 % with KP-10 as compared to NaAsO2 alone (around 38% on average), which is almost the same as our control demonstrating an improvement in the sperm motility. However, the 67% sperm motility in the control mice appears although low but previous reports indicate that even 60.87% motility is considered normal (Reddy et al., 2011).

Presently, intraperitoneal administration of KP-10 might have interfered with water intake thereby decreasing arsenic intake. However, this would have been better justified if arsenic concentration in testes and accessory sex organs was measured and compared between respective experimental groups. Less water intake due to KP-10 administration might be one of the reasons; however, it was ensured during the experiments that the drinking water be replaced with exactly the same dose of $NaAsO_2$ daily while replacing with fresh water, and easily accessible to each animal in the cage. The drinking bottles with stainless steel nozzles were tightly fixed in the cages, and were filled with 250 ml water on daily basis. Each morning the left over water level was measured in the bottles to determine the water intake. This was divided over six animals per cage to get an approximation of how much quantity of water was consumed and to estimate $NaAsO_2$ intake by each animal per day for 35 days. Since the animals remained in good physiological condition until the end of experiments and did not show any untoward signs of lethargy or dehydration, and also were not in a position to waste the water because of tightly fixed bottles and nozzles, we presume that water intake was adequate as per metabolic need of each animal. Thus we cannot presume that the KP, acting directly on water intake neurons or via the HPG axis, suppressed the drinking center

through inhibition of the drinking reflex. Laboratory mouse is although a good and handy model of studying hormones and toxicity, however a prominent limitation is high metabolic rates and that small rodents get into stress very quickly. The factor of stress was however tried to be minimized as much possible by keeping few animals in a cage; the investigators themselves handled all animals during whole of the experiments, and regularly monitored them for any abnormal signs.

**Conclusion**

Keeping in view these findings and our current observations, treatment with KP-10 appears to be a promising countering agent in mitigating the oxidative damage caused by free radicals generated by $NaAsO_2$. This is the first report as regards mitigating effects of KP-10 on metal toxicity. However, it warrants further research to comprehensively evaluate the differential effect of various forms of KP peptide along with administration of antagonist to kisspeptin receptor. Moreover, evaluation of expression level of oxidative stress related genes to study the signalling mechanism involved will provide a better understanding about the role of KP-10 in testicular oxidant-antioxidant system. Finally, current study was conducted using murine animal model but to determine the clinical significance of KP-10 in fertility treatment, comparable results in a primate animal model would be more appropriate.

**Abstract**

Current study was aimed to compare the ameliorative effects of intraperitoneally administered kisspeptin-10 (50 nmol/day once a week administration) and N acetyl cysteine (75 mg/kg b.w. for five alternative days) against testicular toxicity in adult male mice exposed for 35 days to low and high doses of sodium arsenite in drinking water. Mice were divided into tap water control, sodium arsenite alone (4 ppm and 10 ppm), kisspeptin-10 alone, N acetyl cysteine alone, and combined (sodium arsenite + kisspeptin-10; sodium arsenite + N acetyl cysteine; sodium arsenite + kisspeptin+ N acetyl cysteine) treatment groups. Results revealed that both kisspeptin and N acetyl cysteine supplementations are equally effective against sodium arsenite induced testicular damage. This was indicated by an increase ($p<0.001$) in the activity of antioxidant enzymes and a decrease ($p<0.001$) in the levels of oxidative stress biomarkers. Concomitant significant increase was noticeable in the relative organs weight ($p<0.01$), serum testosterone, seminal fructose ($p<0.001$) and significant improvement ($p<0.001$) in sperm parameters. A significant downregulation of lactate dehydrogenase concentration demonstrated further the protective effects of both potential therapeutic agents against tissue damage. Histologically, both kisspeptin and N acetyl cysteine combined with sodium arsenite exposure equally prevented massive germ cell loss and tissue damage, a condition prominent in sodium arsenite alone treated mice. Interestingly, combined supplementation with kisspeptin and N acetyl cysteine along with both 4 ppm and 10ppm exposure to sodium arsenite revealed significant improvement ($p<0.05$) in aforementioned parameters as compared to kisspetin-10 alone supplementation. This is the first comparative study which evaluated the synergistic effect of kisspeptin-10 and N acetyl cysteine for their spermatological, hormonal, biochemical and histoprotective effects against arsenic induced testicular toxicity in male reproductive system.

## 3.1 Introduction

Arsenic is a known reproductive toxicant and a teratogen which has been investigated for its deleterious effects in both animal and clinical research for several decades (Wang et al., 2006). As a carcinogen, it is found to induce tumor formation in several organs including liver, lungs and kidneys etc. (Palma-Lara et al., 2020). Toxic effects of arsenic are not just confined to vital organs as it is also capable to induce pathological changes in male reproductive organs (Sarkar et al., 2003).

Exposure to trivalent arsenic via drinking water and intraperitoneal injections in rats lead to decreased level of circulating gonadotrophins and testicular degeneration (Chinoy et al., 2004). Likewise, deleterious effects of sodium arsenite provided to adult male mice via drinking water (4ppm) for 35 days have also been reported in another study. The findings revealed significant decrease in sperm count, sperm motility and sperm viability upon arsenic exposure. Moreover, lipid peroxidation was increased while activities of antioxidant enzymes were decreased demonstrating increased level of oxidative stress. Activities of testicular steroidogenic enzymes were also decreased in these mice (Reddy et al., 2011).

Keeping in view the numerous *in vivo* outcomes demonstrating toxic effects of sodium arsenite on male reproductive system, several natural therapeutic agents have been proposed recently (Mehrzadi et al., 2018). Since, the mechanism mediating arsenic-induced testicular toxicity involves elevation of oxidative stress (Jomova et al., 2011) thus a potential counteracting agent should fulfill the demand to support physiological antioxidant defense mechanisms. Moreover, the resulting endocrine disruption also requires a potent endocrine regulator reducing oxidative stress level (Mehrzadi et al., 2018).

Previously, melatonin which is a neurohormone has been investigated for its antioxidant potential against arsenic-induced testicular toxicity in rats. It was observed that melatonin counteracted arsenic-induced decrease of testicular antioxidant enzymes and increased malondialdehyde levels. Moreover, cellular apoptosis was significantly reduced while number of germ cells expressing proliferating cell nuclear antigen was elevated upon melatonin treatment (Uygur et al., 2016).

Kisspeptin is another endogenous neuropeptide which has been observed to regulate the HPG axis by initiating the release of GnRH from hypothalamus (Novaira et al., 2009). It is involved in puberty onset and mutations in its receptor gene have been found in patients with congenital hypogonadotropic hypogonadism (Seminara et al., 2003). Its therapeutic application in reproductive dysfunctions have also been investigated in animal models (Thompson et al., 2004) and as well as in the clinical research with no noticeable adverse effects (Dhillo et al., 2007; George et al., 2011). Interestingly, antioxidant properties of kisspeptin have also been suggested in previous *in vivo* studies. It was found to alleviate oxidative stress in rat testes (Güvenç and Aksakal, 2018) and liver (Aydin et al., 2010) by decreasing lipid peroxidation and elevating the activities of antioxidant enzymes.

N acetyl cysteine (NAC) is a well-known therapeutic agent which had long been used as an antidote drug. Recently, its use has been incorporated in several nutraceuticals (Salamon et al., 2019). Being glutathione precursor, its antioxidant role against various types of stressors has also been established through both animals based and clinical research (Pedre et al., 2021). Importantly, it is found to ameliorate toxic effects of sodium arsenite on reproductive system of adult male mice by decreasing levels of oxidative stress biomarkers and increasing the activities of antioxidant enzymes (Reddy et al., 2011).

Taking into account the above mentioned observations and outcomes of our previous experiment (Chapter 2), NAC has been incorporated in the present experiment to further compare and verify the previously suggested mitigating role of kisspeptin against arsenic-induced testicular toxicity in adult mice (Fatima and Qureshi, 2022) through spermatological, biochemical, hormonal and histological evaluation.

## 3.2 Materials and methods

### 3.2.1 Animals and maintenance

Adult male Swiss albino mice were procured from national institute of health (NIH), Islamabad and maintained in the animal house facility of Quaid-i-Azam university under the standard laboratory conditions as mentioned in Chapter 2. After a week of acclimatization, these mice were randomly allocated into different experimental groups according to the current study design.

### 3.2.2 Chemicals

KP-10 (KiSS-1; 112-121; H-YNWNSFGLRF-$NH_2$) was obtained as lyophilized powder from Calbiochem (La Jolla, CA, USA). Sodium pentobarbital (Sigma, Germany) and Heparin (Kota Bharu, Kelantan, Malaysia) were purchased locally. Inorganic sodium arsenite ($NaAsO_2$) and N acetyl cysteine (NAC) were purchased from Sigma-Aldrich (Germany).

### 3.2.3 Dose selection and preparation

Doses of $NaAsO_2$, NAC and KP-10 for current experiment were selected according to previous investigations (Reddy et al., 2011, Ayturk et al., 2017; Guvenc et al., 2018). Moreover, considering the outcomes of our previous study (Fatima and Qureshi, 2021), regimen for KP-10 treatment was also limited to only 'once a week intraperitoneal administration' at the dose of 50nmol/day. Doses of KP-10 and $NaAsO_2$ (4ppm and 10ppm) were prepared from stock solution as mentioned in chapter 2. Whereas, NAC was intraperitoneally administered to experimental mice on 5 alternative days at the dose of 75 mg/kg b.w. All doses were freshly prepared before use.

### 3.2.4 Experimental design

Mice (n=66) were selected randomly and allocated to eleven groups; each contained six animals. Experimental groups were:

- Control having free access to drinking tap water without any treatment
- KP alone+ drinking tap water
- NAC alone + drinking tap water
- $NaAsO_2$ alone 4 ppm in drinking water
- $NaAsO_2$ 4 ppm orally in drinking water + KP
- $NaAsO_2$ 4 ppm orally in drinking water + NAC

- $NaAsO_2$ 4 ppm orally in drinking water + KP+NAC
- $NaAsO_2$ alone 10 ppm in drinking water
- $NaAsO_2$ 10 ppm ppm orally in drinking water + KP
- $NaAsO_2$ 10 ppm + NAC
- $NaAsO_2$ 10 ppm + KP + NAC

### 3.2.5 Necropsy

After completion of experimental time period, mice were overnight fasted, weighed and sacrificed next morning using an overdose of sodium pentobarbital (80 mg/kg b.w.). Venous blood was collected through heart puncture immediately after dissection and sera were collected and stored at –20°C for testosterone determination, after centrifugation at 12204 g for 5 min. Reproductive organs including testes, epididymides and seminal vesicles were also stored after thorough rinse with normal saline. One of the testes was also preserved for histology using 10% buffered formalin as a fixative while the other testis was preserved –20°C for homogenate preparation to measure several biochemical parameters. Sperm parameters and seminal fructose concentration were measured using stored epididymides and seminal vesicles respectively. Following formula was used to calculate relative organ weight:

$$Weight\ of\ the\ organ\ (g) \div Weight\ of\ the\ animal\ body\ (g) \times 100$$

### 3.2.6 Sperm analyses

Epididymal fluid was diluted with normal saline as described by Belsey et al. (1980) in order to determine sperm parameters including total sperm count, sperm motility and viability. Percentage sperm motility was expressed by taking into account the total motile and non-motile sperm. For determination of sperm viability, method described by Talbot and Chacon (1981) was employed using 1% trypan blue solution. Viable sperm remained unstained and percentage of these spermatozoa of the total count was considered as sperm viability.

### 3.2.7 Tissue histology

Standard histology protocol was used for processing of fixed testicular tissues. Briefly, testicular tissues were passed through ascending grades of ethanol for dehydration followed by clearance in xylene and embedding in molten paraffin wax pre-warmed to 56 °C. Using rotary microtome (Shanden, Finesse, Italy), sections of 5 µm thickness

were cut and then dewaxed. Afterwards these were passed through descending grades of alcohol for rehydration. Conventional Hematoxylin and Eosin (H & E) staining of these sections was performed and again dehydration was repeated after washing in tap water. Afterwards, cover slips were mounted using DPX (BDH, Germany) mountant medium. Sections were photographed on a light microscope (OLYMPUS CX41, Japan) with an attached camera (Canon, Japan) at 40x magnification. Photo plates were prepared using the Adobe Photoshop (ver. 7. 0, Microsoft, Inc. USA).

### 3.2.8 Morphometry

Morphometric parameters were quantified from each group using approximately 30 seminiferous tubular sections. Circular tubular sections were given preference during random selection of sections for morphometry. Image J, software (USA) was used for measurement of tubular diameter, lumen diameter and germinal epithelium height at 40x magnification. Following calibration of Image J, appropriate tools were used to take the tubular measurements for calculating the average distance (µm) between respective components. Average distance measured between the opposite points of tubular boundaries was considered as tubular diameter. Likewise, epithelial height was measured as the average distance between tubular and luminal boundaries. Whereas the luminal diameter was measured as distance between opposing points at the inner layer of epithelial membrane.

### 3.2.9 Homogenate preparation

Homogenates of testicular tissues were prepared in 1 ml of phosphate buffered saline (PBS) followed by centrifugation at 12204 *g* for 10 min. Supernatants were collected and stored at –20 °C for further biochemical assays.

#### 3.2.9.1 Oxidative stress biomarkers, antioxidant enzymes and non-enzymatic GSH

Assessment of reactive oxygen species (ROS) and lipid peroxidation (TBARS), levels of reduced glutathione (GSH) and antioxidant enzymes, the superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) was performed using methods described by (Anwar and Qureshi, 2019).

### 3.2.9.2 Lactate dehydrogenase (LDH)

Following protocol provided by the manufacturer of commercially available LDH kit (AMP, diagnostics, Austria), testicular LDH levels were determined from stored supernatants.

### 3.2.9.3 Seminal fructose

Method described by (Ramzan et al., 2013) was employed to homogenize the seminal vesicles. It involved mixing of tissue in 1ml of distilled water followed by centrifugation at 12204 g for 10 min. Collected supernatants were then stored at –20 °C for determination of fructose using a commercial fructose kit (FertiPro NV, Industriepark Noord 32, 8730 Beernem, Belgium). Fructose levels were determined on a spectrophotometer (Agilent 8453, USA) by taking absorbance of control and samples at 505 nm.

### 3.2.9.4 Determination of testosterone concentrations

Enzyme linked immunosorbent assay (ELISA) was performed for determination of serum testosterone level using the commercial kit (Bioactive, Germany). Inter and intra-assay coefficients of variation were respectively 6.4% and 3.8%. The absorbance of control and samples was read at 450 nm.

## 3.3 Statistical analysis

Data were analyzed statistically using the software GraphPad Prism (version 5.0; San Diego, California, USA). One-way analysis of variance followed by Tukey's multiple comparison test was applied to compare the difference between the means of control and treatment groups. The level of significance was $p<0.05$, $p<0.01$ & $p<0.001$. Data are expressed as mean ± standard error of mean (s.e.).

## 3.4 Results

### 3.4.1 Body weight and relative organs weight

All the experimental groups showed no significant change in body weights. Whereas, relative organ weights of testes, epididymides and seminal vesicles ($p<0.01$) were found significantly decreased in mice exposed to 4 ppm and 10 ppm doses of $NaAsO_2$ in drinking water. Treatment with both separate and combined doses of KP-10 and NAC along with exposure to $NaAsO_2$ however revealed no such change in respective parameter as compared to tap water control (Table 3.1).

### 3.4.2 Sperm count, motility and viability assay

Treatment with both 4 ppm (Fig. 3.1-3.3) and 10 ppm (Fig. 3.4-3.6) doses of $NaAsO_2$ significantly ($p<0.001$) decreased the epididymal sperm count, progressive sperm motility and percentage of viable sperm. Whereas, supplementation with both KP-10 and NAC along with exposure to respective doses of $NaAsO_2$ prevented the decline in these parameters thus indicating the protective role of KP-10 and NAC. Moreover, combined treatment of KP-10 and NAC along with $NaAsO_2$ exposure resulted in significant improvement ($p<0.05$) in the sperm parameters as compared to separate treatment with KP-10 along with $NaAsO_2$ exposure.

### 3.4.3 Biochemical analysis

#### 3.4.3.1 Testicular lactate dehydrogenase (LDH)

Testicular damage was indicated by significant increase ($p<0.001$) in LDH levels in mice upon exposure to $NaAsO_2$ alone (4 ppm and 10 ppm), which was alleviated with both separate and combined treatment with KP-10 and NAC as indicated by decrease in testicular LDH to near control level. Additionally, when compared to treatment with KP-10 along with $NaAsO_2$ exposure, combined administration of KP-10 and NAC, further demonstrated significant decrease ($p<0.05$) in testicular LDH levels at both 4ppm (Fig. 3.7) and 10 ppm (Fig. 3.8) doses of $NaAsO_2$.

#### 3.4.3.2 Seminal fructose level

Exposure to $NaAsO_2$ 4 ppm (Fig. 3.9) and 10 ppm (Fig. 3.10) alone led to significant decline in seminal fructose concentrations ($p<0.001$). This decline was prevented by KP-10 and NAC supplementation with both low and high doses of $NaAsO_2$. Combined treatment with KP-10 and NAC was found to be more efficient in improving ($p<0.05$)

seminal fructose levels as compared to KP alone administration along with $NaAsO_2$ exposure.

### 3.4.3.3 Oxidative stress biomarkers and antioxidant enzymes

Significant increase in levels of ROS and TBARS ($p<0.001$) was observed in supernatants procured from homogenized testicular tissue from mice exposed to $NaAsO_2$ alone at doses of 4 ppm (Table 3.2) and 10ppm (Table 3.3). Whereas, levels of antioxidant enzymes (SOD, POD and CAT) were significantly decreased ($p<0.001$) in these treatment groups. In contrast, co-administration of KP-10 and NAC both alone and in combination with $NaAsO_2$ (4 ppm & 10 ppm) exposure led to a significant decline in the ROS and TBARS levels whilst recovering the antioxidant enzymes activities. When compared to KP-10 alone treatment along with $NaAsO_2$ exposure, combined supplementation with KP-10 and NAC, demonstrated significant improvement ($p<0.05$) in testicular oxidant-antioxidant balance of mice treated with low and high doses of $NaAsO_2$.

### 3.4.3.4 Reduced glutathione (GSH)

$NaAsO_2$ 4 ppm (Table 3.2) and 10 ppm (Table 3.3) treated groups showed significant decrease ($p<0.001$) in testicular GSH levels however both alone and combined supplementation with KP-10 and NAC, prevented the decline in testicular GSH as compared to tap water control. Similar to other oxidative stress parameters, testicular GSH was also found to be significantly increased ($p<0.05$) in combined supplementation group (KP-10+NAC) as compared to KP-10 alone treatment along with $NaAsO_2$ exposure.

### 3.4.3.5 Serum testosterone

Treatment with $NaAsO_2$ alone at 4ppm (Fig. 3.11) and 10ppm (Fig. 3.12) doses resulted in significantly decreased ($p<0.001$) serum testosterone levels. Whereas both KP-10 and NAC administration along with respective doses of $NaAsO_2$, prevented this decline thus maintaining the serum testosterone concentration near control level. Significant difference ($p<0.05$) in serum testosterone levels was also noticeable between combined (KP-10 and NAC) and KP-10 alone supplementation groups.

### 3.4.3.6 Histology

Well preserved histological structure was revealed in testes of Tap water control, KP-10 and NAC alone treated groups of mice (Fig. 3. 13 A, B & C). Exposure to low dose of $NaAsO_2$ (4 ppm) (Fig. 3.14 A) resulted in abnormally arranged cells with expanded lumen. In contrast, distribution of germ cells within seminiferous tubules was improved upon both alone and combined supplementation with KP-10 and NAC along with $NaAsO_2$ (4 ppm) exposure (Fig 3.14 B, C & D). Similarly, testicular histology was severely damaged upon treatment with high dose of $NaAsO_2$ (10 ppm) (Fig 3.15 A). The pathological changes were demonstrated as distorted basement membranes, massive depletion of germ cell layers and spermatozoa, disoriented seminiferous tubules, damaged Leydig cells, distorted basement membranes (BM) and disrupted spermatogonia (SG) and elongated spermatids (ES) (similar to previous results Chapter 2). Exposure to $NaAsO_2$ (10 ppm) along with both alone and combined treatments with KP-10 and NAC however significantly restored the germinal epithelium, luminal space, basement membranes and also resulted in replenishment of germ cell and sperm population across seminiferous tubules (Fig. 3.15 B, C and D).

### 3.4.3.7 Morphometry of seminiferous tubules

Similar to previous experiment (Chapter 2), tubular diameters remained unaltered in all experimental groups. When compared to tap water control, luminal diameter was however found significantly increased ($p<0.001$) in $NaAsO_2$ (4 ppm & 10 ppm) alone treated groups. Whereas, $NaAsO_2$ exposure when supplemented with both KP-10 and NAC alone and combined doses prevented the abnormal expansion of luminal diameter. Similarly, significantly decreased ($p<0.001$) germinal epithelial heights due to massive germ cell depletion were also noticeable upon $NaAsO_2$ alone exposure (4ppm & 10ppm). While, KP-10 and NAC administration along with $NaAsO_2$ exposure, prevented the significant decline in epithelial heights and promoted the recovery of germ cell population equivalent to control. Additionally, testicular morphometric parameters demonstrated no significant change upon alone treatment with either KP-10 or NAC as compared to control group (Table 3.4).

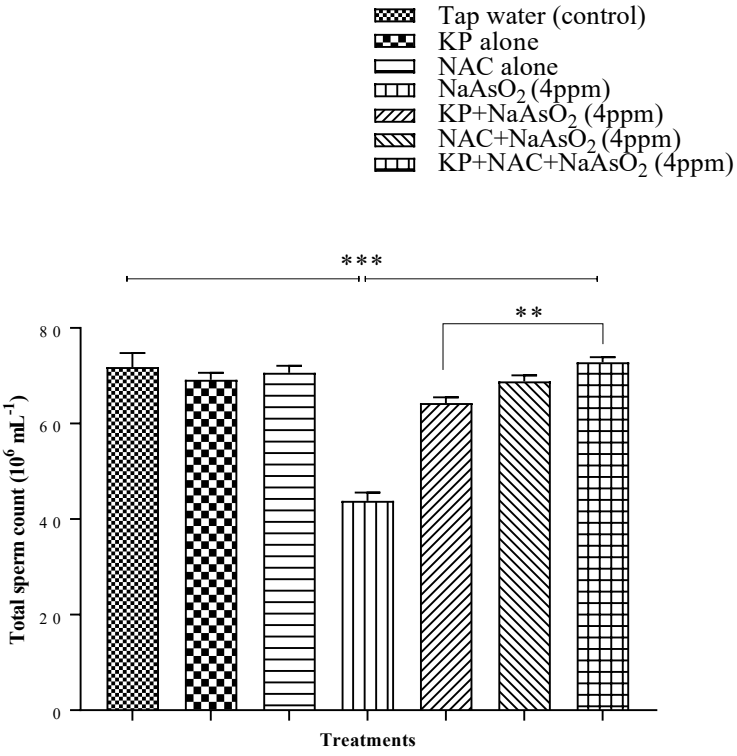


Fig. 3.1 Total sperm count of adult male mice following 35-days exposure to 4 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days). ** $p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups

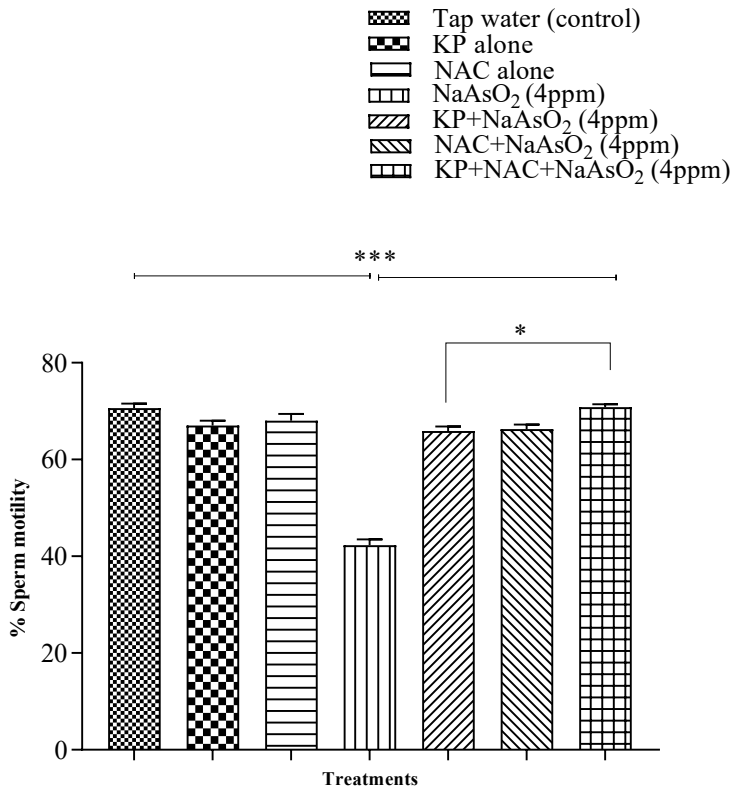


Fig. 3.2 % Sperm motility of adult male mice following 35-days exposure to 4 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days). * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

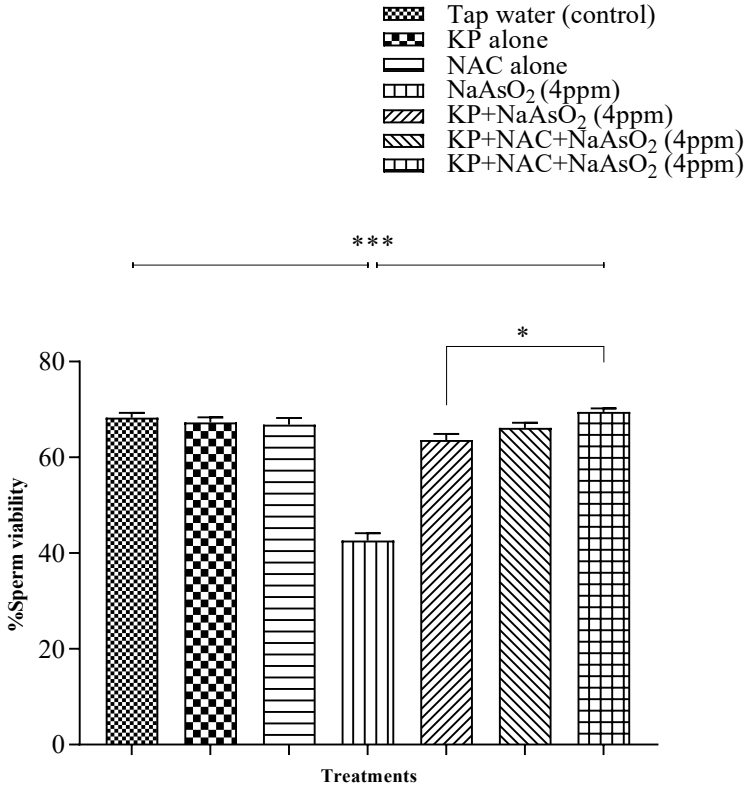


Fig. 3.3 % Sperm viability of adult male mice following 35-days exposure to 4 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days). * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

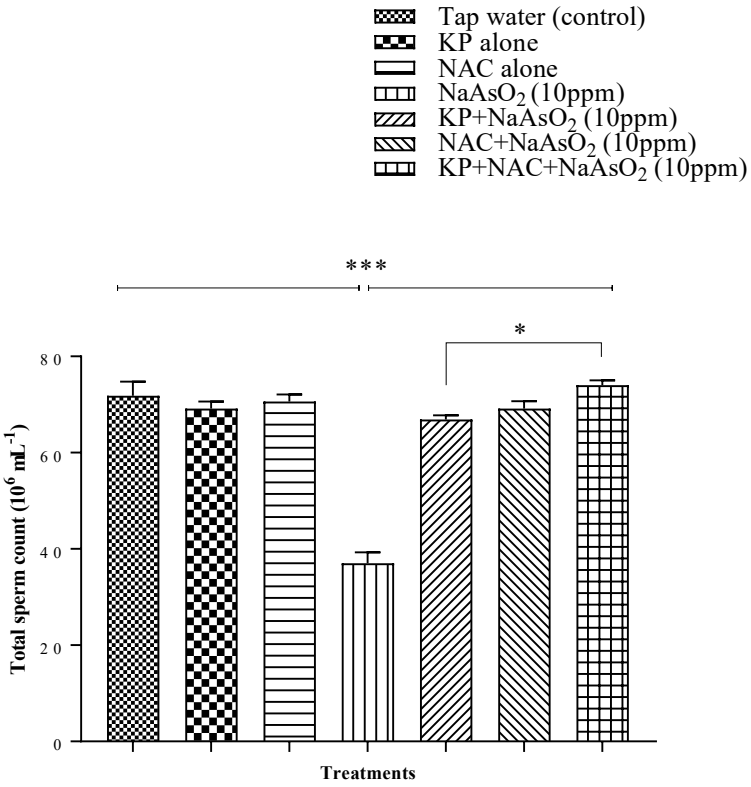


Fig. 3.4 Total sperm count of adult male mice following 35-days exposure to 10 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days). * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

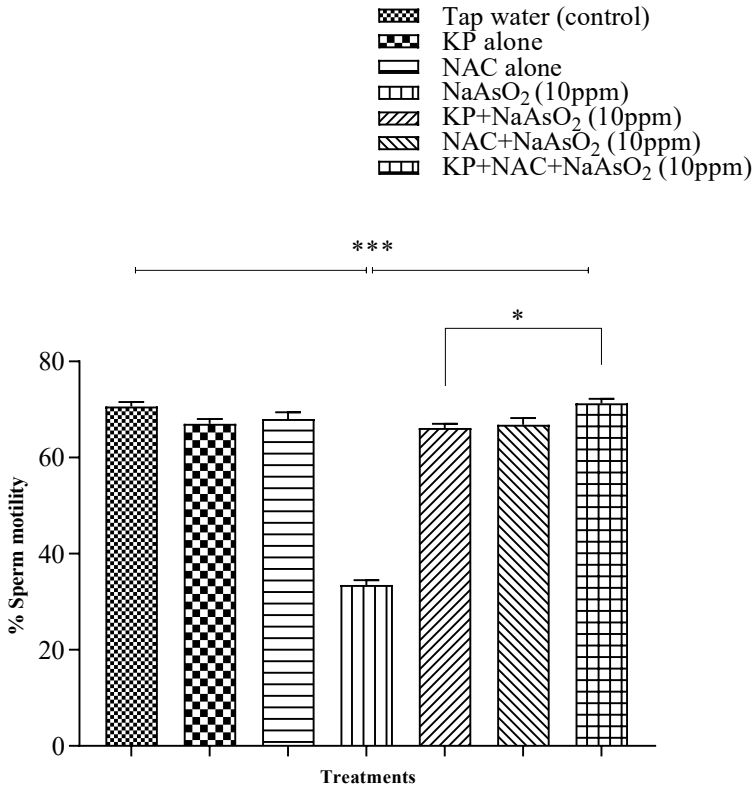


Fig. 3.5 % Sperm motility of adult male mice following 35-days exposure to 10 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days). * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

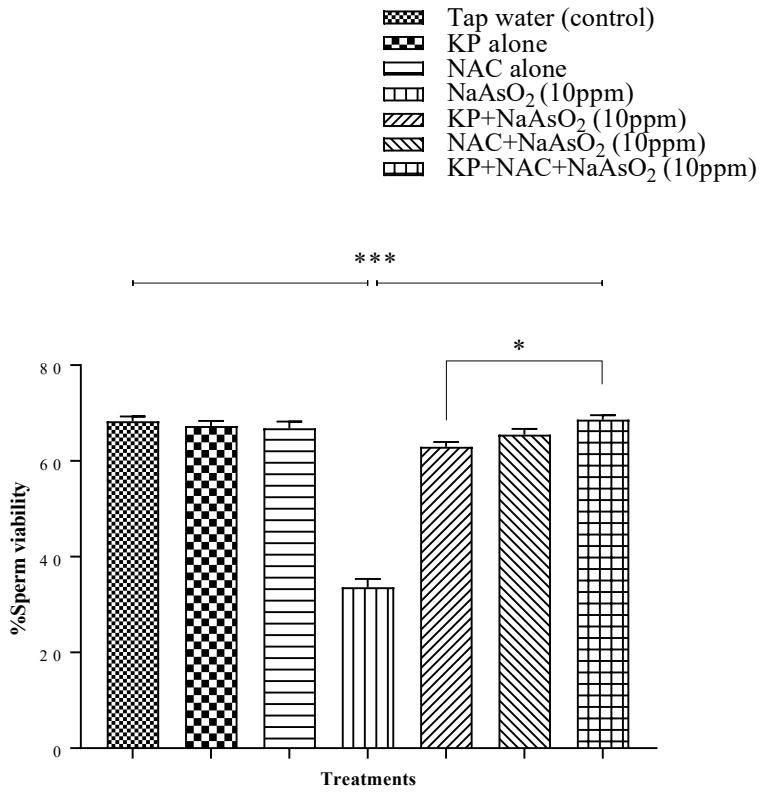


Fig. 3.6 % Sperm viability of adult male mice following 35-days exposure to 10 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days). * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

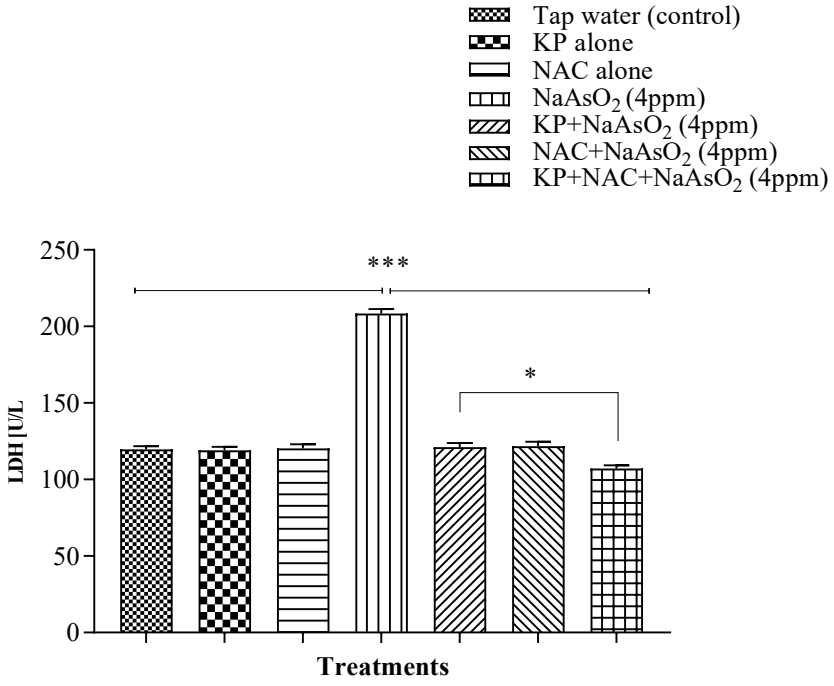


Fig. 3.7 Mean testicular LDH level [U/L] of adult male mice following 35-days exposure to 4 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days). * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

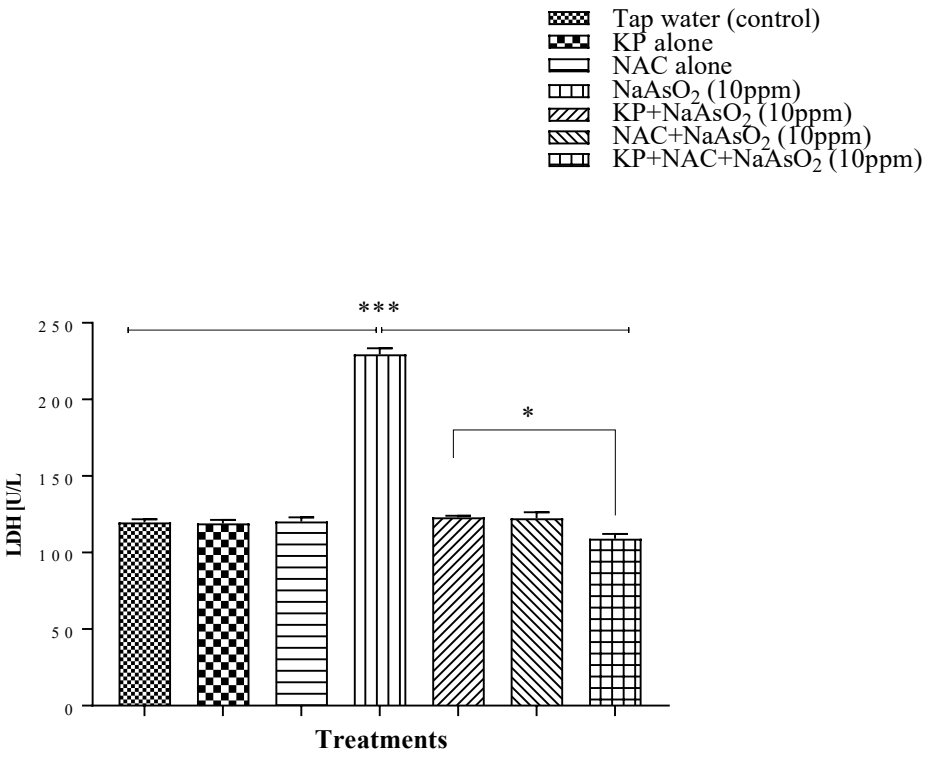


Fig. 3.8 Mean testicular LDH level [U/L] of adult male mice following 35-days exposure to 10 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days). * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

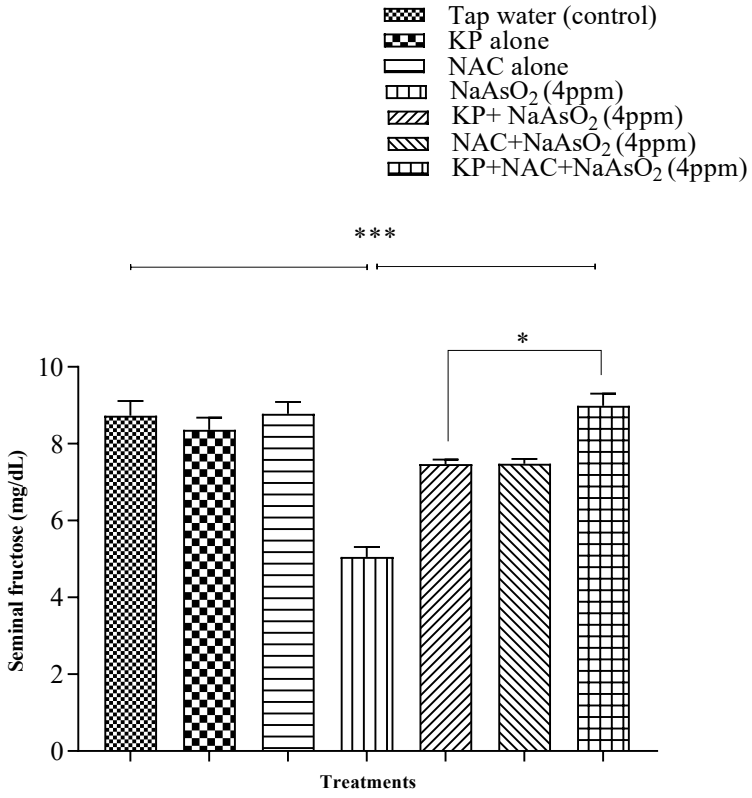


Fig. 3.9 Seminal fructose level (mg/dL) of adult male mice following 35-days exposure to 4 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days). * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

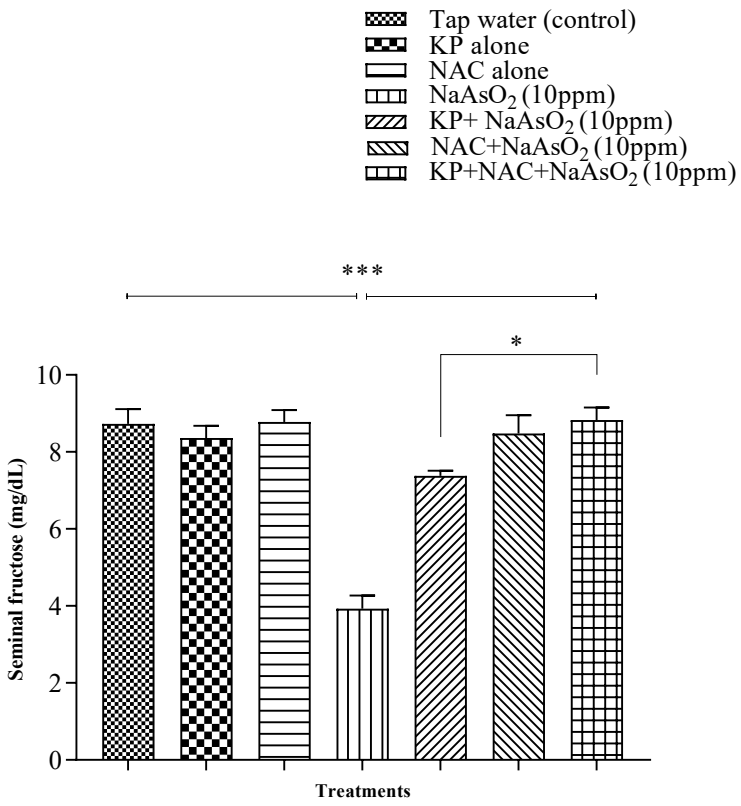


Fig. 3.10 Seminal fructose level (mg/dL) of adult male mice following 35-days exposure to 10 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days). * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

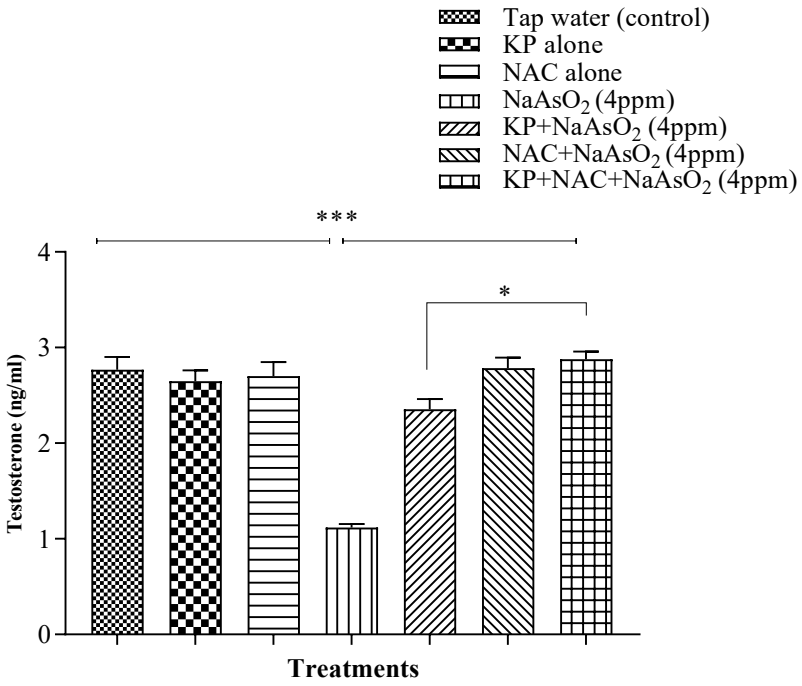


Fig. 3.11 Mean serum testosterone level (ng/ml) of adult male mice following 35-days exposure to 4 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days). * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

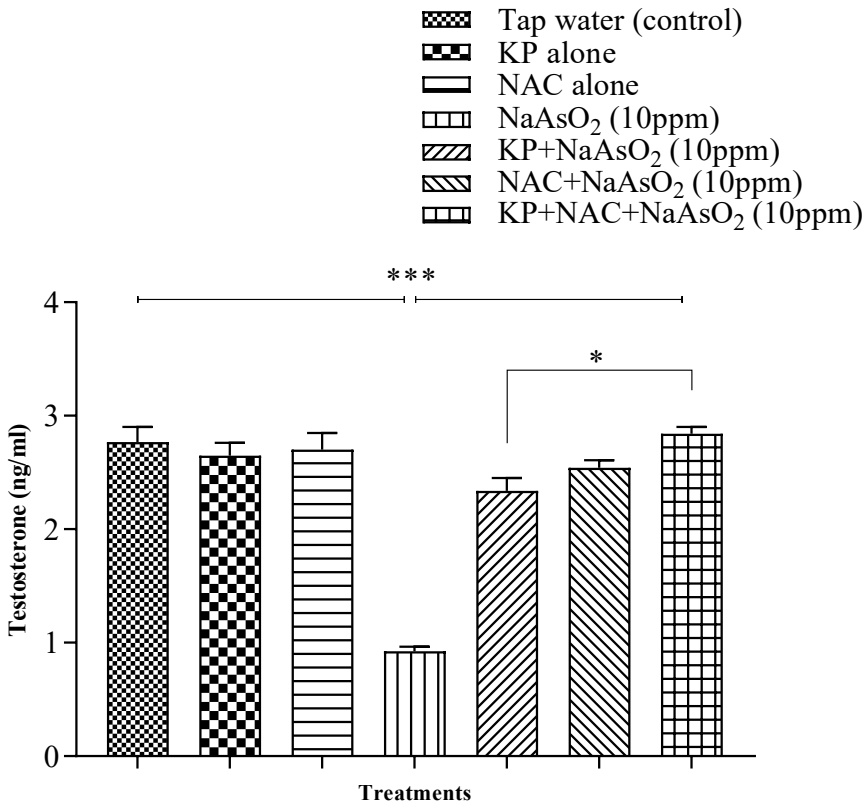


Fig. 3.12 Mean serum testosterone level (ng/ml) of adult male mice following 35-days exposure to 10 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days). * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

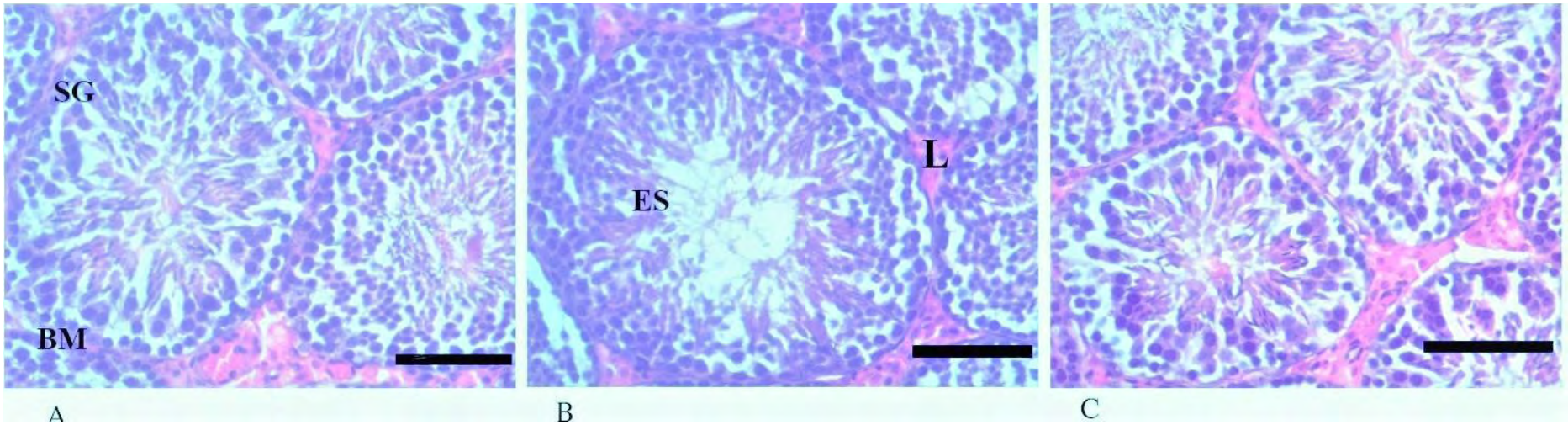


Fig. 3.13 Photomicrographs of testicular sections of adult male mice upon 35 days' exposure to tap water control (A), 50 nmol/day KP-10 once a week (B) 75 mg/kg b.w. NAC for 5 alternative days (C) show normally arranged spermatogonia (SG), elongated spermatids (ES), Leydig cells (L) and basement membrane (BM) Scale bar =50µm

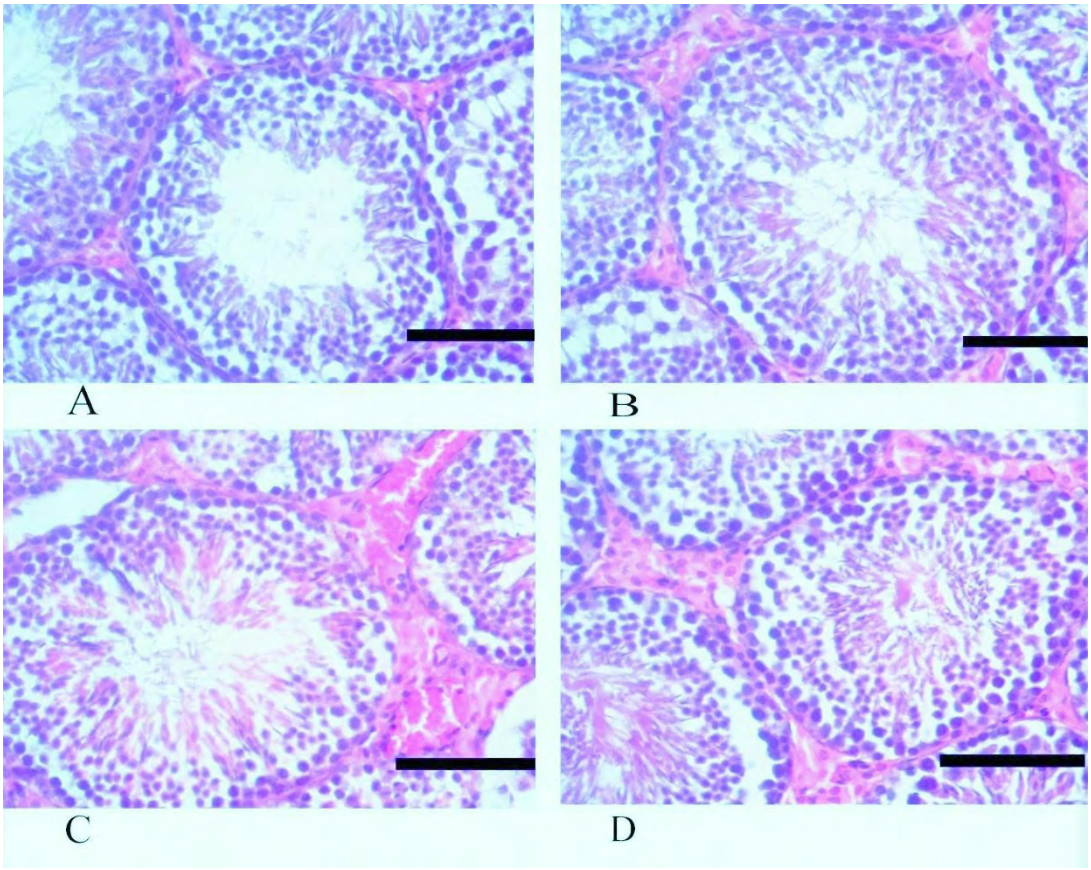


Fig. 3.14 Photomicrographs of testicular sections of adult male mice upon 35 days' exposure to 4 ppm $NaAsO_2$ alone (A) shows abnormally expanded luminal space and prominent germ cell loss; $NaAsO_2$ (4ppm) combined with KP-10 (50 nmol/day once a week) (B) $NaAsO_2$ (4 ppm) combined with NAC (75mg/kg b.w. for 5 alternative days) (C) $NaAsO_2$ (4 ppm) combined with KP-10 and NAC (D) show improvement in luminal space, germ cell distribution and sperm. Scale bar =50 µm

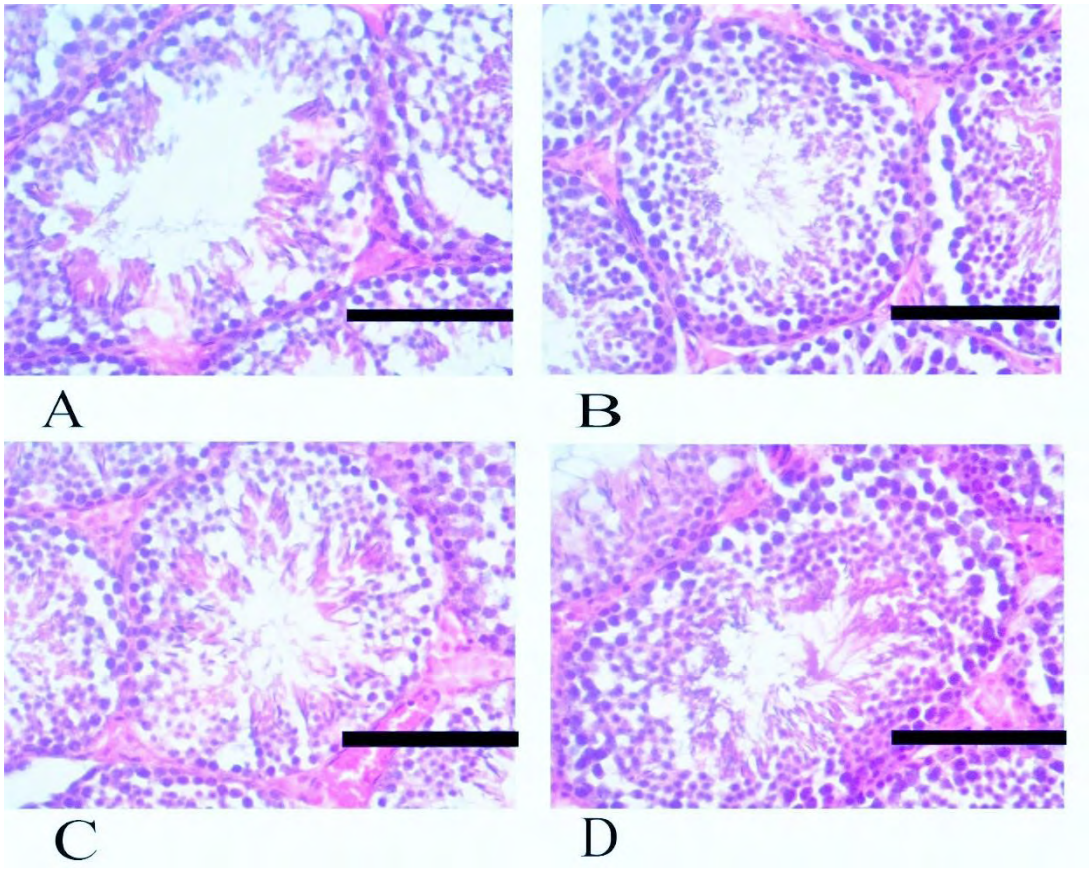


Fig. 3.15 Photomicrographs of testicular sections of adult male mice upon 35 days' exposure to exposure to 10 ppm $NaAsO_2$ alone (A) shows massive germ cell loss and abnormal testicular epithelium; $NaAsO_2$ (10 ppm) combined with KP-10 (50 nmol/day once a week) (B) $NaAsO_2$ (10 ppm) combined with NAC (75mg/kg b.w. for 5 alternative days) (C) $NaAsO_2$ (10 ppm) combined with KP-10 and NAC (D) restored germinal epithelium, lumen, basement membranes and sperm. Scale bar =50 μm

Table 3.1

Body weight and relative organ weights (W/W%) of mice following 35 days exposure to 4 ppm and 10ppm doses of Sodium Arsenite ($NaAsO_2$), Kisspeptin-10 (KP-10) (50 nmol/day once a week) and N acetyl cysteine (NAC) (75mg/kg b.w. for 5 alternative days). Values are mean ± S.E.

| Treatments | Bodyweight(g) | Testes | Epididymides | Seminal vesicles |
|---|---|---|---|---|
| Tap water(control) | $33^{a}$±0.57 | $0.68^{a}$±0.03 | $0.48^{a}$±0.01 | $0.67^{a}$±0.01 |
| KP alone | $32^{a}$±0.98(-3.03) | $0.66^{a}$±0.02(-2.94) | $0.46^{a}$±0.02(-4.16) | $0.66^{a}$±0.03(-1.49) |
| NAC alone | $32^{a}$±0.79 --- | $0.67^{a}$±0.02(1.47) | $0.47^{a}$±0.02(-2.08) | $0.68^{a}$±0.02(1.49) |
| $NaAsO_2$ (4ppm) | $33^{a}$±0.83(0) | $0.54^{b}$±0.02$^{**}$(-20.58) | $0.36^{b}$±0.01$^{**}$(-25) | $0.54^{b}$±0.02$^{**}$(-19.40) |
| $NaAsO_2$ (4ppm)+KP | $32^{a}$±0.83(-3.03) | $0.67^{a}$±0.02(1.47) | $0.46^{a}$±0.01(-4.16) | $0.67^{a}$±0.01(0) |
| $NaAsO_2$ (4ppm)+NAC | $34^{a}$±0.80(3.03) | $0.68^{a}$±0.02(0) | $0.47^{a}$±0.02(-2.08) | $0.67^{a}$±0.02---- |
| $NaAsO_2$ (4ppm)+KP+NAC | $33^{a}$±0.91(0) | $0.67^{a}$±0.01(1.47) | $0.46^{a}$±0.02(-4.16) | $0.65^{a}$±0.01(-2.98) |
| $NaAsO_2$ (10ppm) | $33^{a}$±1.18--- | $0.49^{c}$±0.03$^{***}$(-27.94) | $0.26^{c}$±0.01$^{***}$(-45.83) | $0.40^{c}$±0.02$^{***}$(-40.29) |
| $NaAsO_2$ (10ppm)+KP | $32^{a}$±0.88(-3.03) | $0.67^{a}$±0.02(1.47) | $0.46^{a}$±0.02(-4.16) | $0.66^{a}$±0.01(-1.49) |
| $NaAsO_2$ (10ppm)+NAC | $32^{a}$±1.01--- | $0.67^{a}$±0.01---- | $0.48^{a}$±0.01(0) | $0.67^{a}$±0.02(0) |
| $NaAsO_2$ (10ppm)+KP+NAC | $33^{a}$±1.22(0) | $0.68^{a}$±0.02(0) | $0.47^{a}$±0.01(-2.08) | $0.67^{a}$±0.02---- |

Different superscripts in a column show significant difference at $^{**}p < 0.01$ & $^{***}p < 0.001$. values in parentheses represent %change from Tap water control. (n=6/group)

Table 3.2

Oxidative stress parameters in mouse testicular homogenate following 35-day exposure to 4 ppm dose of Sodium Arsenite ($NaAsO_2$), Kisspeptin-10 (KP-10) (50 nmol/day once a week) and N acetyl cysteine (NAC) (75mg/kg b.w. for 5 alternative days). Values are mean ± S.E.

| Groups | ROS (abs) | TBARS (nM $min^{-1}$ mg $protein^{-1}$) | SOD unit $mg^{-1}$ | POD unit $min^{-1}$ | CAT unit $min^{-1}$ | GSH µM/g |
|---|---|---|---|---|---|---|
| Tap water control | $1.16^{a}$±0.01 | $0.81^{a}$±0.07 | $12.33^{a}$±1.25 | $7.17^{a}$±0.56 | $6.48^{a}$±0.37 | $5.21^{a}$±0.26 |
| KP alone | $1.17^{a}$±0.02 | $0.74^{a}$±0.05 | $12.41^{a}$±0.75 | $6.89^{a}$±0.40 | $6.41^{a}$±0.41 | $5.38^{a}$±0.33 |
| NAC alone | $1.19^{a}$±0.01 | $0.80^{a}$±0.06 | $12.38^{a}$±0.78 | $6.73^{a}$±0.67 | $6.49^{a}$±0.39 | $5.25^{a}$±0.47 |
| $NaAsO_2$ (4ppm) | $1.48^{b}$±0.02*** | $1.31^{b}$±0.01*** | $4.36^{b}$±0.16*** | $2.42^{b}$±0.10*** | $2.64^{b}$±0.32*** | $1.85^{b}$±0.28*** |
| $NaAsO_2$ (4ppm)+KP | $1.20^{a}$±0.01▲ | $0.96^{a}$±0.06▲ | $9.40^{a}$±0.23▲ | $5.92^{a}$±0.26▲ | $5.67^{a}$±0.19▲ | $4.33^{a}$±0.16▲ |
| $NaAsO_2$ (4ppm)+ NAC | $1.18^{a}$±0.02 | $0.80^{a}$±0.05 | $10.59^{a}$±0.83 | $7.56^{a}$±0.59 | $5.88^{a}$±0.23 | $4.55^{a}$±0.15 |
| $NaAsO_2$ (4ppm)+KP+NAC | $1.11^{a}$±0.02▲ | $0.68^{a}$±0.04▲ | $13.54^{a}$±0.57▲ | $8.22^{a}$±0.45▲ | $6.99^{a}$±0.19▲ | $5.79^{a}$±0.33▲ |

Similar superscripts in a column show no significant change from tap water control, letter b presents significant difference from all groups at ***$p < 0.001$ and symbol ▲ presents significant difference between marked groups at $p<0.05$. (n=6/group)

Table 3.3

Oxidative stress parameters in mouse testicular homogenate following 35-day exposure to 10 ppm dose of Sodium Arsenite ($NaAsO_2$), Kisspeptin-10 (KP-10) (50 nmol/day once a week) and N acetyl cysteine (NAC) (75mg/kg b.w. for 5 alternative days). Values are mean ± S.E.

| Groups | ROS (abs) | TBARS (nM $min^{-1}$ mg $protein^{-1}$) | SOD unit $mg^{-1}$ | POD unit $min^{-1}$ | CAT unit $min^{-1}$ | GSH µM/g |
|---|---|---|---|---|---|---|
| Tap water control | $1.16^{a}$±0.01 | $0.81^{a}$±0.07 | $12.33^{a}$±1.25 | $7.17^{a}$±0.56 | $6.48^{a}$±0.37 | $5.21^{a}$±0.26 |
| KP alone | $1.17^{a}$±0.02 | $0.74^{a}$±0.05 | $12.41^{a}$±0.75 | $6.89^{a}$±0.40 | $6.41^{a}$±0.41 | $5.38^{a}$±0.33 |
| NAC alone | $1.19^{a}$±0.01 | $0.80^{a}$±0.06 | $12.38^{a}$±0.78 | $6.73^{a}$±0.67 | $6.49^{a}$±0.39 | $5.25^{a}$±0.47 |
| $NaAsO_2$ (10ppm) | $1.79^{b}$±0.01*** | $1.34^{b}$±0.02*** | $3.46^{b}$±0.42*** | $1.94^{b}$±0.22*** | $1.63^{b}$±0.19*** | $1.25^{b}$±0.15*** |
| $NaAsO_2$ (10ppm)+KP | $1.19^{a}$±0.01▲ | $0.96^{a}$±0.04▲ | $10.08^{a}$±0.56▲ | 5.57±0.18▲ | $5.60^{a}$±0.15▲ | $4.27^{a}$±0.13▲ |
| $NaAsO_2$ (10ppm)+NAC | $1.17^{a}$±0.02 | $0.73^{a}$±0.05 | $12.16^{a}$±0.87 | 6.30±0.49 | $6.70^{a}$±0.17 | $5.12^{a}$±0.28 |
| $NaAsO_2$ (10ppm)+KP+NAC | $1.11^{a}$±0.01▲ | $0.71^{a}$±0.04▲ | $13.91^{a}$±1.00▲ | 7.83±0.59▲ | $6.99^{a}$±0.10▲ | $5.61^{a}$±0.31▲ |

Similar superscripts in a column show no significant change from tap water control, letter b presents significant difference from all other groups at ***$p < 0.001$ and symbol ▲ presents significant difference between marked groups at $p<0.05$. (n=6/group)

Table 3.4

Morphometric measurements of seminiferous tubules of mice testes following 35 days exposure to 4 ppm and 10 ppm doses of Sodium Arsenite ($NaAsO_2$), Kisspeptin-10 (KP-10), (50 nmol/day once a week) and N acetyl cysteine (NAC) (75mg/kg b.w. for 5 alternative days). Values are Mean ±S.E.

| Treatments | Tubular diameter (µm) | Lumen diameter (µm) | Epithelial height (µm) |
|---|---|---|---|
| Tap water(control) | $201.16^{a}$±3.11 | $47.83^{a}$±3.44 | $82.66^{a}$±1.96 |
| KP alone | $202.50^{a}$±3.81 | $48.50^{a}$±3.33 | $82.00^{a}$±2.80 |
| NAC alone | $201.83^{a}$±3.89 | $49.16^{a}$±3.23 | $80.66^{a}$±2.91 |
| $NaAsO_2$ (4ppm) | $204.16^{a}$±2.56 | $68.83^{b}$±1.40*** | $53.16^{b}$±2.73*** |
| NaAsO2 (4ppm)+KP | $203.83^{a}$±3.66 | $50.16^{a}$±1.20 | $72.33^{a}$±2.37 |
| NaAsO2 (4ppm)+NAC | $203.66^{a}$±4.06 | $48.66^{a}$±7.03 | $74.66^{a}$±2.85 |
| $NaAsO_2$ (4ppm)+KP+NAC | $204.83^{a}$±4.65 | $50.66^{a}$±3.32 | $72.00^{a}$±2.48 |
| $NaAsO_2$ (10ppm) | $205.50^{a}$±2.74 | $72.33^{c}$±1.45*** | $44.33^{c}$±2.96*** |
| NaAsO2 (10ppm)+KP | $208.83^{a}$±3.54 | $49.66^{a}$±2.51 | $72.16^{a}$±2.38 |
| NaAsO2 (10ppm)+NAC | $205.16^{a}$±4.55 | $51.16^{a}$±1.74 | $74.83^{a}$±2.21 |
| $NaAsO_2$ (10ppm)+KP+NAC | $202.83^{a}$±2.95 | $50.83^{a}$±2.13 | $73.83^{a}$±1.88 |

Different superscripts in a column show significant difference at ***$p < 0.001$. (n=30 sections/group)

## 3.5 Discussion

Outcomes of our previous experiment (Chapter 2) suggested that kisspeptin may bear the antioxidant properties or somehow activates the antioxidant defense mechanisms which prevent the oxidative damage in testicular tissue caused by arsenic toxicity in adult mice. Considering the need for comprehensive evaluation and further verification of these observations, a comparative study had been conducted incorporating the N acetyl cysteine (NAC) which has long been known due to its antioxidant properties against several types of stressors including arsenic. Previous treatment plan of KP-10 dosing was also modified on the basis of obtained results as both intermittent and continuous administration proved their efficacy against 35-days exposure to 4ppm and 10ppm doses of $NaAsO_2$. Therefore, to limit the unnecessary physical stress for experimental animals, only once a week intermittent intraperitoneal administration was selected for current experiment.

Experimental mice were supplemented with KP-10 and NAC through intraperitoneal injections along with exposure to $NaAsO_2$ via drinking water. In addition to separate administration of both potential therapeutics, their combined treatment was also provided for evaluation of their synergistic effect against arsenic-induced testicular toxicity. The obtained results revealed the decrease in ROS and TBARS, increase in the activities of antioxidant enzymes including SOD, POD, CAT and that of non-enzymetic GSH, restoration of circulating testosterone and other biochemical parameters including testicular LDH and seminal fructose levels within normal range upon supplementation with KP-10 and NAC in treatment groups exposed to both 4ppm and 10ppm doses of $NaAsO_2$. Concomitantly, sperm parameters including total sperm count, progressive sperm motility and sperm viability also demonstrated significant improvement. Besides, histological and morphometric evaluation of testicular sections further exhibited the germ cell protection and replenishment within seminiferous tubules thus preventing the reproductive dysfunction.

Apparently, both KP-10 and NAC prevented the toxic effects of $NaAsO_2$ alone exposure which included decreased tissue indices, increased ROS, TBARS and testicular LDH levels, decreased activities of SOD, POD, CAT and massive germ cell depletion within seminiferous tubules. Importantly, synergistic effect of concomitant administration of KP-10 and NAC against $NaAsO_2$ exposure at both 4ppm and 10ppm

doses was significantly greater than KP alone supplementation along with $NaAsO_2$ exposure.

Similar to our previous findings (Chapter 2), no significant difference was observed in the body weights of control and treatment groups. However, relative weights of testes, epididymides and seminal vesicles demonstrated significant decline upon exposure to 4pmm and 10 ppm doses of $NaAsO_2$. However, both alone and combined supplementation with KP-10 and NAC restored the relative organ weights near to control thus indicating their protective effects against arsenic toxicity. These findings are in agreement with previous study performed by Reddy et al., (2011) which demonstrated similar decrease in testicular weights along with other reproductive parameters of adult male mice including serum testosterone level, sperm count, motility and viability upon 35-days exposure to 4ppm dose of $NaAsO_2$. Significant improvement was however observed in these parameters upon intraperitoneal administration of NAC for five alternative days along with $NaAsO_2$ exposure. Likewise, similar effects of KP-10 have been previously reported against both 4ppm and 10ppm doses of $NaAsO_2$ in adult male mice (Fatima and Qureshi, 2022). Taking into account the previously reported protective effects of KP-10 and NAC against arsenic-induced testicular toxicity, currently observed synergistic effect of KP-10 and NAC against arsenic-induced toxic effects can be well-justified.

Similar to our previous study, tissue damage caused by arsenic toxicity was also indirectly assessed in current experiment via measurement of testicular LDH level which is considered a standard biochemical biomarker of several diseases (Klein et al., 2020). Current findings also revealed significantly elevated testicular LDH levels in mice exposed to both 4 &10 ppm doses of $NaAsO_2$ alone. KP-10 and NAC supplementation however equally prevented this increase and maintained the LDH levels near control. Furthermore, concomitant supplementation of KP-10 and NAC was found to be more efficient in lowering the LDH level as compared to KP-10 alone supplementation with $NaAsO_2$ exposure. This can be reasoned as being a strong antioxidant, NAC has been well-studied for its protective effects against testicular toxicity induced by heavy metals including arsenic (Reddy et al., 2011) cadmium, and lead (Kumar et al., 2013). Kumar et al. (2013) have reported the significant decline in testicular LDH level upon NAC supplementation in rats treated with respective heavy metals. Therefore, protective effects of both NAC and KP-10 could have been

cumulated upon combined supplementation with $NaAsO_2$ exposure during current experiment. Although underlying mechanisms mediating these effects may vary for KP-10 and NAC which need further investigation.

The results obtained in current comparative study reveal the histoprotective effects of NAC supplementation along with $NaASO_2$ exposure. These include significant improvement in testicular histology demonstrated by restored germ cell population, normal arrangement of basement membranes and germ cell layers within seminiferous tubules. Similar observations were noticed upon KP-10 supplementation. Moreover, Histomorphometry of testicular sections also showed normalization of epithelial heights and luminal diameters to near control upon supplementation with both KP-10 and NAC. However, no difference was observed between combined or alone supplementation of NAC and KP-10 along with $NaAsO_2$ exposure.

Likewise, Khalil et al., (2021) have recently compared the protective effects of NAC and melatonin (also a neuropeptide like KP-10) against obesity-induced testicular functions in adult rats which revealed significant improvement in hormonal, spermatological and histological parameters etc. Both melatonin and NAC supplementation were found to equally enhance the testicular architecture in treated rats.

Although reason for selecting KP-10 for our study as a potential therapeutic agent against arsenic-induced metal toxicity has been previously explained (Chapter 2). However, the outcomes of above mentioned recent study provide further support for investigating the mitigating potential of KP-10 and its comparison with a known therapeutic agent i.e. NAC. Further evaluation regarding the pathway mediating protective effects of KP-10 is still needed.

As regards the KP-10 treatment plan, continuous administration of KP-10 was excluded from current experiment. As mentioned before, both intermittent and continuous administration revealed similar results thus increasing the interventional stress for experimental mice seemed unnecessary. Additionally, the risk of receptor desensitization was also minimized by employing intermittent KP-10 administration for 35-days. Previously, twice a week administration (Jayasena et al., 2010) of kisspeptin has been found to be more efficient in maintaining LH secretion for longer duration as compared to twice a day dosing (Jayasena et al., 2009) in women with

hypothalamic amenorrhoea. Therefore, continuous stimulation by exogenous KP-10 seems unnecessary for the current experimentations.

Like our previous experiment, KP-10 and NAC alone treatments revealed no significant difference in spermatological, biochemical, hormonal and histological parameters not only from tap water control but also from each other. These included un-altered levels of oxidative stress parameters, testicular LDH and seminal fructose levels. In addition, serum testosterone level and tissue histology also revealed no significant change upon either KP alone or NAC alone administration in mice provided with drinking tap water. These findings are in agreement with our previous results and findings reported by Reddy et al. (2011) who found no significant change in respective parameters upon NAC alone treatment when compared to control.

**Conclusion**

As previously mentioned, KP-10 is an endogenous neuropeptide which regulates puberty onset and reproductive functions (Tena-Sempere, 2006), is also found to exhibit antioxidant properties which have been reported in previous studies (Aydin et al., 2010; Güvenç and Aksakal 2018). NAC is another well-known therapeutic agent with established antioxidant effects at an advanced molecular level (Sun et al., 2014). However, molecular assessment of KP-10 via evaluation of oxidative stress related genes such as caspases and its effect on expression of steroidogenesis regulating genes could further assist in thorough comprehension of our previous and current results which reveal the protective effect of KP-10 against arsenic-induced deleterious effects on male reproductive organs of mice.

**Abstract**

Mitigating role of the both KP-10 and NAC against arsenic-induced toxic effects on male reproductive system is evident through previous *in vivo* literature. However, no such study using organ culture system has been reported so far. Additionally, reports regarding comparative evaluation of the effects of these therapeutic agents through *in vitro* experiments are lacking. Presently, equally sliced fragments of mice testes were incubated with culture media containing $NaAsO_2$ (100µM) alone and supplemented with either alone or combined doses of KP-10 (1µM) and NAC (50µM) for 2 and 24 h. Whereas, treatment free culture media served as control for current experiments. Each respective testicular culture was later evaluated for biochemical, hormonal and histological parameters. Current results revealed that both KP and NAC supplemented groups equally decreased ($p<0.001$) the testicular oxidative stress level by reducing ROS and TBARS while increasing ($p<0.001$) the activities of antioxidant enzymes; SOD, POD, CAT and non-enzymatic GSH after 2 and 24 h. of incubation, when compared with $NaAsO_2$ alone exposure. Significant decline ($p<0.001$) in testicular LDH in supplemented groups indicated KP and NAC- induced prevention of tissue damage inflicted by arsenite toxicity as compared to $NaAsO_2$ alone treatment. In addition, synergistic effect of combined treatment with KP and NAC was significantly greater ($p<0.05$) for these biochemical parameters as compared to KP alone supplementation along with $NaAsO_2$ treatment for both 2 and 24 h. of exposure.

Likewise, histological evaluation of testicular cultures also demonstrated significant improvement in the tubular architecture upon alone and combined (KP+NAC) treatment along with $NaAsO_2$ exposure at 2 and 24 h. Whereas, tissue fragments incubated with $NaAsO_2$ alone revealed decreased germ cell population and expanded lumens within seminiferous tubules. Furthermore, prolonged incubation for 24 h. caused comparatively more tissue damage as compared to 2 h. exposure. Testicular testosterone level also decreased significantly upon incubation with $NaAsO_2$ alone for both 2 ($p<0.05$) and 24 ($p<0.001$) h. when compared with $NaAsO_2$ alone group. While normalization to near control was observed upon supplementation with both KP and NAC, either alone or in combination along with $NaAsO_2$ treatment. This is the first comparative study which evaluated the kisspeptin-10 and N acetyl cysteine for their hormonal, biochemical and histoprotective effects against toxic effects of trivalent arsenic on mice testicular cultures.

## 4.1 Introduction

*In vivo* assessment of male reproductive toxicity caused by known toxicants is limited, as it is time consuming and requires large quantities of compounds to be tested (Nakamura et al., 2018). *In vitro* models have been therefore alternatively introduced which either employ use of isolated testicular cell culture (Yin et al., 2017) or organ culture techniques (Steinberger and Steinberger, 1970). Additionally, it has been suggested that known toxicants evaluated through *in vivo* models should also be tested through these culture models for better comparisons thus emphasizing the significance of these methods in evaluating the male reproductive toxicity (Nakamura et al., 2018).

Toxic effects of arsenic poisoning on various physiological functions have long been investigated using both *in vivo* and *in vitro* model systems. Arsenic-induced reproductive toxicity however lacks comprehensive *in vitro* evaluation. Recent reports on *in vitro* effects of sodium arsenite on pre-pubertal and adult mice testicular cultures suggest further comprehensive evaluation (Anwar and Qureshi, 2019; Anwar et al., 2020).

As regards the remedial studies, outcomes of previous *in vivo* investigations have frequently reported the therapeutic potential of various natural compounds including vitamin C (Im Chang et al., 2007), Quercetin (Baltaci et al., 2016) and N-acetyl cysteine (NAC) (Reddy et al., 2011) against arsenic-induced testicular toxicity in rats and mice. However, data regarding comparative experiments using testicular cultures for both short and long incubation periods are scant.

N-acetyl cysteine, which is a well-known antioxidant and found to be potent against sodium arsenite-induced reproductive toxicity in adult male mice (Reddy et al., 2011), needs to be further evaluated through *in vitro* experiments. Recently, Baetas et al. (2019) have reported the protective effect of NAC in human sperms incubated with etoposide drug which is used in chemotherapy of testicular cancers and is known to exert cytotoxic effects on testes. Incubation with 50µM NAC pre- and post- etoposide treatment revealed increased GSH levels confirming the antioxidant properties of NAC. However, differential effect of pre- and post- treatments on sperm DNA fragmentation requires further testing of NAC in combination with other antioxidants. As regards arsenite toxicity, protective effect of NAC has been found in cells from anterior pituitary glands of rats which were first incubated for 2 h. with NAC (20nmol/L)

followed by sodium arsenite (25µmol/L) for 6 and 24 h. without NAC. These *in-vitro* experiments fully prevented the increased expression of oxidative stress biomarker genes including HMOX1 and MT1 (Ronchetti et al., 2016).

Likewise, KP-10 which has been recently reported as a potent counteracting agent against arsenic-induced testicular damage in adult mice (Fatima and Qureshi, 2022), should also be tested on testicular cultures exposed to sodium arsenite doses. Considering these earlier findings and previous *in vivo* results (Chapter 3), further *in vitro* experiments were conducted for the first time to verify not only the antioxidant effect of KP-10 through biochemical analyses but also to evaluate its direct stimulatory/compensatory effect on testosterone secretion in the presence of $NaAsO_2$ which is an endocrine disruptor and strong pro-oxidant. In addition, protective effect of NAC against arsenic-induced reproductive toxicity will be further verified and compared through alone and combined treatment with KP-10.

## 4.2 Materials and methods

### 4.2.1 Animals and maintenance

Adult male Swiss albino mice were obtained from National Institute of Health (NIH), Islamabad and maintained in the animal house facility of Quaid-i-Azam university under standard laboratory conditions (temperature 25±4 °C, photoperiod of 12:12 Light: Dark hours and 40% humidity). They were given free access to standard rodent chow and fresh drinking water *ad libitum.*

### 4.2.2 Chemicals

KP-10 (KiSS-1; 112-121; H-YNWNSFGLRF-$NH_2$) was obtained as lyophilized powder from Calbiochem (La Jolla, CA, USA). Sodium pentobarbital (Sigma, Germany) was purchased locally. Inorganic sodium arsenite ($NaAsO_2$), N acetyl cysteine (NAC), Pencillin and Streptomycin were purchased from Sigma-Aldrich (Germany). Dulbecco's modified eagle's medium/Ham F12 (DMEM/Ham F12) was purchased from Caisson labs, CA, USA.

### 4.2.3 Dose selection and preparation

Doses of KP-10 (Mei et al., 2013**)** NAC (Baetas et al., 2019) and $NaAsO_2$ (Muller et al., 1986; Anwar and Qureshi, 2019) were selected considering the previous *in vitro* investigations. For both $NaAsO_2$ and NAC, stock solutions (0.05M) were prepared in distilled water and required concentrations of 100µM and 50µM respectively were obtained through serial dilutions in DMEM/Ham F12 culture medium. Whereas, 1mg/ml stock solution of lyophilized KP-10 was prepared in DMSO and final doses of 1µM concentration were prepared using culture medium. Doses were freshly prepared and immediately used for each set of experiment.

### 4.2.4 Testes preparation for organ culture

*In vitro* evaluation of biochemical, histological and hormonal parameters was performed using organ culture according to Moundipa et al. (2006) and Freyberger et al. (2010). For this purpose, testes were dissected out and thoroughly rinsed with physiological saline. These were then de-capsulated and cut into four equal pieces. Each piece was randomly placed into already labelled and filled culture tube with 2ml of culture media containing respective doses and supplemented with penicillin (50IU/ml) and streptomycin (50µg/ml).

### 4.2.5 Experimental design

Eighteen adult male mice were used for obtaining testes for organ cultures. In each culture set up, at least six mice were dissected to obtain testes which were further cut into 4 equal pieces (6 × 2 testes × 4 pieces = 48 pieces). Culture tubes were labelled for following seven treatment groups (6 tubes/ group). Each piece was separately placed in single culture tube accordingly. three replicates were run for each treatment.

1. Control having treatment free culture media
2. KP-10 (1μM) alone prepared in culture media
3. NAC alone (50 μM) prepared in culture media
4. $NaAsO_2$ (100 μM) alone prepared in culture media
5. $NaAsO_2$ containing media supplemented with KP-10
6. $NaAsO_2$ containing media supplemented with NAC
7. $NaAsO_2$ containing media supplemented with KP + NAC

### 4.2.6 Incubation and tissue collection

Two sets of *in vitro* experiments were performed depending on the incubation time period 2 or 24 h. For each experiment, prepared culture tubes were placed in a humidified incubator at 5% $CO_2$ in air at 37 $^{\circ}$C for desired duration. Following respective incubation period, culture media were removed and testicular fragments were washed with physiological saline. These fragments were further processed for both histology and homogenate preparation.

### 4.2.7 Fragment histology

Testicular tissues from each treatment group were fixed in 10% buffered formalin and processed for standard histology. Briefly, these were dehydrated in ascending ethanolic grades, cleared in xylene and embedded in molten paraffin wax prewarmed to 56 °C. Thick sections (5 μm thickness) were cut on a rotary microtome (Shanden, Finesse, Italy). Sections were then dewaxed and rehydrated with descending grades of alcohol and then stained with conventional Hematoxylin and Eosin (H & E) stains, washed in tap water, dehydrated again and cover slipped with DPX (BDH, Germany) mountant medium. Sections were photographed on a light microscope (OLYMPUS CX41, Japan) with an attached camera (Canon, Japan) at 40x magnification. Photo plates were prepared using the Adobe Photoshop (ver. 7. 0, Microsoft, Inc. USA). Numeric

evaluation through Histomorphometry was avoided due to the reason that testicular explants were decapsulated before incubation followed by fixation. Therefore, if morphometry was performed on such photomicrographs, results would be ambiguous.

### 4.2.8 Homogenate preparation

Testicular tissues were homogenized in 1 ml phosphate buffered saline (PBS) and centrifuged at 12204 *g* for 10 min. Supernatants were collected and stored at –20 °C to conduct later the biochemical assays.

### 4.2.9 Oxidative stress biomarkers, antioxidant enzymes and non-enzymatic GSH

Testicular homogenates were prepared according to the methods as described (Anwar and Qureshi, 2019), for the assessment of reactive oxygen species (ROS) and lipid peroxidation (TBARS), levels of reduced glutathione (GSH) and antioxidant enzymes, the superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT).

#### 4.2.9.1 Lactate dehydrogenase (LDH)

Testicular LDH levels were determined from stored supernatants using a commercial LDH kit (AMP, diagnostics, Austria) and following the protocol given therein by the manufacturer.

#### 4.2.9.2 Determination of testosterone concentrations

Intra-testicular testosterone levels were determined through enzyme linked immunosorbent assay (ELISA) using the commercial kit (Bioactive, Germany). Inter and intra-assay coefficients of variation were respectively 6.4% and 3.8%. The absorbance of control and samples was read at 450 nm.

#### 4.2.9.3 Statistical analysis

Data were analyzed statistically using the software GraphPad Prism (version 5.0; San Diego, California, USA). One-way analysis of variance followed by Tukey's multiple comparison test was applied to compare the difference between the means of control and treatment groups. The level of significance was $p<0.05$ & $p<0.001$. Data are expressed as mean ± standard error of mean (s.e).

## 4. 4. Results

### 4.4.1 Biochemical analyses

#### 4.4.1.1 Testicular lactate dehydrogenase (LDH)

Significantly increased ($p<0.001$) LDH levels were observed in testicular cultures upon incubation with $NaAsO_2$ alone (100 µM) containing media for both 2 (Fig. 4.1) and 24 h. (Fig. 4.2) However, both alone and combined supplementation with KP-10 (1 µM) and NAC (50 µM) effectively lowered the LDH levels near treatment free control. Additionally, significant decrease ($p<0.05$) in testicular LDH was observed when $NaAsO_2$ containing media were concomitantly supplemented with both KP-10 and NAC as compared to KP alone supplementation. Whereas, no significant change from control was observed in testicular LDH level in KP alone and NAC alone treatment groups following 2 and 24 h. incubation.

#### 4.4.1.2 Oxidative stress biomarkers and antioxidant enzymes

Testicular cultures incubated with $NaAsO_2$ alone (100 µM) for 2 and 24 h. demonstrated significant increase ($p<0.001$) in ROS (Fig. 4.3, Fig. 4.4) and TBARS concentrations (Fig. 4.5, Fig. 4.6). Whereas, activities of antioxidant enzymes including SOD (Fig. 4.7, Fig. 4.8) POD (Fig. 4.9, Fig. 4.10) and CAT (Fig. 4.11, Fig. 4.12) were found significantly declined ($p<0.001$) when compared to treatment free control group. Whereas, treatment with either alone or combination of KP and NAC following both 2 and 2 hrs. of incubation along with $NaAsO_2$ exposure significantly decreased ($p<0.001$) ROS and TBARS concentrations while increased ($p<0.001$) the activities of SOD, POD and CAT. However, combined supplementation was found to be more efficient ($p<0.05$) in lowering the oxidative stress level when compared to KP-alone treatment along with $NaAsO_2$ exposure for respective time durations. Furthermore, KP and NAC alone treated testicular cultures showed no significant change in the oxidative stress parameters from treatment free control.

#### 4.4.1.3 Reduced glutathione (GSH)

Similar to antioxidant enzymes, non-enzymatic GSH was also significantly decreased ($p<0.001$) upon 2 (Fig. 4.13) and 24 h. (Fig. 4.14) exposure to $NaAsO_2$ (100 µM) alone. Whereas, supplementation with KP (1µM) and NAC (50µM) significantly improved the GSH level to near treatment free control group. In addition, significant difference

($p<0.05$) was observed between GSH levels of alone KP and NAC + KP combined supplemented groups along with $NaAsO_2$ treatment. While, testicular cultures incubated with KP and NAC alone in $NaAsO_2$-free media revealed no significant change in GSH levels as compared to control group.

### 4.4.2 Testicular testosterone

Intra-testicular testosterone level was significantly decreased in cultures incubated with $NaAsO_2$ (100µM) containing media for both 2 (Fig. 4.15) and 24 h. (Fig. 4.16). Although 2 h. exposure with $NaAsO_2$ resulted in significantly declined ($p<0.05$) testosterone levels, however more profound effect ($p<0.001$) was observed following 24 h. treatment. Whereas, $NaAsO_2$ treated groups supplemented with either alone or combination of KP and NAC, demonstrated no such change from treatment free control. Likewise, incubation with both KP and NAC alone in $NaAsO_2$ free media also demonstrated no significant change following 2 and 24 h. exposure. Moreover, combined treatment with KP + NAC along with $NaAsO_2$ exposure resulted in significantly increased ($p<0.05$) testosterone level as compared to KP alone supplementation for 24 h. However, no such difference was observed in cultures incubated for 2 hr.

### 4.4.3 Histology

Testicular fragments incubated with treatment free control, KP alone (1 µM) and NAC alone (50 µM) for both 2 (Fig. 4.17) and 24 (Fig. 4.19) h. revealed normal luminal spaces within seminiferous tubules with abundant germ cell population. In contrast, exposure to 100 µM concentration of $NaASO_2$ alone for 2 and 24 h. resulted in expanded tubular lumens with loss of spermatocytes and abnormally arranged spermatogonia along the basement membranes (Fig. 4.18 A, Fig. 4.20 A). Whereas, supplementation with KP and NAC alone (Fig. 4.18 B, C; Fig. 4.20 B, C) significantly prevented the germ cell loss. Likewise, KP+NAC combined treatment also improved the tubular histology (Fig. 4.18 D; Fig. 4.20 D).

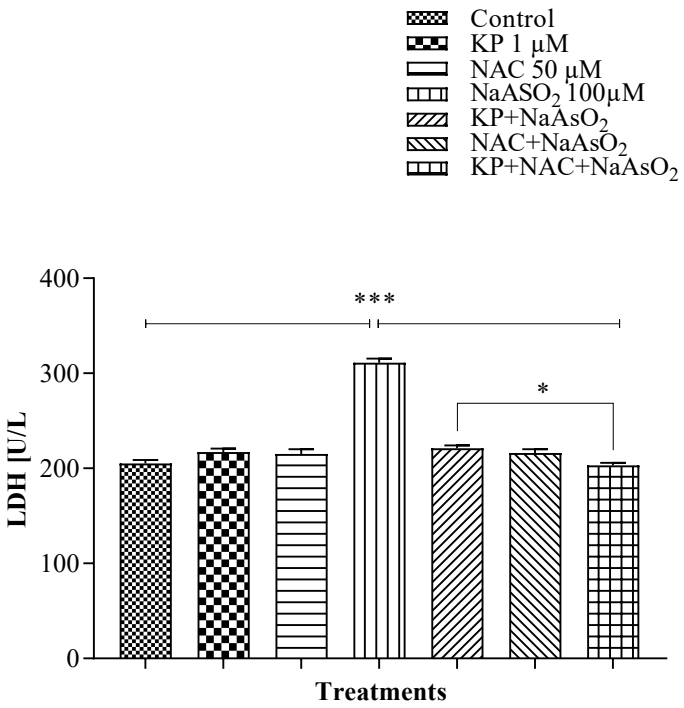


Fig. 4.1 Mean testicular LDH concentration following 2 hr. incubation with $NaAsO_2$, KP-10 and NAC doses. * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

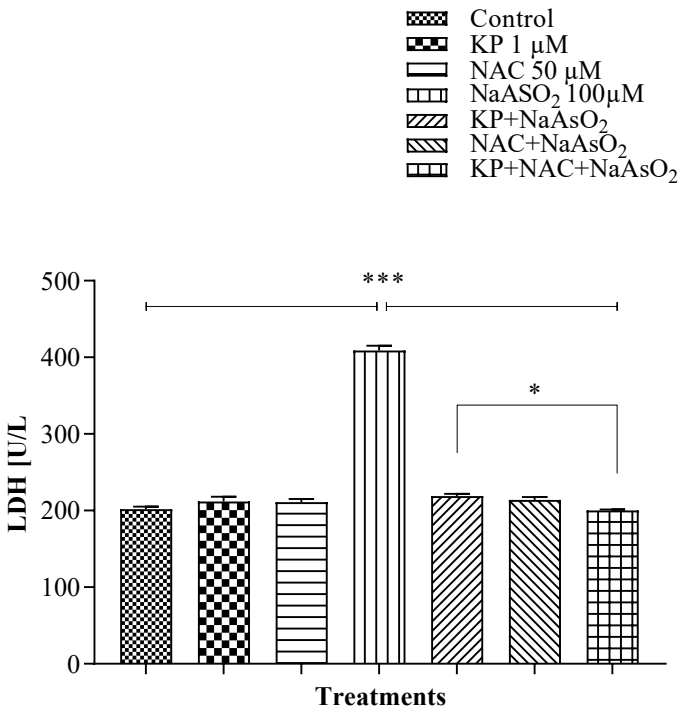


Fig. 4.2 Mean testicular LDH concentration following 24 hr. incubation with $NaAsO_2$, and KP-10 and NAC doses. * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

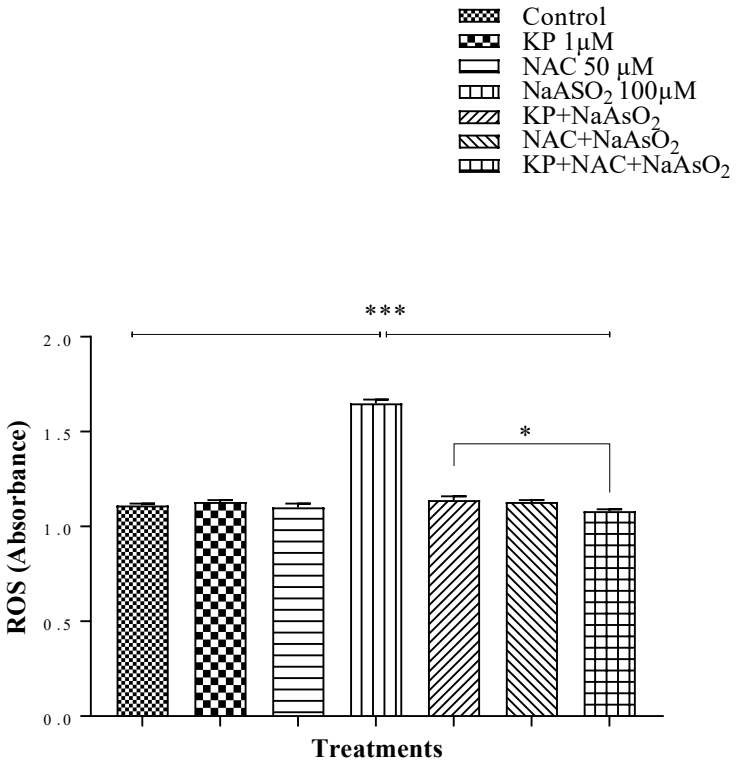


Fig. 4.3 Mean testicular ROS following 2 hr. incubation with $NaAsO_2$, KP-10 and NAC doses. * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

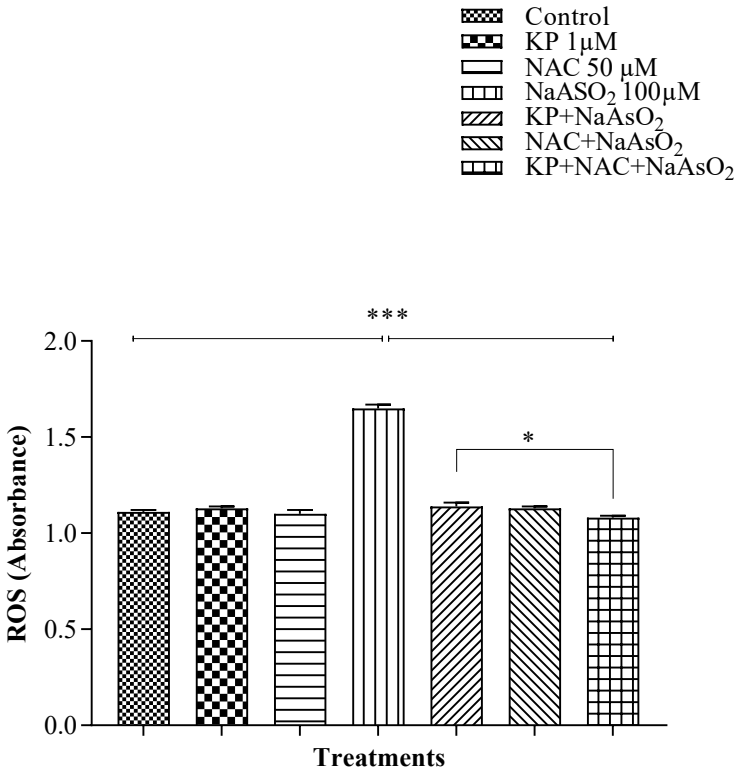


Fig. 4.4 Mean testicular ROS following 24 hr. incubation with $NaAsO_2$, KP-10 and NAC doses. * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

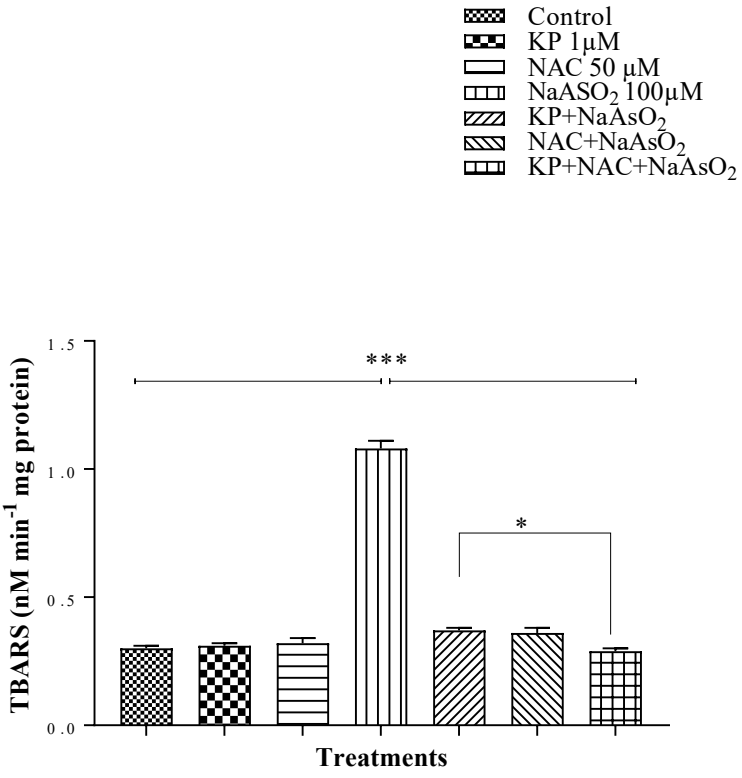


Fig. 4.5 Mean testicular TBARS following 2 hr. incubation with $NaAsO_2$, KP-10 and NAC doses. * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

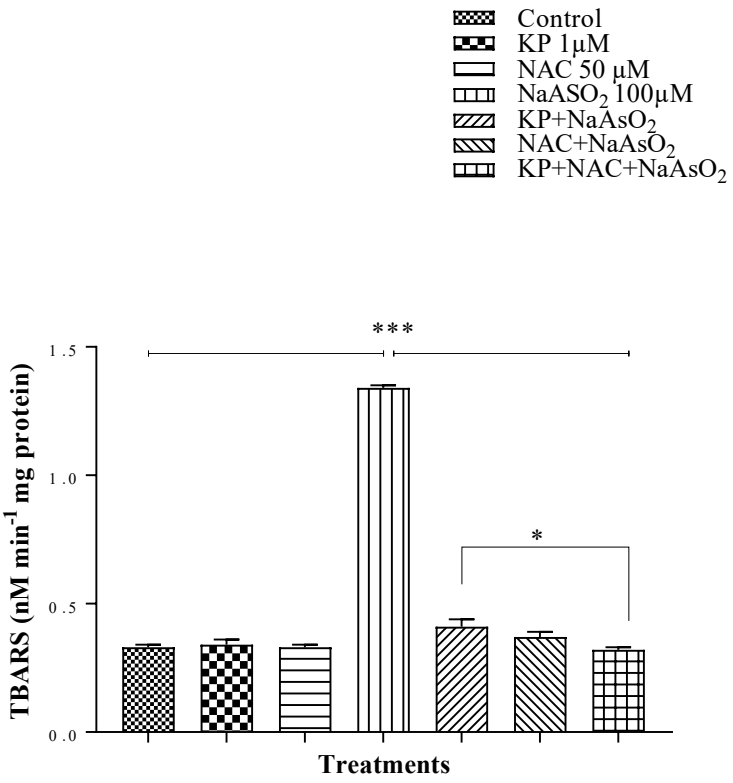


Fig. 4.6 Mean testicular TBARS following 24 hr. incubation with $NaAsO_2$, KP-10 and NAC doses. * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

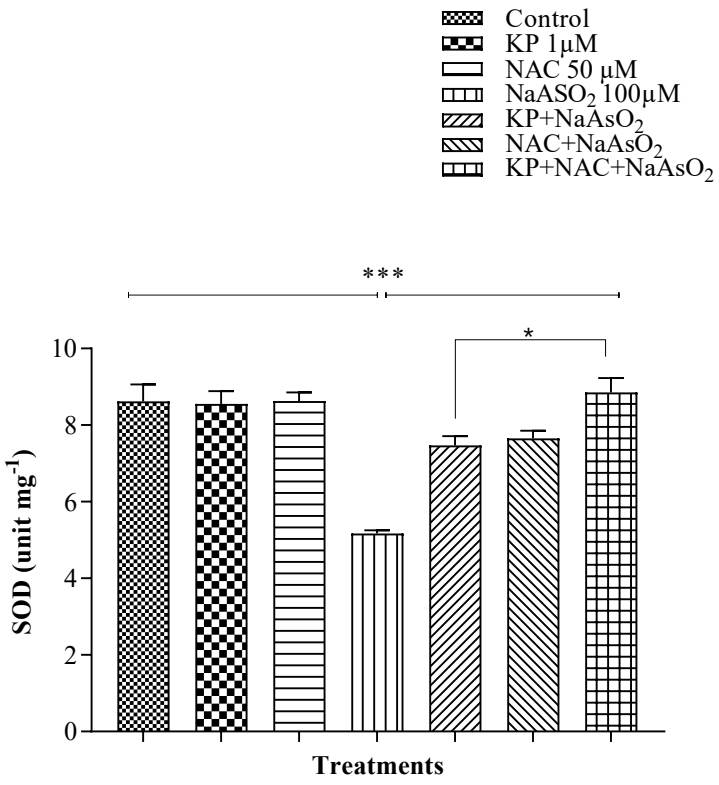


Fig. 4.7 Mean testicular SOD following 2 hr. incubation with $NaAsO_2$, KP-10 and NAC doses. * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

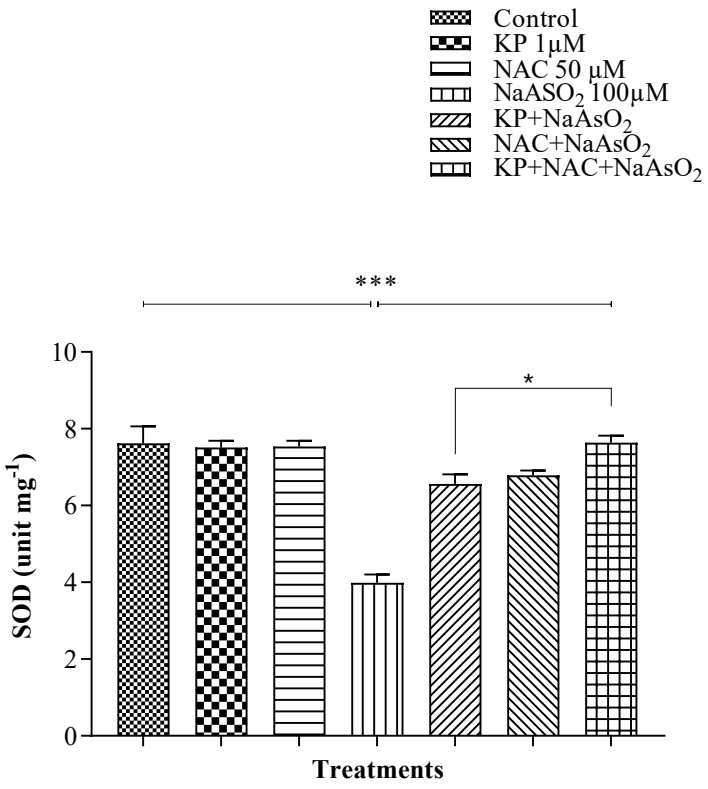


Fig. 4.8 Mean testicular SOD following 24 hr. incubation with $NaAsO_2$, KP-10 and NAC doses. * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

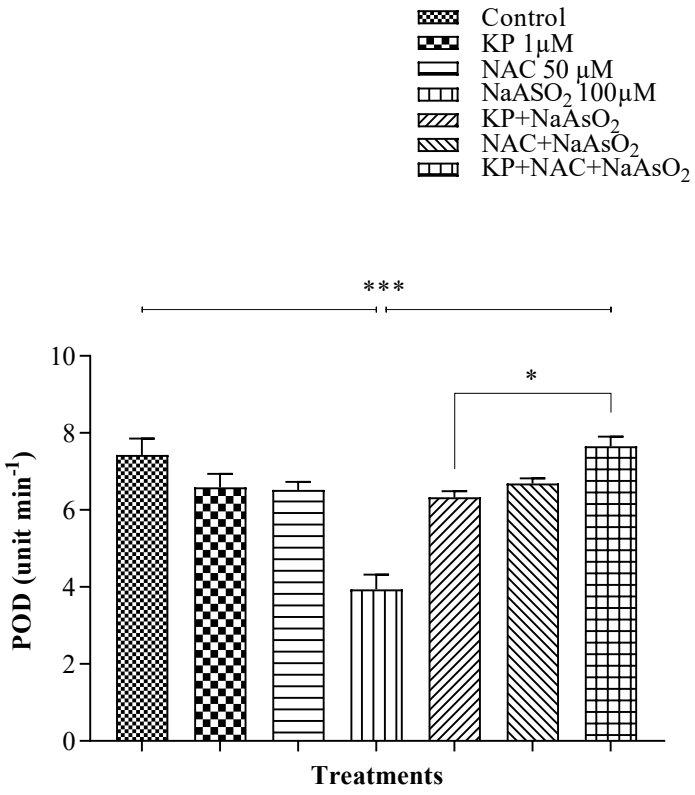


Fig. 4.9 Mean testicular POD following 2 hr. incubation with $NaAsO_2$, KP-10 and NAC doses. * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

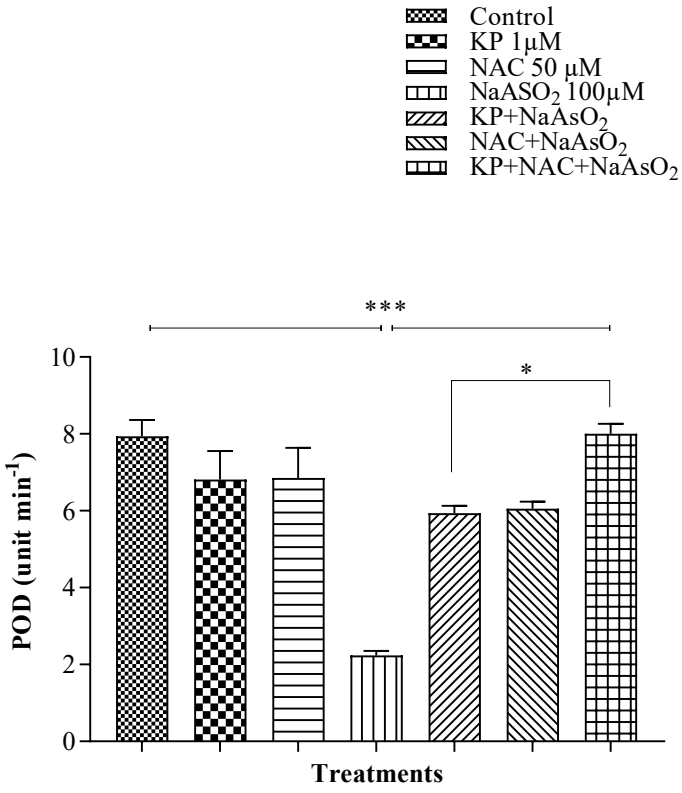


Fig. 4.10 Mean testicular POD following 24 hr. incubation with $NaAsO_2$, KP-10 and NAC doses. * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

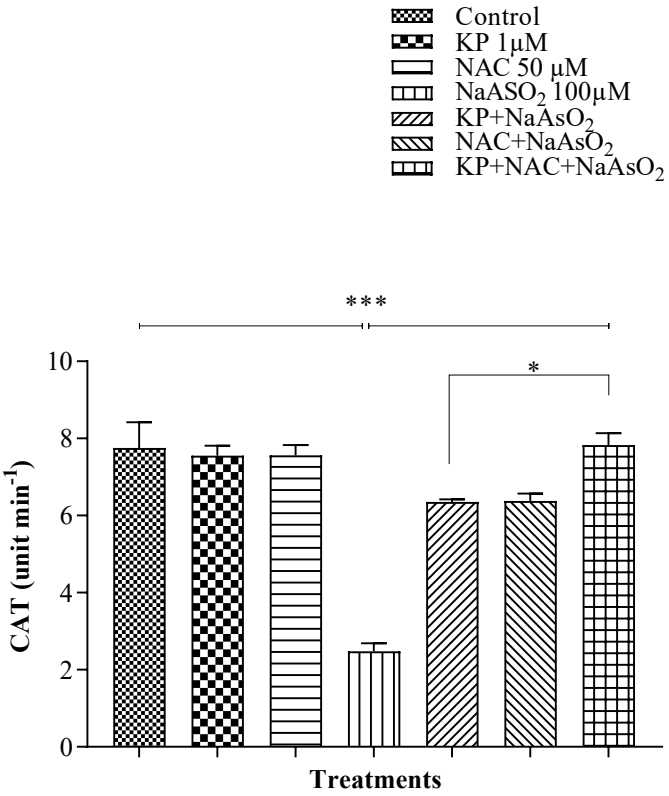


Fig. 4.11 Mean testicular CAT following 2 hr. incubation with $NaAsO_2$, KP-10 and NAC doses. * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

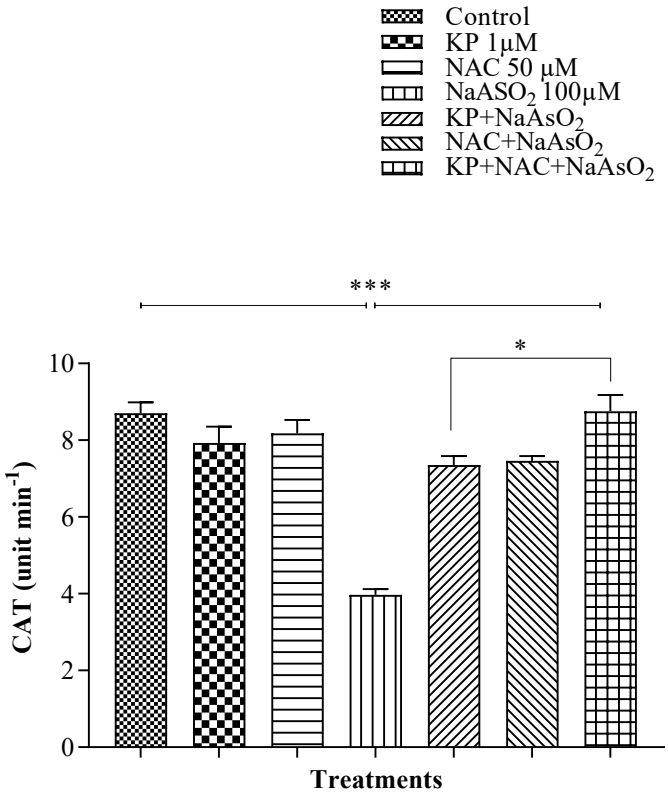


Fig. 4.12 Mean testicular CAT following 24 hr. incubation with $NaAsO_2$, KP-10 and NAC doses. * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

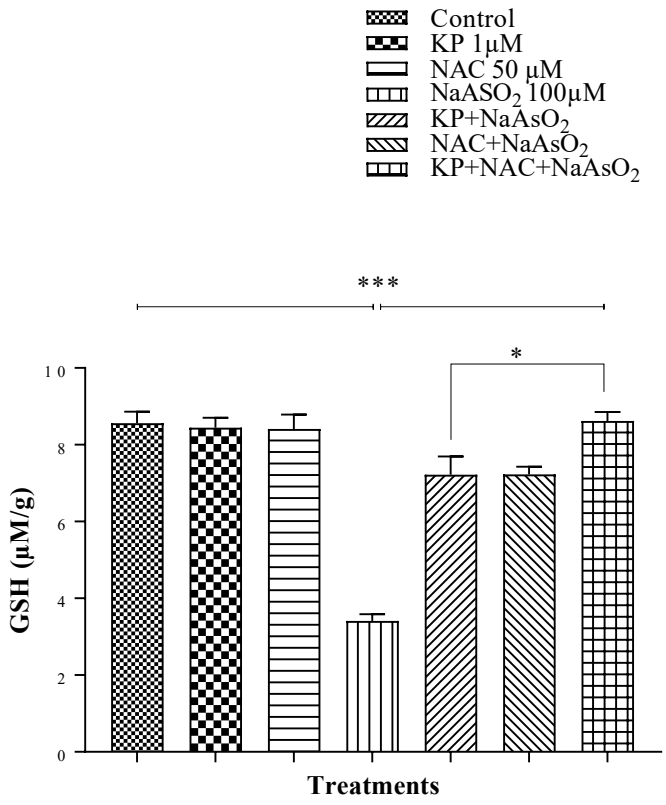


Fig. 4.13 Mean testicular GSH following 2 hr. incubation with $NaAsO_2$, KP-10 and NAC doses. * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

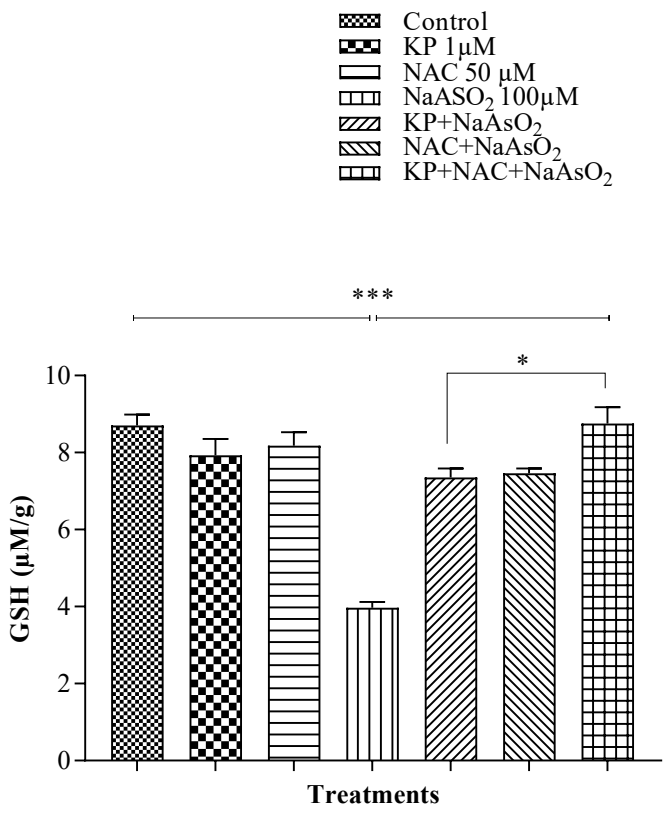


Fig. 4.14 Mean testicular GSH following 24 hr. incubation with $NaAsO_2$, KP-10 and NAC doses. * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

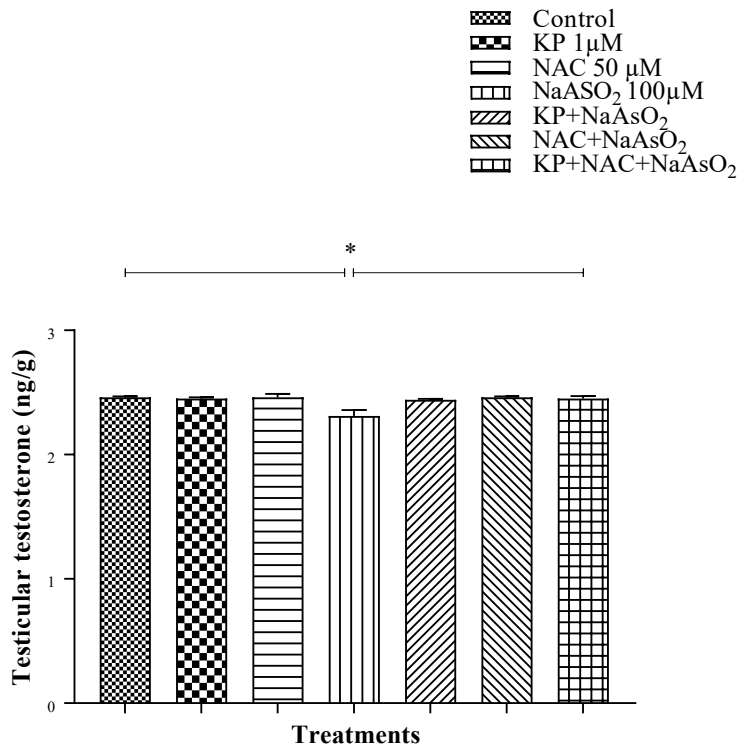


Fig. 4.15 Mean testicular testosterone concentration following 2 hr. incubation with $NaAsO_2$, KP-10 and NAC doses. * $p<0.05$ show significant difference between respective treatment groups

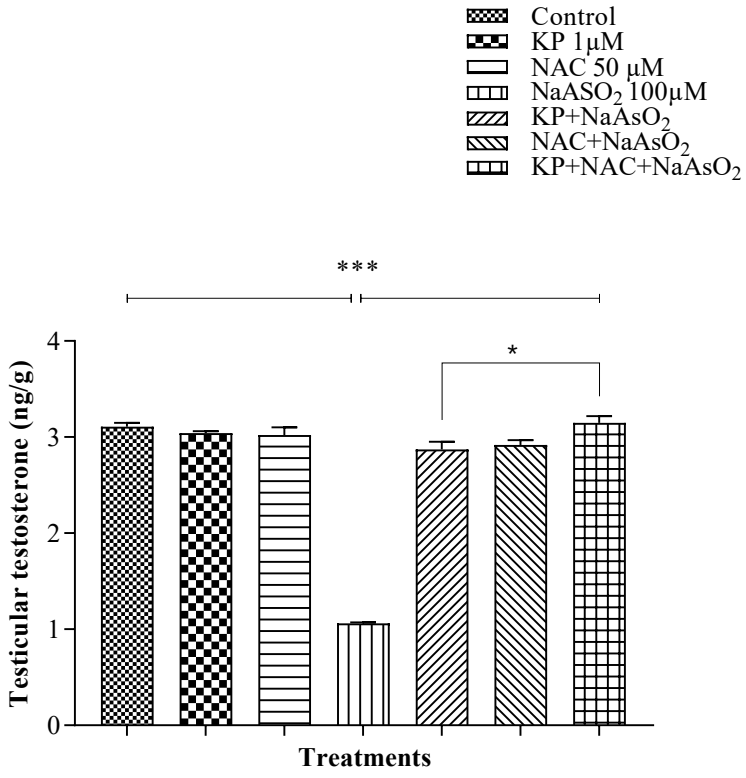


Fig. 4.16 Mean testicular testosterone concentration following 24 hr. incubation with $NaAsO_2$, KP-10 and NAC doses. * $p<0.05$ & *** $p<0.001$ show significant difference between respective treatment groups

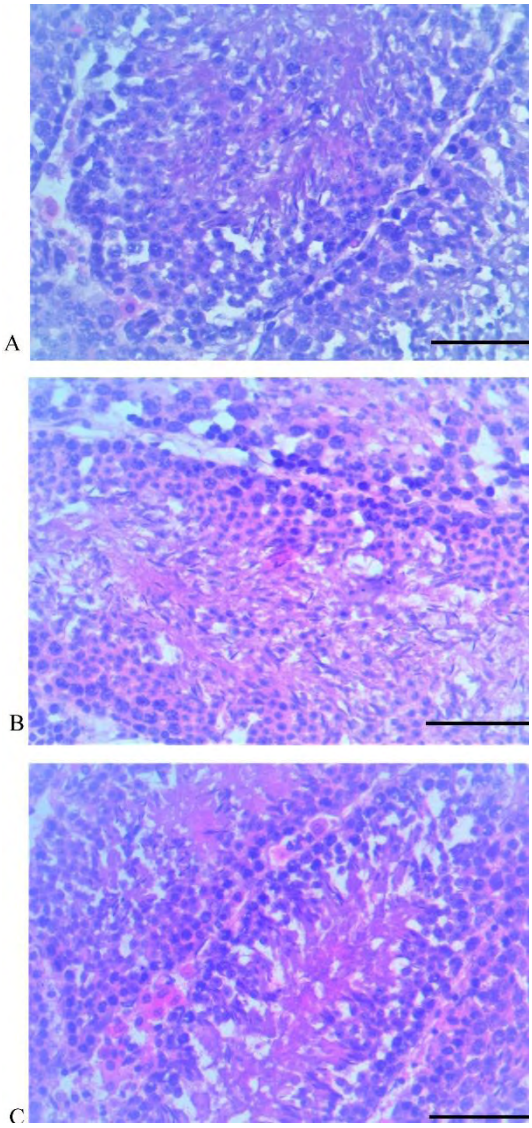


Fig. 4.17 Photomicrographs of testicular sections of adult male mice following 2 hr. incubation with treatment free control (A), KP-10 (1µM) (B) and NAC (50µM) (C) show normally arranged germ cell layers Scale bar =50µm

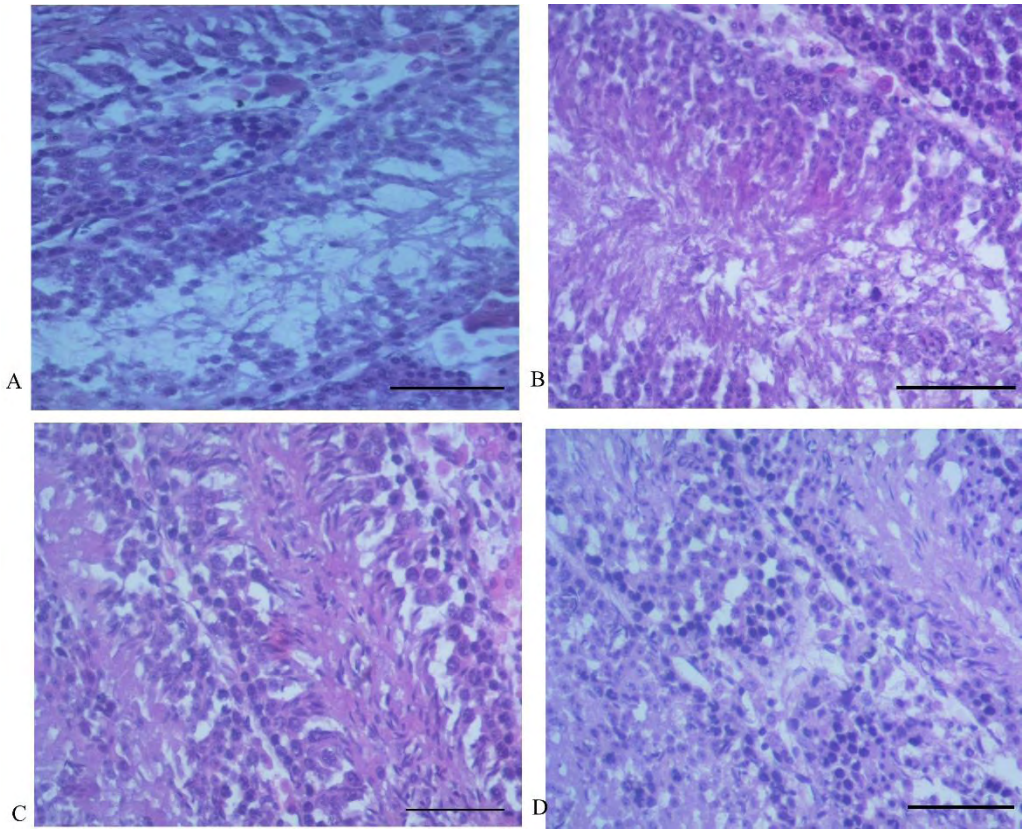


Fig. 4.18 Photomicrographs of testicular sections of adult male mice following 2 hr. incubation with $NaAsO_2$ (100µM) alone shows abnormally expanded luminal space and prominent germ cell loss (A), (B) KP-10 + $NaAsO_2$ (C) NAC + $NaAsO_2$ and (D) KP + NAC + $NaAsO_2$ show normal luminal space, improved germ cell distribution and sperm Scale bar =50µm

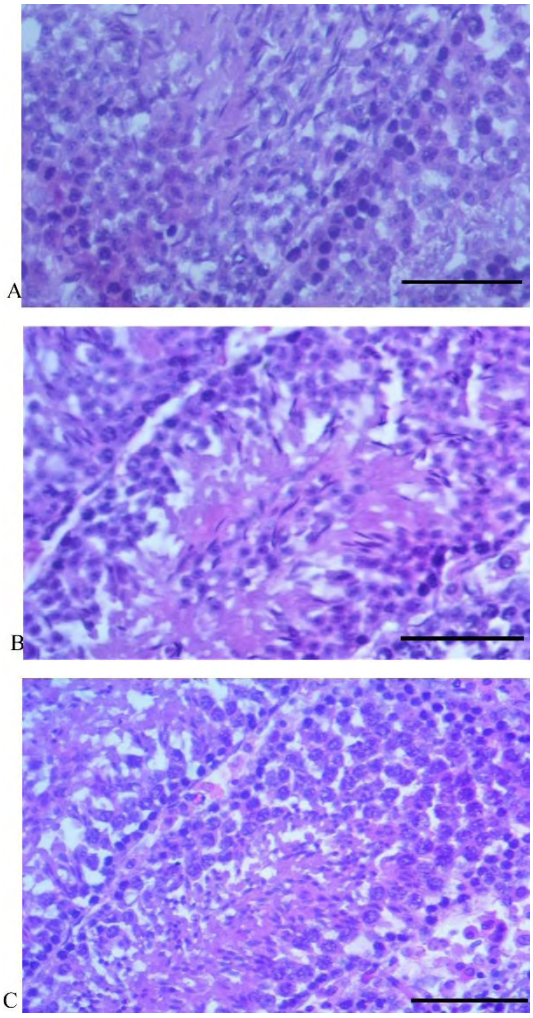


Fig. 4.19 Photomicrographs of testicular sections of adult male mice following 24 hr. incubation with treatment free control (A), KP-10 (1µM) (B) and NAC (50µM) (C) show normally arranged germ cell layers Scale bar =50µm

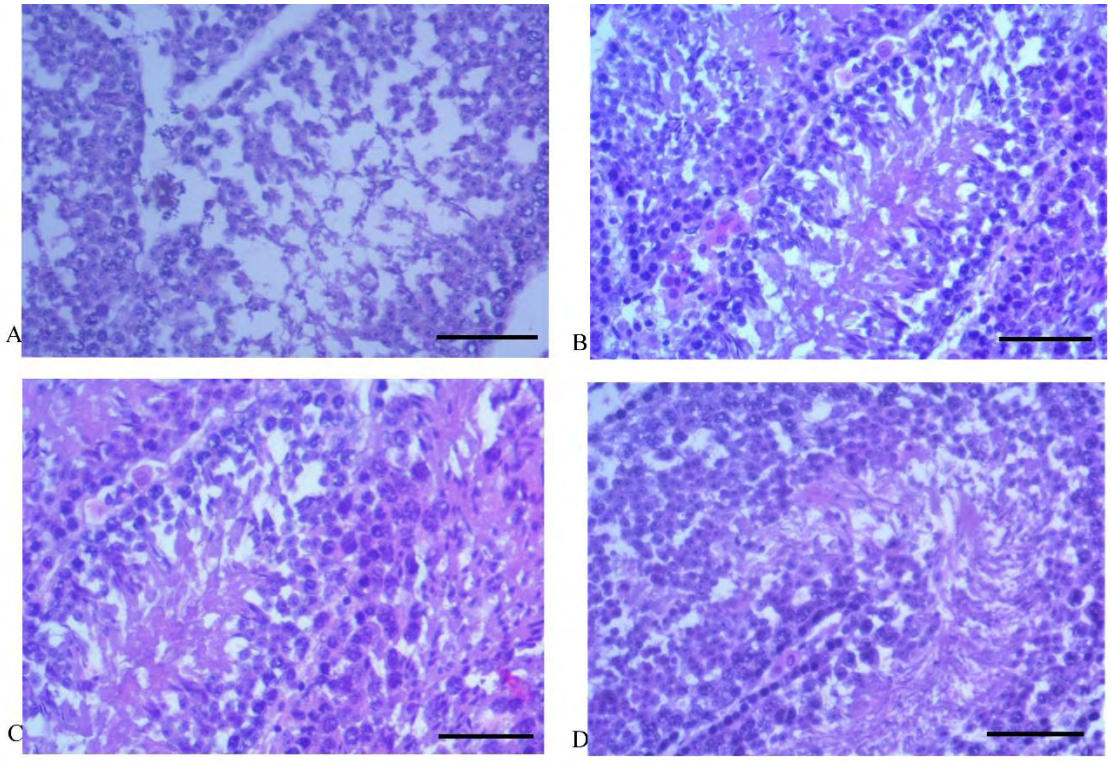


Fig. 4.20 Photomicrographs of testicular sections of adult male mice following 24 hr. incubation with $NaAsO_2$ (100µM) alone shows abnormally expanded luminal space and prominent germ cell loss (A), (B) KP-10 + $NaAsO_2$ (C) NAC + $NaAsO_2$ and (D) KP + NAC + $NaAsO_2$ show normal luminal space, improved germ cell distribution and sperm Scale bar =50µm

## 4.5 Discussion

Toxic effects of inorganic arsenic on male reproductive system have been previously evaluated through both *in vivo* and *in vitro* experiments on adult mice. These include reduced reproductive performance indicated by endocrine disruption resulting in decreased circulating and intra-testicular testosterone levels, deteriorated semen quality, tubular degeneration within testes and imbalance of testicular oxidant-antioxidant system (Reddy et al., 2011; Anwar and Qureshi, 2019; Fatima and Qureshi; 2022).

Considering the adverse effects upon arsenic exposure, potential therapeutic agents (previously mentioned) have also been investigated which significantly improved the overall reproductive performance in mice and rats challenged with arsenic exposure according to respective study designs (Im Chang et al., 2007; Reddy et al., 2011; Baltaci et al., 2016; Fatima and Qureshi, 2022). In addition, comparative evaluation of KP and NAC (Chapter 3) was conducted for the first time to investigate their protective effects against sodium arsenite-induced testicular toxicity in adult mice.

Taking into account the requirement of direct assessment of therapeutic potential of KP-10 and NAC against toxic effects of arsenic, series of *in vitro* experiments were currently designed employing short durations of arsenic exposure. Testicular fragments were incubated with treatment free culture media, $NaASO_2$ (100 µM), KP-10 (1 µM) and NAC (50 µM) according to experimental design (mentioned earlier) for 2 and 24 h. Biochemical evaluation of testicular cultures treated with $NaAsO_2$ alone for both 2 and 24 h. demonstrated significantly increased LDH, ROS and TBARS levels along with decreased activities of antioxidant enzymes including SOD, POD and CAT. Non-enzymetic GSH also exhibited similar trend upon arsenite exposure. Whereas, supplementation with either KP-10 or NAC alone along with $NaAsO_2$ treatment, equally prohibited these pathological changes thus protecting against arsenite-induced testicular damage. Importantly, similar to earlier *in vivo* findings, synergistic effect of combined treatment with KP and NAC was found to be more profound as compared to KP alone supplementation.

As regards the testicular testosterone levels, significant decline was observed following both 2 and 24 h. exposure to $NaAsO_2$ alone unlike previous *in vitro* findings reported by Anwar and Qureshi (2019) who observed no such decline in intra-testicular

testosterone levels following 2 hr. of incubation. Currently observed decrease following 2 h. incubation ($p<0.05$) was however lower than significantly greater decrease ($p<0.001$) observed after 24 h. exposure to $NaAsO_2$ alone. Similar to biochemical parameters, testosterone level was also normalized to near control upon supplementation with either KP alone and NAC alone or KP+NAC combined treatment along with $NaAsO_2$ exposure for both 2 and 24 hrs. Moreover, combined treatment with KP+NAC for 24 h. resulted in significant improvement in testosterone levels when compared with KP alone supplementation. Such difference in testosterone level was however not observed upon 2 h. incubation with respective treatments thus presenting the time dependent effect of both toxicant and counteracting agents.

Previously, contradictory outcomes were demonstrated regarding the regulatory role of kisspeptin on testicular functions through both *in vivo* and *in vitro* experiments. Such as, kisspeptin has a profound effect on testosterone secretion through central axis but no such effect was observed after direct treatment of macaque testicular cultures. It was suggested therefore, it might be involved in some other regulatory pathway within the testes (Tariq and Shahab, 2017). Moreover, use of appropriate dose is also critical to get desired therapeutic effects of KP-10. For this purpose, previous *in vitro* studies were thoroughly analysed. Such as, Mei et al. (2013), evaluated whether KP-10 at the dose of 1 μM of high dose was able to stimulate testosterone release from MA-10 cell line as well as testes fragments. They found no evidence of direct stimulation of testosterone release or enhancing LH actions. These outcomes are in contrast with the findings reported by Irfan et al. (2013), who found significantly increased hCG-stimulated testosterone levels in acyline (an antagonist of GnRH receptor) treated rhesus monkeys as compared to the responses with hCG alone treatment. In addition, comparatively higher dose of KP-10 was selected for current experiments in order to counteract the previously reported (Anwar and Qureshi, 2019) toxic effects of high dose of $NaAsO_2$ 100 μM.

Another explanation in the context of prevention of testicular damage could be the general effect of $NaAsO_2$ treatment which is reported in a recent study by Zhou et al. (2017). This explanation cannot be used to justify the current findings as they used low dose of $NaAsO_2$ (<2 μM) in order to evaluate its suppressing effect on UV-induced cellular apoptosis in skin cells. Whereas, high doses (100 μM) of $NaAsO_2$ were used in current experiment according to our hypothesis. Apoptotic effect of arsenite on Leydig

cell number was although not directly evaluated in current experiments, but the expression of apoptosis related genes were evaluated currently and have also been evaluated in previous *in vitro* experiments (Anwar et al., 2020) at 100 µM dose in testicular explants of mice.

Presently, testicular cultures of adult mice incubated with KP-10 alone (1 µM) in arsenite free culture media also demonstrated no significant change from basal testosterone concentration determined in treatment free control group. These observations further emphasize the previous *in vivo* outcomes (Chapter 2) that kisspeptin is capable to regulate the testosterone secretion upon exogenous intervention, however it does not alter the basal testosterone levels as evidenced by an earlier study where in the presence of acyline-induced receptor inhibition, exogenous KP increased testosterone secretion in rhesus monkeys. In addition, differential responses could be reasoned as the physiological differences between non-human primate and murine experimental models.

Like the earlier *in vivo* outcomes (Chapter 3), histological evaluation of testicular fragments also demonstrated disrupted testicular morphology upon 2 and 24 h. incubation with 100µM dose of $NaAsO_2$ alone. Seminiferous tubules revealed time dependent depletion of germ cells, expanded lumens and showed comparatively greater tissue damage following 24 h. incubation. Similar observations have been reported by Anwar et al. (2020) who incubated infantile testicular fragments with high doses of $NaAsO_2$ (50 and 100 µM) alone. Whereas, testicular cultures placed in $NaASO_2$ treated groups supplemented with both KP and NAC restored the germ cell population and normalized the luminal spaces near control. Moreover, KP and NAC alone treatment without $NaAsO_2$ did not reveal any pathological changes in testicular histology upon 2 and 24 h. incubations.

As regards the mechanism mediating the protective effect of NAC demonstrated through previous and current observations, it was found to exhibit both direct and indirect antioxidant properties by either acting as GSH precursor/acting as GSH in case of its depletion or by breaking thiolated proteins which may act as antioxidants, respectively (Aldini et al., 2018). In addition, NAC has been recently found to downregulate testicular expression of apoptosis related genes including caspase 3, 8 and Bax etc. along with an improvement in sperm parameters in rats exposed to

continuous doses of lead (Abedini et al., 2022). Considering these recent findings, current *in vitro* observations which for the first time revealed mitigating role of NAC against arsenic-induced oxidative stress in testicular cultures cannot only be well-explained but also suggest further genetic evaluation through both i*n-vivo* and *in-vitro* experiments.

KP-10 which is considered an important endocrine regulator has also been previously investigated for its therapeutic potential in fertility related disorders (Dhillo et al., 2005; (Dhillo et al., 2007; Jayasena et al., 2009; Jayasena et al., 2010). Additionally, its protective effect on male reproductive system against a few oxidizing endocrine disruptors has also been recently reported (Güvenç and Aksakal, 2018; Fatima and Qureshi, 2022). These previous *in vivo* findings support the currently observed mitigating role of KP-10 against arsenite toxicity induced in testicular cultures. Taking into account the previous studies, synergistic effect of both NAC and KP-10 in current experiments is also well justified (previously mentioned).

**Conclusion**

Like previous *in vivo* findings, comparative evaluation through *in vitro* experiments revealed that both KP-10 and NAC are equally effective against sodium arsenite-induced testicular damage. Additionally, molecular mechanisms mediating protective effects of NAC have been previously reported however respective literature regarding KP-10 is still deficient. Therefore, there is need to further analyse at molecular level whether KP-10 protects against arsenite-induced oxidative damage indirectly through promoting steroidogenesis or directly through suppressing expression of oxidative stress related genes. Moreover, incubation of testicular cultures for longer periods will further support the current observations.

## Abstract

Both *in vivo* and *in vitro* studies have frequently demonstrated that redox imbalance caused by $NaAsO_2$ promotes apoptosis in several tissues including testes, ovaries, placenta and brain with an indication of inhibitory effect on the expression of steroidogenic genes in testicular tissue. Considering the anti-apoptotic and steroidogenic effects of both KP-10 and NAC, current study compared the protective effect of KP and NAC against arsenite toxicity through assessment of mRNAs for Caspase 3, StAR and Cyp11a1 in mice testicular tissues obtained from both *in vivo* and *in vitro* experiments (Chapter 3 and 4). Both experiments included treatment free controls, $NaAsO_2$ alone treated groups with and without KP and NAC supplementation. Following completion of the experimental time period, testicular tissues were collected and stored for RNA extraction. *In vivo* findings obtained from RT-PCR revealed that $NaAsO_2$ alone exposure caused significant elevation ($p<0.05$ at 4ppm; $p<0.001$ at 10 ppm) in the expression of Caspase 3 mRNA in a dose dependent pattern while decreasing the expression of StAR ($p<0.05$ at 4ppm; $p<0.001$ at 10ppm) and Cyp11a1 mRNAs ($p<0.001$) as compared to tap water control. However, both alone and combined supplementation with KP and NAC significantly downregulated the expression of Caspase 3 concomitantly upregulating the StAR and Cyp11a1 mRNAs. Likewise, *in vitro* results also demonstrated significantly increased expression of Caspase 3 mRNA ($p<0.01$ at 2 h; $p<0.001$ at 24 h) while decrease occurred in StAR ($p<0.05$ at 2 h; $p<0.01$ at 24 h) and Cyp11a1 mRNAs ($p<0.01$ at 2 h; $p<0.001$ at 24 h) expression in time dependent manner. Supplementation with KP and NAC either alone or in combination however, significantly increased expression of StAR and Cyp11a1 mRNAs along with a decreased Caspase 3 expression. In addition, combined supplementation with these therapeutic agents in both experimental regimens demonstrated their synergistic effects as indicated by significantly greater suppression of Caspase 3 and up regulation of StAR and Cyp11a1 mRNAs when compared to $NaAsO_2$ alone treatment. The present novel findings provide evidence of the anti-apoptotic and steroidogenic effects of KP-10 against heavy metal-induced testicular toxicity.

## 5.1Introduction

One of the established mechanisms underlying arsenic toxicity involves increased production of ROS which ultimately leads to oxidative damage thus exerting its deleterious effects on various organs. It is also known to elevate plasma glucocorticoid level which disrupts the hormonal pathways by desensitizing the gonadotroph cells which results in reduced gonadotropin secretion. Ultimately, these changes lead to gonadal toxicity as indicated by poor semen quality, reduced LH, FSH and testosterone levels and germ cell loss (Zubair et al., 2017).

Recent assessment of apoptotic effects of arsenic compounds on testicular tissue at genetic level have revealed that $NaAsO_2$ treatment to murine Leydig tumor cell line results in an increased expression of caspases-3, 8 and 9 which are considered authentic biomarkers of apoptosis (Mu et al., 2019). Previously, arsenic trioxide ($As_2O_3$) (3mg/kg b.w.) exposure to male mice also demonstrated significantly decreased mRNA levels of genes encoding steroidogenic enzymes such as cytochrome P450 side chain cleavage, 3 β and 17 – hydroxysteroid dehydrogenases (Chiou et al., 2008).

Recently, *in vitro* exposure of TM3 Leydig cells to high dose of sodium arsenite (384,8 μM) for 24 h along with LH stimulation, showed reduced viability and decreased expression level of CYP11A1 (Cytochrome P450), whereas expression of StAR (steroidogenic acute regulatory protein) gene was found to be significantly increased as compared to treatment free control. In addition, mRNA expression levels of genes encoding 3 β and 17-hydroxysteroid dehydrogenases also demonstrated significant decrease upon exposure to high dose of sodium arsenite (Taşçi et al., 2019). Similarly, sodium arsenite exposure has been found to decrease the expression of StAR, CYP11A1 and CYP19A1 resulting in follicular degeneration in female rats (Chen et al., 2022).

As regards NAC, it has been extensively studied at molecular level for its antioxidant role against toxic effects of arsenic. It has been found to suppress the oxidative stress related genes including HMOX1 and MT1 in anterior pituitary cultures of rats treated with sodium arsenite (Rochentii et al., 2016). In addition, NAC is found to affect the expression of steroidogenic and apoptosis related genes in a dose dependent manner in porcine placental trophoblast cells. It was found to decrease the caspase 3 mRNA

expression while upregulating the CYP11A1 mRNA expression at low dose. Opposite effects were observed at high doses of NAC. Whereas, StAR remained unaffected at all doses (Ding et al., 2021). Furthermore, it is also found to downregulate the expression of caspase 3 and 9 in $H_2O_2$ treated male germ cells thus protecting against oxidative stress-induced apoptosis (Maheshwari et al., 2011). Similar observations have been found in rat hepatocytes where it demonstrated significant decrease in protein levels of caspase 3 and 9 upon exposure to cadmium acetate (Liu et al., 2016).

Although, molecular research related to antioxidant potential of KP-10 against metal-induced reproductive toxicity is still lacking, however a recent study on rats with sub arachnoid hemorrhage has demonstrated the suppression of neuronal apoptosis by intra-nasal treatment with KP54 (1.0 nmol/kg). The findings revealed that treatment significantly decreased the cleaved caspase 3 and BAX levels which are considered apoptotic biomarkers (Huang et al., 2021).

These findings suggest that at molecular level, arsenic-induced oxidative stress may promote expression of apoptotic genes and also downregulate the steroidogenic genes ultimately leading to testicular damage. Additionally, protective effect of NAC at molecular level has been previously studied against several oxidizing agents however literature on KP-10 is still deficient in this regard. Therefore, this study was aimed at molecular assessment of KP-10 against arsenic toxicity and its comparison with NAC to propose its therapeutic role.

## 5.2 Materials and methods

### 5.2.1 Animals and maintenance

Adult male Swiss albino mice were obtained from National Institute of Health (NIH), Islamabad and maintained in the animal house facility of Quaid-i-Azam university under standard laboratory conditions (temperature 25±4 °C, photoperiod of 12:12 Light: Dark hours and 40% humidity). They were given free access to standard rodent chow and fresh drinking water *ad libitum.*

### 5.2.2 Chemicals

KP-10 (KiSS-1; 112-121; H-YNWNSFGLRF-$NH_2$), Sodium pentobarbital (Sigma, Germany), inorganic sodium arsenite ($NaAsO_2$), N acetyl cysteine (NAC), Pencillin, Streptomycin and Dulbecco's modified eagle's medium/Ham F12 (DMEM/Ham F12) were purchased from respective manufacturers as described in chapter 4. Various chemicals used for real time PCR including TRIzol, Chloroform, Isopropanol, 70% Ethanol, DEPC water, RNA template, dNTPs, M-MuLV reverse transcriptase, M-MuLV buffer, template RNA and primers oligod $(T)_{18}$ and nuclease free water (NF $H_2O$) were purchased from Sigma Aldrich (St. Louis, Missouri, USA).

### 5.2.3 Experiment design

Both *in vivo* and *in vitro* experiments were conducted following previously explained experimental design in chapter 3 and 4 respectively (Fig. 5.1). Tissue samples from all experimental groups were immediately collected in Eppendorf tubes containing RNA latter to prevent RNA degradation. Following experimental groups were selected for evaluation of mRNA expression of target genes.

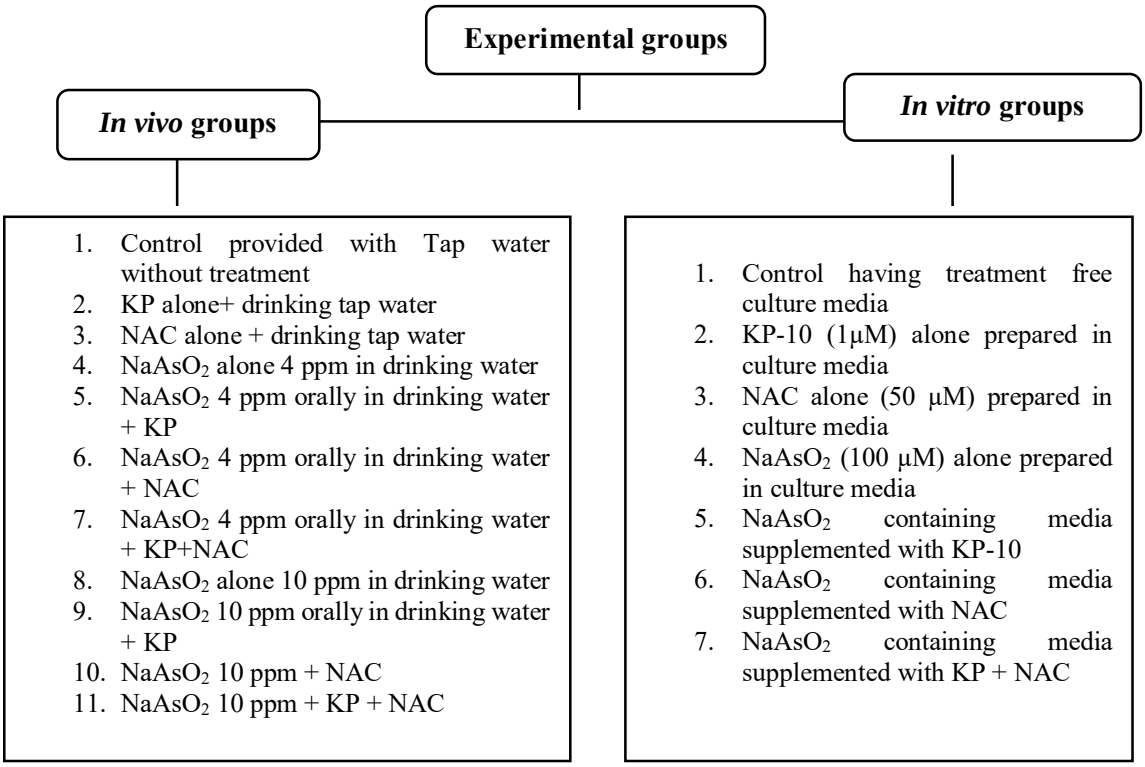


Fig. 5.1 Schematic representation of experimental groups

### 5.2.4 Tissue collection and preparation

After completion of *in vivo* experimental duration, testes were dissected out from anesthetized mice, rinsed with physiological saline and decapsulated. Likewise, *in vitro* testicular cultures were also collected following respective incubation periods, rinsed and stored in RNA latter at 4°C for further processing.

### 5.2.5 RNA extraction

For extraction of total RNA from testicular tissues, 100 mg tissue was collected and homogenized in 1ml of TRIzol solution following incubation at room temperature for 5 min. Chloroform was added and incubated at room temperature for further 3 min. For phase separation, homogenate was then centrifuged at 12,000 rpm for 10 min at 4°C. Aqueous layers were collected, mixed with an equal ratio of chilled isopropanol and further incubated on ice for 10 min for RNA precipitation. Samples were again centrifuged and supernatants were discarded. Pellets were washed with 70% ethanol and completely air dried. Afterwards, 40µl of DEPC was added and stored at −20 °C

for later use. RNA extraction for each sample was performed in triplicates followed by evaluation of its quality and quantity using a Nanodrop plate (Skanit RE 4.1, Thermo Scientific).

### 5.2.6 cDNA synthesis

cDNA synthesis kit (Vivantis cDSK01–050) was used to synthesize complementary DNA (cDNA). RNA-primer mixture was prepared for each sample by mixing oligod(T) primers, dNTPS and nuclease free water (NF $H_2O$). This mixture was incubated at 65°C for 5 min and then chilled on ice for 2 min and then vortexed. cDNA synthesis mixture was separately prepared by mixing M-MuLV Reverse Transcriptase enzyme, M-MuLV buffer and NF $H_2O$. This mixture was then added to RNA-primer mixture following manufacturer's instructions. Afterwards, final mixture was homogenized, centrifuged and incubated at 42 °C for 1 h. The enzyme catalyzed reaction was then inactivated by incubating the mixture at 85 °C for 5 min and then chilled on ice and centrifuged briefly. The synthesized cDNA was then stored at -20 °C for later use.

### 5.2.7 Primer sequences

Following primer sequences for Caspase 3, StAR and Cyp11a1 designed by Wang et al. (2015) were selected for current study.

| | |
|---|---|
| Caspase 3 | F: CCTCAGAGAGACATTCATGG |
| | R: GCAGTAGTCGCCTCTGAAGA |
| StAR | F: AGCATGTTCCTCGCTACGTT |
| | R: GCACAGCTTGGTGCCTTAATC |
| Cyp11a1 | F: TGGCACACAGAAAATCCATTACC |
| | R: TTGGGGTCCACGATGTAAACT |

### 5.2.8 Real-time polymerase chain reaction (RT-PCR)

Real-time polymerase reaction was performed with Mic PCR (BioMolecular System). Reaction mixture was prepared with cDNA, forward and reverse primers, 2X SYBR green Amplifyme Universal Mix (Blirt AM02-020) and nuclease free water. PCR amplification was performed at 95 °C for 20 seconds, and 40 cycles of 95 °C for 3

seconds and 60 °C for 30 seconds, (Fig. 5.2) followed by melting curve analysis (Fig. 5.3). Gene expression levels were presented as fold changes.

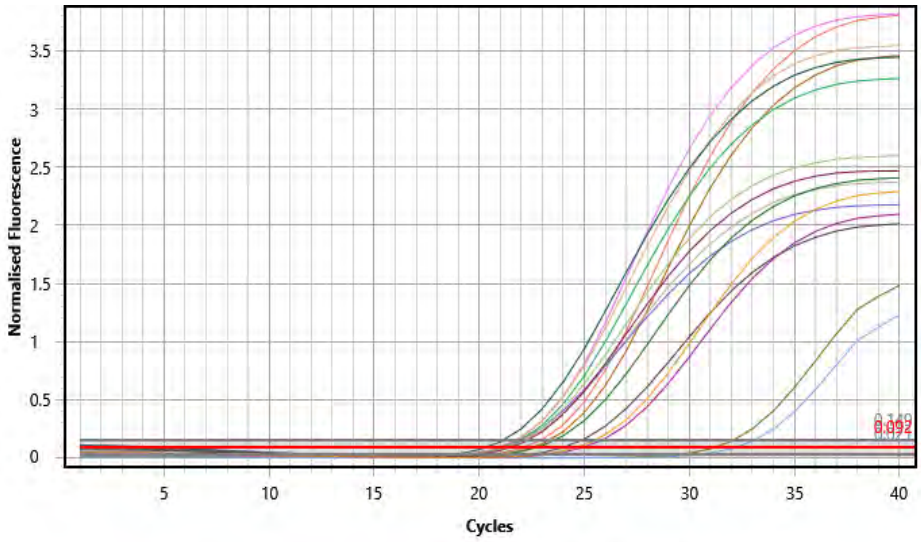


Fig. 5.2 Real time cycling graphs depicting normalized fluorescence on Y axis and Cycles (Cq) at X axis, samples against threshold are demonstrated by different color peaks.

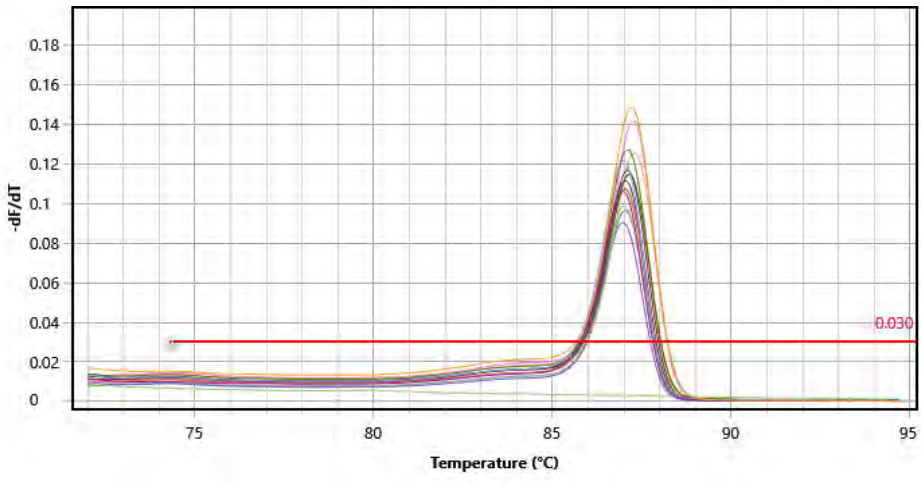


Fig. 5.3 Melt analysis curve plotted as $dF/dT$ (y-axis) against temperature (ºC, x-axis), demonstrating the amplification of desired products. Double peaks between 75ºC to 80ºC of temperature range depicting dimer formation.

### 5.2.9 Data analysis

Data were analyzed statistically using the software GraphPad Prism (version 5.0; San Diego, California, USA). One-way analysis of variance followed by Tukey's multiple comparison test was applied to compare the difference between the means of control and treatment groups. The levels of significance were $p<0.05$, $p<0.01$ & $p<0.001$. Data are expressed as mean ± standard error of mean (s.e).

## 5.3 Results

### 5.3.1 *In vivo* experiments

#### 5.3.1.1 Testicular caspase 3 mRNA

The expression of caspase 3 mRNA was significantly elevated in a dose dependent manner in the testicular tissue of mice exposed to 4ppm ($p<0.05$) (Fig. 5.4) and 10 ppm ($p<0.001$) (Fig. 5.5) $NaAsO_2$ as compared to tap water control. In contrast, supplementation with KP-10 and NAC alone along with $NaAsO_2$ exposure at both doses resulted in a significant decline ($p<0.05$) in caspase 3 expression. Moreover, synergistic effect of KP and NAC combined supplementation was revealed as significantly greater suppression of caspase 3 ($p<0.01$) when compared to $NaAsO_2$ alone treated groups. No significant change was however observed upon KP and NAC alone treatments in comparison with tap water control.

#### 5.3.1.2 Testicular StAR mRNA

Exposure to $NaAsO_2$ alone significantly suppressed the testicular StAR expression at both 4ppm ($p<0.05$) (Fig. 5.6) and 10ppm ($p<0.001$) (Fig. 5.7) doses when compared to tap water control. Supplementation with KP-10 and NAC alone along with $NaASO_2$ exposure however prevented this decline and significantly upregulated ($p<0.01$) the StAR expression when compared to $NaAsO_2$ alone treated groups at both doses. In addition, combined effect of KP and NAC against both doses of $NaAsO_2$ was significantly higher ($p<0.001$).

#### 5.3.1.3 Testicular Cyp11a1 mRNA

The expression of testicular Cyp11a1 mRNA was significantly declined ($p<0.001$) upon $NaAsO_2$ exposure irrespective of low (4ppm; Fig. 5.8) and high (10 ppm; 5.9) dosing, when compared to tap water control. However, treatment with both alone and combination of KP and NAC along with $NaAsO_2$ exposure at both 4pp and 10ppm significantly increased ($p<0.001$) the Cyp11a1 mRNA. In addition, no significant change was observed in testicular Cyp11a1 mRNA expression upon KP and NAC alone treatment when compared to tap water control group.

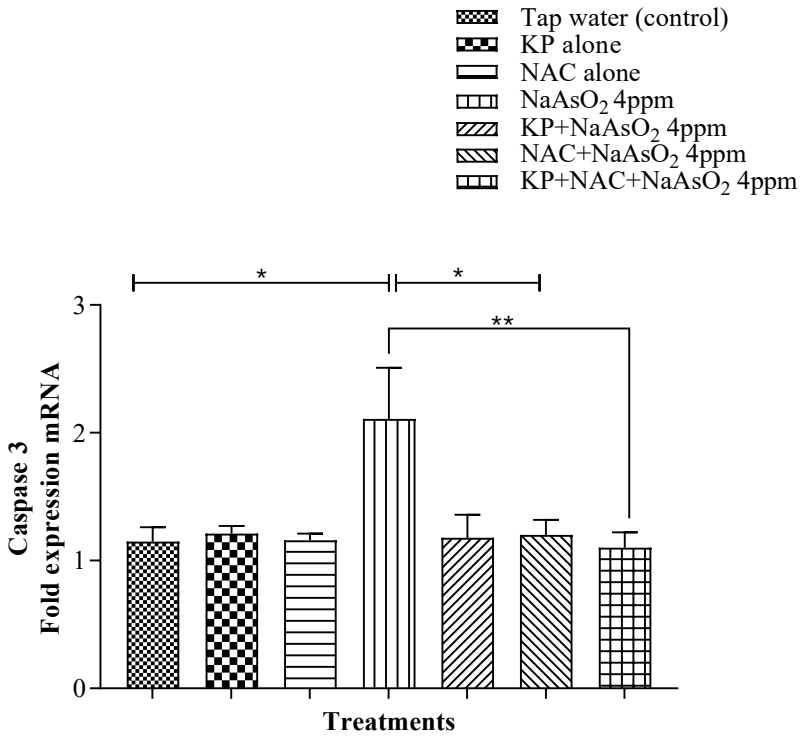


Fig. 5.4 The expression of testicular caspase 3 mRNA of adult male mice following 35-days exposure to 4 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days). * $p<0.05$ & ** $p<0.01$ show significant difference between respective treatment groups

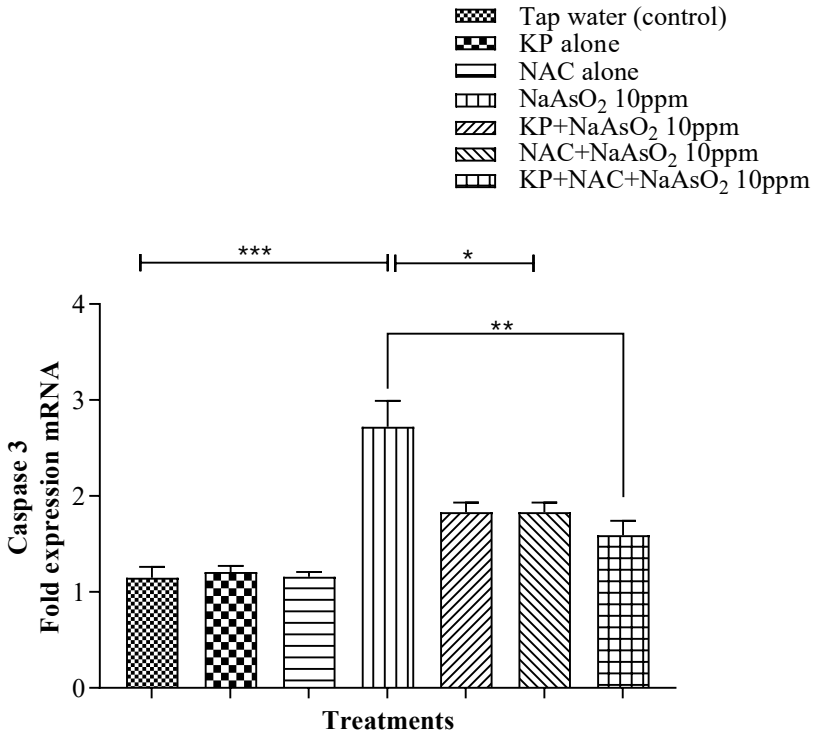


Fig. 5.5 The expression of testicular caspase 3 mRNA of adult male mice following 35-days exposure to 10 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days) * $p<0.05$, ** $p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups

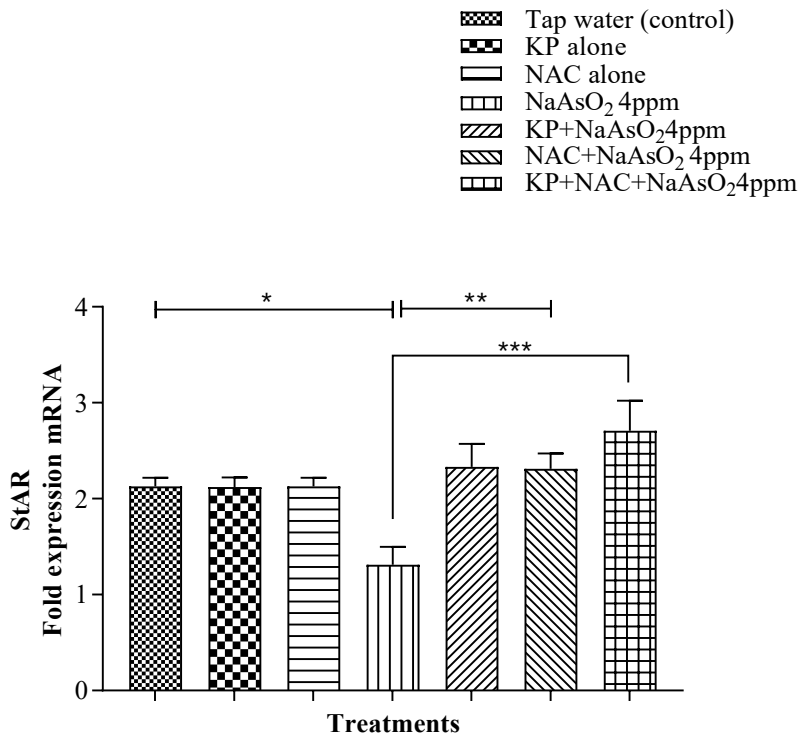


Fig. 5.6 The expression of testicular StAR mRNA of adult male mice following 35-days exposure to 4 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days) * $p<0.05$, ** $p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups

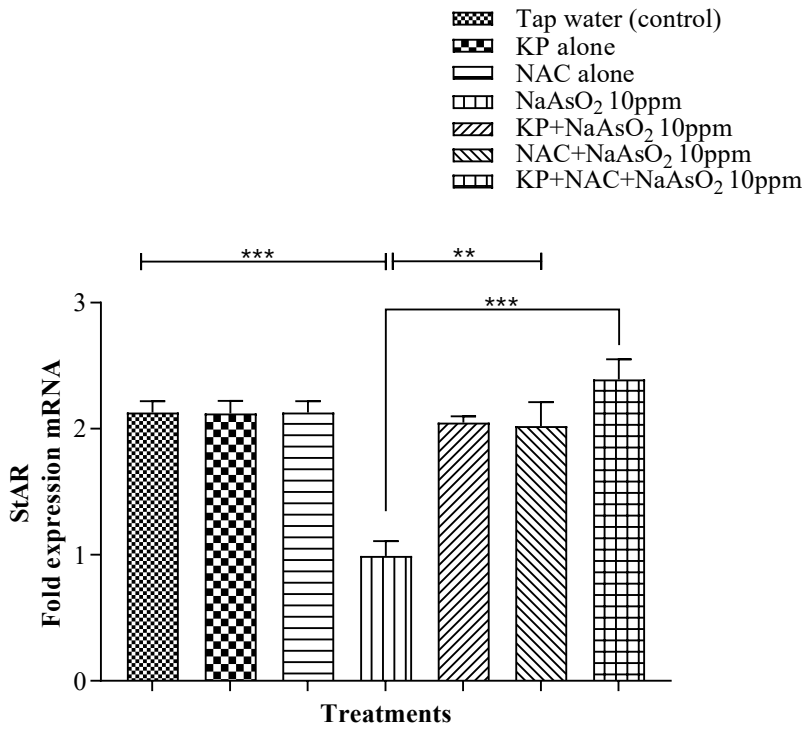


Fig. 5.7 The expression of testicular StAR mRNA of adult male mice following 35-days exposure to 10 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days) * $p<0.05$, ** $p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups

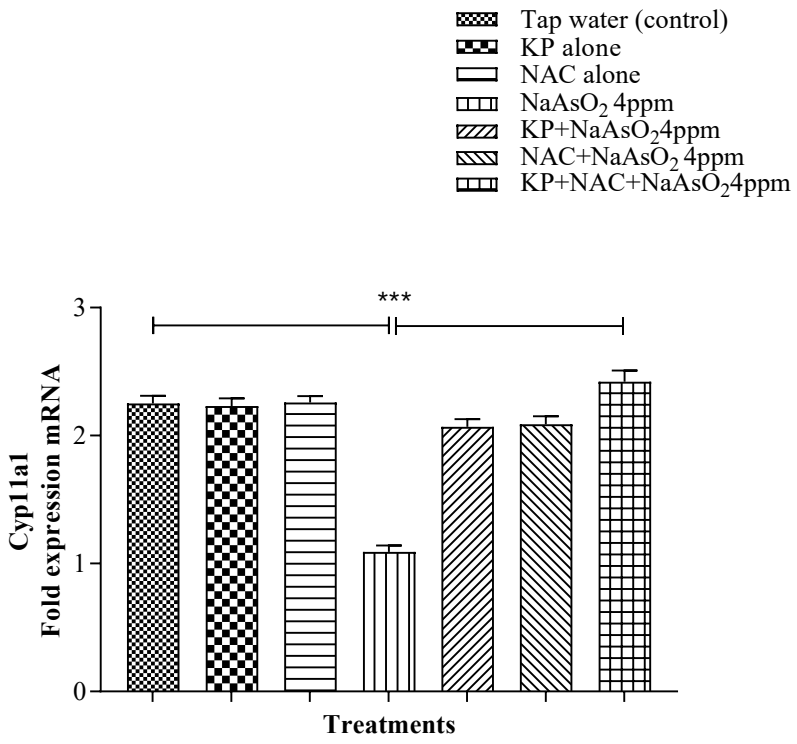


Fig. 5.8 The expression of testicular Cyp11a1 mRNA of adult male mice following 35-days exposure to 4 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days) *** $p<0.001$ shows significant difference between respective treatment groups

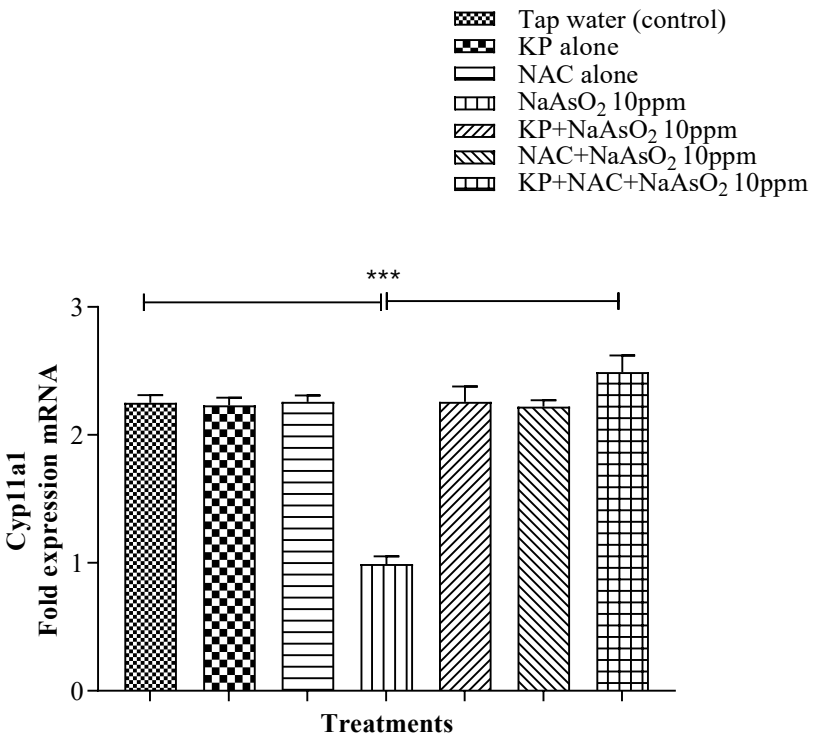


Fig. 5.9 The expression of testicular Cyp11a1 mRNA of adult male mice following 35-days exposure to 10 ppm dose of $NaAsO_2$, KP-10 (50 nmol/day once a week) and NAC (75 mg/kg b.w. for 5 alternative days) *** $p<0.001$ shows significant difference between respective treatment groups

### 5.3.2 *In vitro* experiments

#### 5.3.2.1 Testicular caspase 3 mRNA

Testicular cultures incubated with $NaAsO_2$ alone revealed time dependent increase in caspase 3 mRNA expression following 2 h ($p<0.01$) (Fig. 5.10) and 24 h ($p<0.001$) (Fig. 5.11) incubation, when compared with treatment free control. In contrast, supplementation of $NaAsO_2$ containing media with both KP-10 and NAC alone significantly decreased the caspase 3 expression following 2 h ($p<0.05$) and 24 h ($p<0.01$) incubation. Additionally, combined supplementation with KP-10 and NAC along with $NaAsO_2$ treatment more effectively suppressed the caspase 3 expression following 2 h ($p<0.01$) and 24 h ($p<0.001$) exposure thus revealing their synergistic effect when compared to $NaASO_2$ alone incubated testicular cultures.

#### 5.3.2.2 Testicular StAR mRNA

Significantly decreased expression of StAR mRNA was observed in a time dependent manner in testicular cultures incubated with $NaAsO_2$ alone following 2 h ($p<0.05$) (Fig. 12) and 24 h ($p<0.01$) (Fig. 5.13) incubation when compared to treatment free control. In contrast, both KP-10 and NAC alone supplementation significantly elevated ($p<0.05$ at 2 h & $p<0.01$ at 24 h) the StAR expression as compared to $NaAsO_2$ alone treatment. Moreover, combined supplementation with KP and NAC following both 2 ($p<0.01$) and 24 h ($p<0.001$) incubation was found to be more effective thus depicting synergistic effect of both therapeutics. Additionally, no significant change in StAR expression was observed upon exposure to KP and NAC alone following both 2 and 24 h incubation as compared to treatment free control.

#### 5.3.2.3 Testicular Cyp11a1 mRNA

Testicular cultures incubated with $NaAsO_2$ alone also revealed time dependent significant decline in Cyp11a1 mRNA expression following 2 h ($p<0.01$) (Fig. 5.14) and 24 h ($p<0.001$) (Fig. 5.15) incubation as compared to treatment free control. In contrast, supplementation with KP and NAC alone significantly elevated ($p<0.01$) the Cyp11a1 expression following both 2 and 24 h incubations, when compared to $NaAsO_2$ alone treatment. Moreover, combined supplementation with KP and NAC more

effectively ($p<0.001$) upregulated the Cyp11a1 expression following both 2 and 24 h incubations.

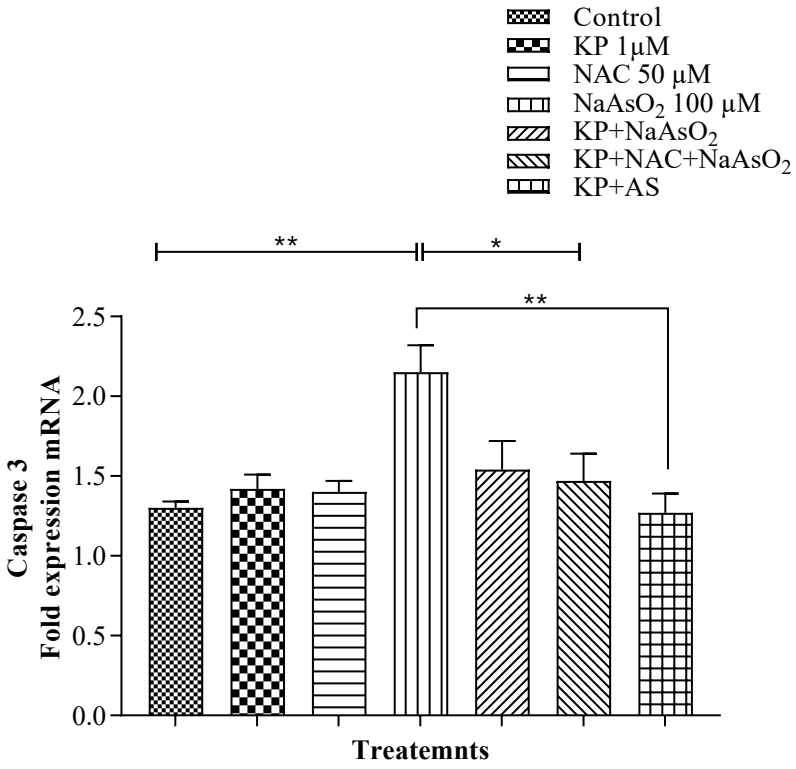


Fig. 5.10 The expression of caspase 3 mRNA following 2 h. incubation with $NaAsO_2$, KP-10 and NAC doses. * $p<0.05$ & ** $p<0.01$ show significant difference between respective treatment groups

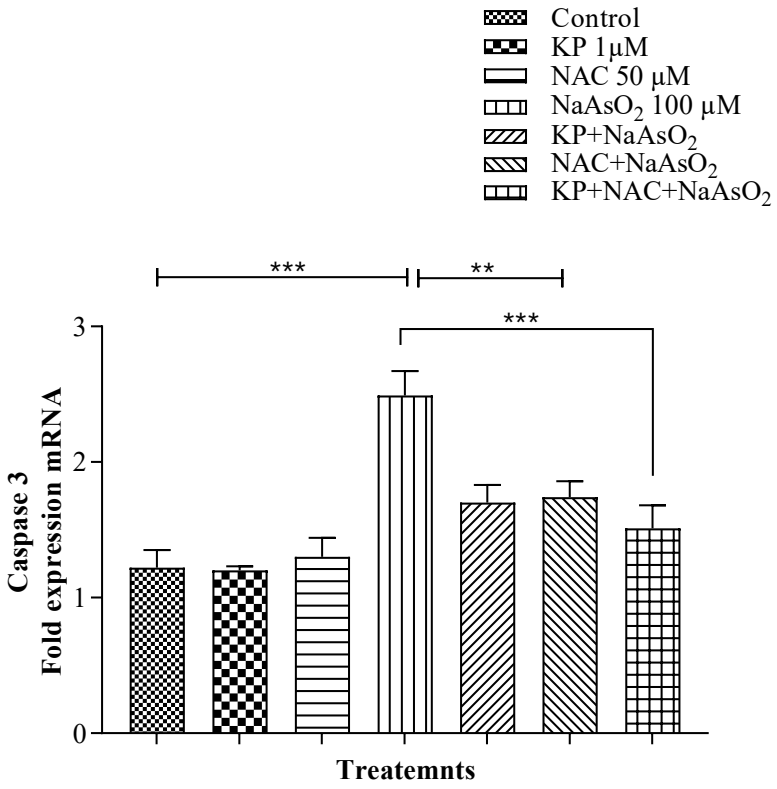


Fig. 5.11 The expression of caspase 3 mRNA following 24 h. incubation with $NaAsO_2$, KP-10 and NAC doses. ** $p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups

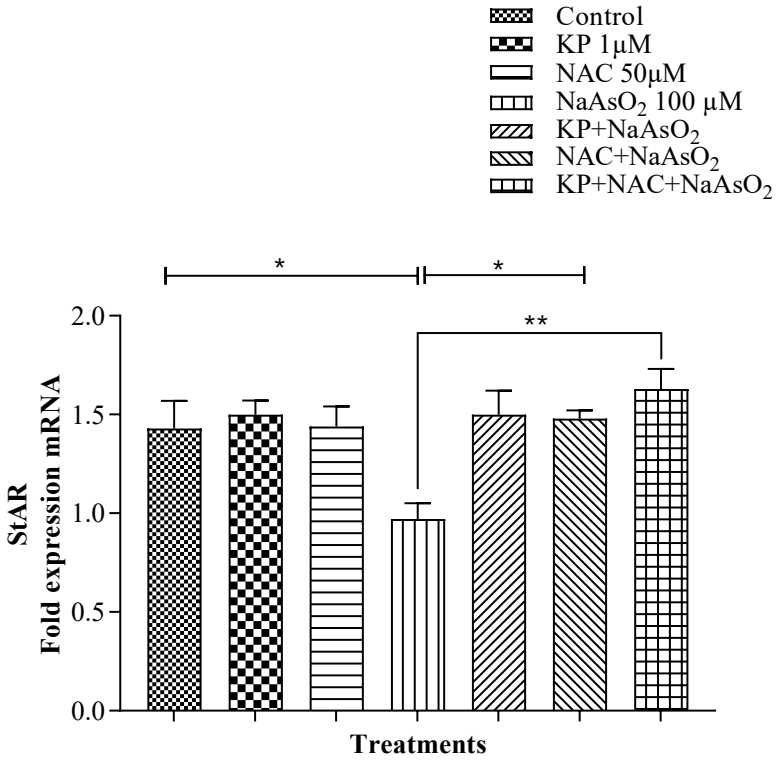


Fig. 5.12 The expression of StAR mRNA following 2 h. incubation with $NaAsO_2$, KP-10 and NAC doses. ** $p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups

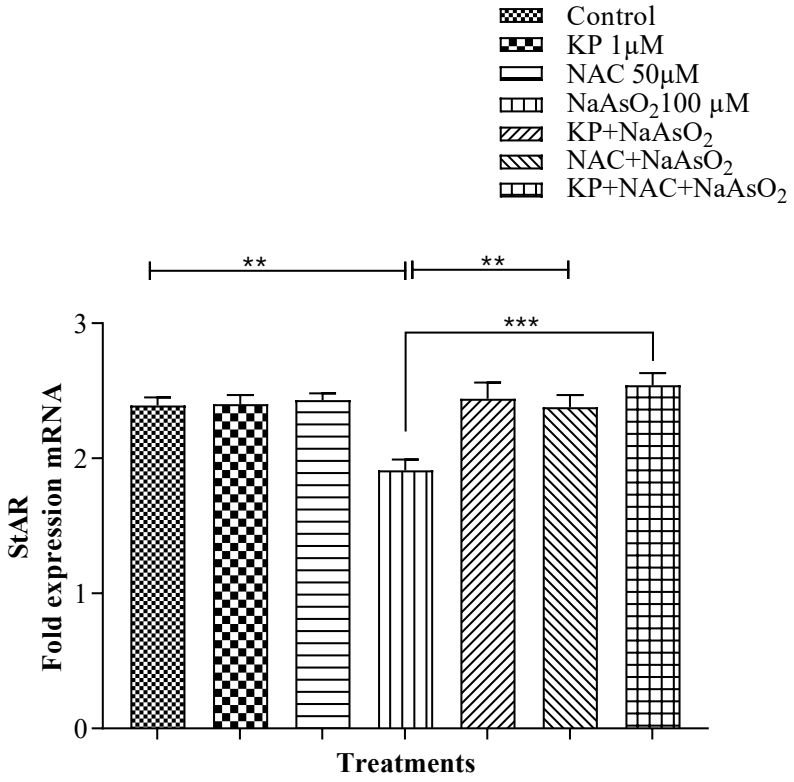


Fig. 5.13 The expression of StAR mRNA following 24 h. incubation with $NaAsO_2$, KP-10 and NAC doses. ** $p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups

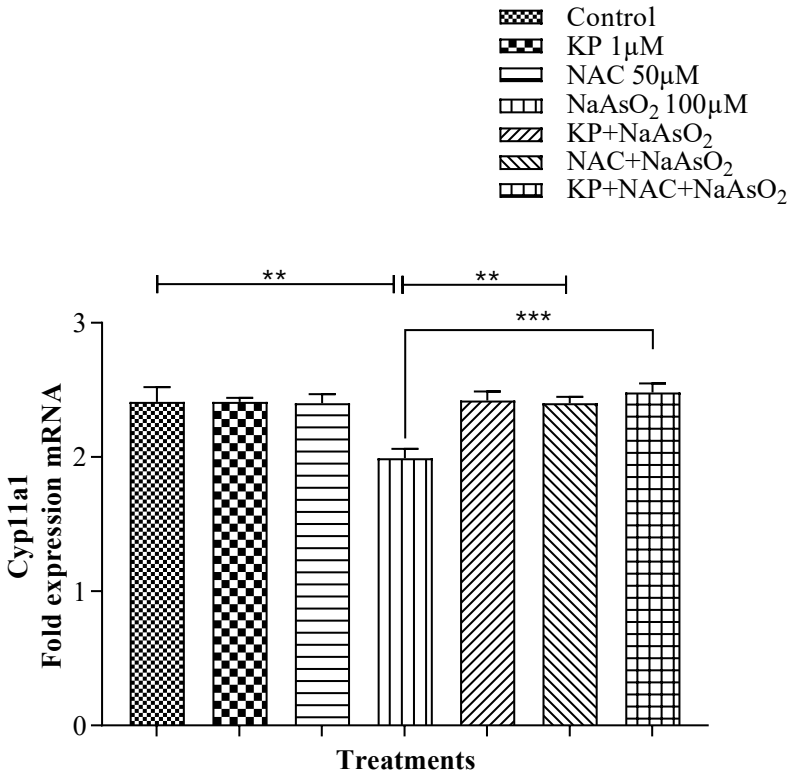


Fig. 5.14 The expression of Cyp11a1 mRNA following 2 h. incubation with $NaAsO_2$, KP-10 and NAC doses. ** $p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups

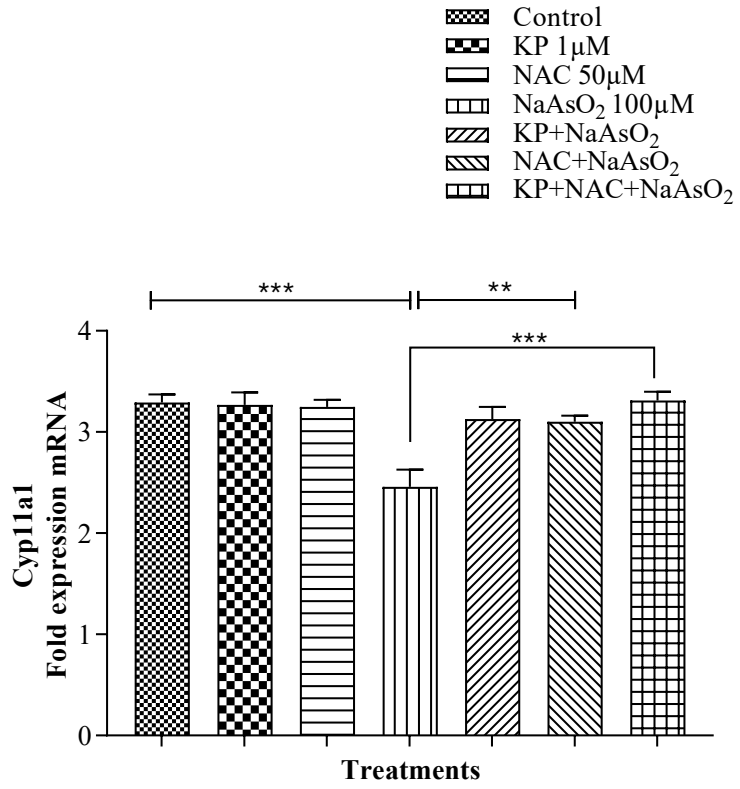


Fig. 5.15 The expression of Cyp11a1 mRNA following 24 h. incubation with $NaAsO_2$, KP-10 and NAC doses. ** $p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups

## 5.4 Discussion

Previously, both animal and clinical studies have frequently reported the toxic effects of arsenic compounds on male reproductive system including decreased weights of reproductive organs, poor seminal quality, histopathological changes and hormonal dysfunctions (Ahmad et al., 2008; Das et al., 2009). As regards the molecular mechanisms involved in mediating the arsenic-induced gonadal dysfunction, increased level of oxidative stress promoting apoptosis and suppression of the steroidogenesis have also been demonstrated in earlier studies (Khan et al., 2013; Shen et al., 2013).

In previous chapters (2-4) protective effects of both KP-10 and NAC were evaluated and compared through seminal, biochemical, hormonal and histological evaluation through both *in vivo* and *in vitro* experiments. In a quest for potential therapeutics against arsenic toxicity, evaluation at cellular and molecular level was necessary to establish their protective roles. That is why, current chapter aims at molecular evaluation of arsenic-induced apoptosis and steroidogenic suppression and its attenuation through KP-10 and NAC supplementation in both *in vivo* and *in vitro* experimental groups.

In the current study, expression levels of Caspase 3, StAR and Cyp11a1 mRNAs were evaluated through reverse transcription PCR in mice testicular tissues collected from both *in vivo* and *in vitro* experiments. Present findings revealed significant increase in the expression of caspase 3 in dose dependent manner upon 35-days exposure to both low and high doses of $NaAsO_2$ in drinking water as compared to tap water control. Likewise, testicular cultures incubated with $NaAsO_2$ alone containing media for 2 and 24 h also demonstrated significant elevation of caspase 3 mRNA expression in a time dependent manner when compared to treatment free control. These outcomes are in agreement with previously reported *in vivo* and *in vitro* studies which demonstrated the arsenic-induced upregulation of apoptotic markers including caspases. For instance, Zheng et al. (2019) have recently found that chronic exposure to $NaAsO_2$ at 5 or 50 ppm doses leads to dose dependent increase in expression of Bax and caspase 3 in mice testes. Similar observations have been noticed by Anwar et al. (2020) in testicular cultures of pre-pubertal mice incubated with 10, 50 or 100 μM doses of $NaAsO_2$. They also found significant increase in cleaved caspase 3 expression along with decreased

germ cell density at higher doses. The current observations further verify that caspase 3 is an important molecular biomarker of arsenic-induced testicular toxicity.

As regards the attenuating role of NAC against redox imbalance caused by oxidizing agents, earlier studies have suggested the suppression of apoptosis related genes to be the underlying mechanism. NAC has been found to counteract $H_2O_2$-induced apoptosis in male germ cells by significantly downregulating of caspase 3 and 9 (Maheshwari et al., 2011). Similarly, its protective effect against cadmium acetate has been reported in rat hepatocytes where it significantly decreased the activities of caspase 3 and 9 (Liu et al., 2016). Furthermore, NAC has also been reported to suppress the expression of oxidative stress related genes in rat pituitary cells exposed to $NaAsO_2$ doses (Rochenti et al., 2016). Considering these outcomes, current observations are well-justified as NAC supplementation along with $NaAsO_2$ exposure significantly prevented the upregulation of testicular caspase 3 in both *in vivo* and *in vitro* conditions.

Likewise, it was also observed that KP-10 significantly counteracted the apoptotic effect of $NaAsO_2$ by decreasing the caspase 3 mRNA expression *in vivo* and in testicular cultures incubated for 2 and 24 h. In addition, current results also revealed the equal effectiveness of KP and NAC in mitigating arsenite-induced oxidative stress through downregulation of caspase 3 which further justifies the previously mentioned results in chapter 3 and 4. These novel findings can be reasoned in the context of a recently reported study by Huang et al. (2021), who found that KP54 attenuates oxidative damage leading to neuronal apoptosis in rats suffering from subarachnoid hemorrhage (SAH) through activation of GPR54/ARRB2/PI3K/AKT/GSK3β signaling pathway. It was observed that intra-nasal administration (1nmol/kg) of KP-54 to SAH rats significantly decreased the protein levels of apoptotic biomarker proteins including BAX and cleaved caspase 3 (CC3) in the rat brain following 24 h of SAH. Furthermore, antioxidant properties of KPs as increased level of SOD have also been observed in the uterus and ovary of rats injured with ischemia-reperfusion (Aslan et al., 2017). Similar observations have been noticed in young rat livers (Aydin et al., 2010). Considering the contradictory findings as regards proliferative effect of KPs (Golzar et al., 2015; Liu et al., 2017), use of appropriate dosage and isoform of KP is necessary to ensure its benefits against oxidative damage (Huang et al., 2021). In addition, recent studies have reported the protective effects of KP-10 against $NaAsO_2$ (Fatima and Qureshi, 2022)

and methotrexate-induced (Güvenç and Akaskal, 2018) testicular toxicities through biochemical, hormonal and histological evaluation. Current results are logically explained taking into account these considerations. Like the earlier *in vivo* and *in vitro* findings (Chapter 3 and 4 respectively), combined supplementation of NAC and KP resulted in a statistically greater attenuation of the toxic effects of $NaAsO_2$ treatment as compared to alone treatments with these therapeutics.

For indirect assessment of protective effects of KP-10 and NAC against $NaAsO_2$-induced oxidative damage, testicular expression of steroidogenic genes including StAR and Cyp11a1 was also evaluated in the present study. Current findings from *in vivo* experiment revealed significant decline in the expression of both StAR and Cyp11a1 upon 35 days' exposure to drinking water containing $NaAsO_2$ alone at 4ppm and 10 ppm doses. However, StAR presented the dose dependent decline with greater suppression at high dose (10 ppm) whereas, Cyp11a1 was equally suppressed at both doses of $NaAsO_2$. Previously, an *in vivo* study on male mice subcutaneously treated with arsenic trioxide ($As_2O_3$) has also demonstrated arsenic-induced suppression of steroidogenic genes. Significant decline was observed in circulating LH, FSH and testosterone levels along with expression of mRNAs involved in testosterone synthesis including Cyp17, P450scc and 3$\beta$HSD (Chiou et al., 2008).

Expression levels of StAR and Cyp11a1 mRNA were also found downregulated in testicular cultures incubated with $NaAsO_2$ alone containing media for 2 and 24 h in time dependent manner as compared to treatment free control. These findings are supported by previous *in vitro* studies which also demonstrate significant decline in the expression of StAR, Cyp11a1 and Cyp19 mRNAs in rat ovarian tissue (Chen et al., 2022) and decreased StAR mRNA in TM3 Leydig cells upon exposure to $NaAsO_2$ doses (Taşçi et al., 2019).

As regards the KP-10 and NAC supplementation, both therapeutics equally upregulated the testicular expression of steroidogenic genes in both *in vivo* and *in vitro* experiments when compared to $NaAsO_2$ alone treated groups. In addition, synergistic effect was demonstrated in testicular cultures incubated for both 2 and 24 h with combined doses of KP and NAC as comparatively higher upregulation of StAR and Cyp11a1 when compared to $NaAsO_2$ alone treated cultures. Whereas, 35 days' exposure to both 4 and 10 ppm doses of $NaAsO_2$ in drinking water along with either alone or combined

supplementation with NAC and KP demonstrated equally upregulated Cyp11a gene in testicular tissues, when compared to $NaAsO_2$ alone treatment groups. The expression of StAR mRNA was however synergistically increased upon combined supplementation in testicular tissues following 35 days' exposure.

These findings can be reasoned by considering the steroidogenic effect of NAC in porcine placental trophoblast cells in which it significantly elevated the Cyp11a1 mRNA expression (Ding et al., 2021). Similar observations have been reported by Jallouli et al. (2016) who found that NAC restored the testicular mRNA levels for StAR protein, cytochrome p450scc and other steroidogenic enzymes in rats treated with dimethoate which is an organophosphate compound. Currently observed increase in testicular StAR and Cyp11a1 upon KP-10 supplementation for both 2 and 24 h incubation can be reasoned as steroidogenic role of KP has also been recently suggested in Leydig cells (Hsu et al., 2020). In addition, the presence of Kiss1R on spermatocytes of adult mice also suggest the possibility of paracrine signaling pathway between sperm and Leydig cells (Sharma et al., 2020). Taking into account these observations, direct steroidogenic effect of KP-10 supplementation is suggested against arsenic-induced suppression of StAR and Cyp11a1. In addition, an indirect effect of KP supplementation on steroidogenic genes can also explain the currently observed *in vivo* findings. Since KP is an endogenous endocrine regulator and its peripheral administration has long been found to stimulate GnRH secretion (Thompson et al., 2004) which ultimately leads to an increase in circulating levels of LH and testosterone (Dhillo et al., 2005). Therefore, upregulation of steroidogenic genes in current experiments could have indirectly involved the HPG-axis against endocrine disrupting effects of $NaAsO_2$ treatment.

## Conclusion

Molecular evaluation of KP-10 and NAC against sodium arsenite-induced testicular damage demonstrates for the first time that underlying mechanisms of these therapeutics might involve apoptotic and steroidogenic signalling pathways. In addition, *in vivo* and *in vitro* findings suggest both indirect and direct steroidogenic effect of KP-10 against arsenite induced suppression of steroidogenic genes. Furthermore, synergistic effect of KP-10 and NAC has also been observed at molecular level justifying their therapeutic efficacies. These outcomes support the findings mentioned in Chapter 3 and 4 however, evaluation of other oxidative stress/apoptosis related and steroidogenic genes and their proteins is necessary for comprehensive assessment. Moreover, longer incubation periods and addition of KP-10 isoforms and antagonists will further provide an insight regarding underlying signalling pathway.

# General Discussion

Toxic effects of arsenic compounds on male reproductive system have been comprehensively evaluated in various mammalian species including rats (Khan et al., 2013), mice (Reddy et al., 2011), rabbit bucks (Zubair et al., 2014) and teddy goat bucks (Zubair et al., 2016) etc. Endocrine disruption resulting in decreased levels of LH, FSH and testosterone, poor seminal parameters including decreased sperm quality and count, testicular degeneration, redox imbalance leading to increased level of oxidative stress and impaired activities of steroidogenic enzymes are among those observations which have been frequently reported by these investigations. In addition, recent *in vitro* findings have further highlighted the testicular damage caused by $NaAsO_2$ exposure in both pre-pubertal and adult mice. Incubation with varying doses of $NaAsO_2$ demonstrated elevated levels of oxidative stress and germ cell loss in adult mice testes while increased cellular apoptosis in infantile testicular cultures at high doses (Anwar and Qureshi, 2019; Anwar et al., 2020). Recently, de Araújo-Ramos et al. (2021), have further investigated the mechanism underlying arsenic-induced endocrine disruption in male rats. They observed that arsenic does not alter the testicular macrophage function leading to hormonal dysfunction instead it involves other complex interactions. There is still need for further experiments to answer the following question; Whether arsenic is a strong toxicant or a potent endocrine disruptor?

Considering the outcomes of ongoing research on arsenite-induced reproductive toxicity, it was necessary to explore novel therapeutics of both exogenous and endogenous origin. In quest for discovering such a counteracting agent, current study was designed to comprehensively and comparatively evaluate the therapeutic potential of Kiss-1 peptide (KP-10) with NAC (a well-known therapeutic) at biochemical, spermatological, hormonal, histological and genetic level against $NaAsO_2$-induced testicular damage through both *in vivo* and *in vitro* experiments.

Pharmaceutical status of KP-54 and KP-10 has been approved as new drugs against several reproductive disorders by U.S. Food and Drug Administration (FDA). Currently, they are under investigation of phase 1 or 2 of clinical trials (d'Anglemont de Tassigny et al., 2017). Moreover, protective effects of KPs have also been observed in neurological diseases like Alzheimer's (Milton et al., 2012) and other brain injuries caused by increased level of oxidative stress (Akkaya et al., 2014). Importance of KPs

in current therapeutic research can be reasoned as endogenous KPs/GPR54 signalling is known to play regulatory role in metastasis (Murphy, 2005), puberty onset (Abreu and Kaiser, 2016) and other andrological functions via HPG axis (Dhillo et al., 2005), whereas exogenous KPs treatments have recently demonstrated the antioxidant and attenuating properties against oxidative damage in liver, brain and testicular tissues of rats and mice (Aydin et al., 2010; Akkaya et al., 2014; Güvenç and Akaskal, 2018 Huang et al., 2021). Taking into account these earlier findings, series of *in vivo* and *in vitro* experiments were conducted for the first time to evaluate the antioxidant properties of KP-10 against an inorganic compound of arsenic; sodium arsenite ($NaAsO_2$).

Outcomes of the first pilot experiment (Chapter 2, Fatima and Qureshi, 2022) revealed that both continuous (Once a day) and intermittent intraperitoneal administration (once a week) of KP-10 (50 nmol/day) affectively attenuates the $NaAsO_2$-induced testicular damage in adult mice at 4ppm and 10 ppm doses via drinking water. Protective effect of KP-10 was revealed by decreased level of oxidative stress, improvement in spermatological parameters, normalized circulating testosterone levels and germ cell replenishment in the semniferous tubules and prevention of testicular deterioration. These findings also demonstrated that both regimens of KP-10 treatment have equal efficacy in mitigating the deleterious effects of arsenic exposure. Additionally, unaltered basal testosterone level and other experimental parameters upon KP-10 alone treatment (both continuous and intermittent) as compared to tap water control, further verified that exogenous KP-10 at 50 nmol dose is safe enough to be used without the risk of acute testicular degeneration. Previously, Thompson et al. (2006) have compared the different isoforms of KP and found that subcutaneous administration of KP-54 causes testicular degeneration in male rats at 50 nmol dose. In contrast, the attenuating role of KP-10 at 50nmol dose has been recently reported against oxidative damage caused by methotrexate drug in rats (Güvenç and Aksakal, 2018). Considering these findings, it is therefore concluded that factors including peptide isoform, dose and duration, route of administration, model animal species and additional intervention (exposure to arsenite as an EDC) are the main determinants of final outcomes observed in current experiment. Moreover, these *in vivo* findings also suggest the indirect effects of KP-10 through modulation of gonadotrophin levels as indicated by normalization of circulating testosterone. Measurement of LH levels in current study could have

provided better explanation in this regard. However, it was skipped considering the already available literature verifying the stimulatory effect of KP-10 on LH secretion. Such as *in vivo* studies on goats (Hashizume et al., 2010) and cattle (Ezzat et al., 2009) have previously confirmed that KP-10 stimulates gonadotrophin secretion in respective species. Taking into account the inhibitory effect of inorganic arsenic observed in animal studies (Kim and Kim, 2015), regulatory role of KP-10 against $NaAsO_2$-induced endocrine disruption is well justified. Additionally, KP-10 alone treatment did not alter the basal circulating testosterone levels in mice having access to treatment free drinking water. Considering the regulatory role KP-10 on HPG-axis, this finding needs some explanation. Such as, the substantial time interval between KP-10 administration and sampling might be the reason for such outcomes. As regards the reason for comparative evaluation of KP-10 with NAC (Chapter 3) abundant literature is available on the therapeutic potential of NAC against several oxidative stressors including the $NaAsO_2$ (Reddy et al., Salamon et al., 2019; Pedre et al., 2021). Especially, it has been found to attenuate the toxic effects of etoposide (an anti-cancer drug used for treatment of testicular cancers) on human spermatozoa (Baetas et al., 2019). Therefore, comparing the efficacy of a recently explored antioxidant with a well-established therapeutic was necessary to further strengthen the current research hypothesis. Previously, remedial effects of melatonin (another endogenous neuropeptide) and NAC have been reported against Tramadol-induced hepatotoxicity in rats (Adikwu et al., 2017).

Taking into account these considerations, experimental mice were provided with both KP-10 (once a week at 50noml dose) and NAC (for five alternative days at 75mg/kg dose) supplementation through intraperitoneal injections. Keeping in view our previous outcomes, only intermittent dosing of KP-10 was selected for comparative evaluation with NAC. The results thus obtained demonstrated that KP-10 and NAC had comparable efficacy in attenuating the oxidative damage and resulting in improved spermatological, biochemical, hormonal and histopathological changes in testicular tissue caused by 35-day exposure to low and high doses of $NaAsO_2$ exposure via drinking water. When compared to $NaAsO_2$ alone treatment, both therapeutics were found equally effective against arsenic toxicity when separately supplemented following exposure to 4ppm and 10 ppm doses of $NaAsO_2$. Combined supplementation (KP+NAC) however, produced more profound effect on all parameters as compared to alone supplementation with KP-10 thus demonstrating a synergistic effect of KP and

NAC in current experiment. Additionally, both KP-10 and NAC alone treatment without $NaAsO_2$ exposure exhibited no significant change in any parameter from tap water control group thus eliminating the risk of potential side effects of these therapeutics. These findings are in agreement with previously reported studies on NAC (Reddy et al., 2011) and KP-10 (Fatima and Qureshi, 2022), which demonstrate their protective effects against $NaAsO_2$-induced reproductive toxicity in adult male mice. In conclusion, the outcomes of respective experiment significantly verified the efficacy of currently used treatment plan, KP-10 and NAC dosing to be used in animal research.

The assessment of potential toxicants affecting male reproductive system is considered an essential part of drug development protocol (ICH Draft guideline S5(R3), 2017). In this regard, *in vivo* screening puts a few limitations on the speed of respective procedure including time consumption and requirement of large amount of test compound (Nakamura et al., 2018). Therefore, *in vitro* methods have been proposed for this purpose which either employ organ culture (Steinberger and Steinberger, 1970) or isolated cell culture (Yin et al., 2017) techniques. Moreover, several easy to evaluate reliable testicular parameters can be assessed during *in vitro* toxicity screening including histology and spermatogenesis (Sasaki et al., 2011). However, comparative testing of a potential toxicant with both *in vivo* and *in vitro* is essential for thorough evaluation (Nakamuru et al., 2018).

It is because of these reasons and considering the feasibility and requirement, organ culture technique was employed to evaluate the currently proposed research hypothesis (Chapter 4). Following the 2 and 24 h incubation with $NaAsO_2$ (100 µM), KP-10 (1 µM) and NAC (50 µM), biochemical, hormonal and histological parameters were evaluated from testicular fragments collected from respective treatment groups. The obtained findings revealed significant toxicity of 100 µM dose of $NaAsO_2$ following both 2 and 24 h incubation as compared to treatment free control. However, both KP-10 and NAC supplementation either alone or in combination equally attenuated the deleterious effects of $NaAsO_2$ exposure. Interestingly, synergistic effect of combined dose of KP and NAC was significantly higher as compared to KP-10 alone supplementation thus further verifying the earlier *in vivo* outcomes which also demonstrated similar effect. In addition, alone incubation with KP-10 or NAC without $NaAsO_2$ treatment, revealed no significant change in intra-testicular testosterone level, tissue histology or other biochemical parameters when compared to treatment free

control. These findings are supported by previous literature as $NaAsO_2$ is found to induce testicular degeneration in organ cultures incubated with high doses (Anwar et al., 2019).

Moreover, it is also evident through the current experimentation that KP-10 treatment does not alter the intra-testicular testosterone secretion if provided without any additional intervention. These findings can be reasoned with the outcomes reported by Irfan et al. (2013), who found significant increase in hCG stimulated testosterone secretion in monkeys treated with acylin which is a GnRH inhibitor. Whereas, alone treatment with hCG did not alter the basal testosterone level. Moreover, Mei et al. (2013) reported no significant change in testosterone release from testicular fragments upon treatment with KP-10. Therefore, currently demonstrated *in vitro* findings affectively support the previous *in vivo* results mentioned in chapter 3.

In conclusion, both *in vivo* and *in vitro* findings of current study revealed that $NaAsO_2$ exerts deleterious effects on male reproductive system of adult mice. Its role as an endocrine disruptor was indicated by significantly decreased circulating and intra-testicular testosterone levels. Whereas, as a strong oxidizing agent, it was found to increase the testicular ROS and TBARS levels while on the other, decreasing the activities of antioxidant enzymes SOD, POD and CAT along with non-enzymatic GSH. Indirectly, its damaging effect was demonstrated by increased testicular LDH levels resulting in tissue damage observed through testicular histology and morphometry. Moreover, reproductive performance was directly impaired revealed as decreased spermatological parameters including sperm count, motility and viability along with decreased seminal fructose levels. Whereas, supplementation with both KP-10 and NAC either alone or combined, significantly prevented the aforementioned pathological changes and improved the spermatological, biochemical, hormonal and histological parameters near to control.

Considering the recent advancements in toxicology and drug discovery research, molecular assessment of underlying mechanisms through which Kiss1-peptide (KP-10) might protect against $NaAsO_2$-induced testicular damage was necessary to establish its therapeutic role. Previous studies have reported the oxidative damage accelerating the cellular apoptosis and suppressing the steroidogenesis as the underlying mechanisms mediating the deleterious effects of arsenic compounds on reproductive system (Khan

et al., 2013; Shen et al., 2013). As regards the apoptosis, caspases are considered authentic biomarkers as have been mentioned in chapter 5. Moreover, both *in vivo* and *in vitro* treatments with arsenic have been found to upregulate the Caspase 3 expression in mice testes (Zheng et al., 2019; Anwar et al., 2020) thus promoting cellular apoptosis. Likewise, downregulation of steroidogenic genes including StAR and Cyp11a1 has also been reported recently in rat ovarian cells (Chen et al., 2022) and TM3 Leydig cells (Taşçi et al., 2022) following $NaAsO_2$ treatment.

Considering the recent literature on Caspase 3, StAR and Cyp11a1 as molecular markers of arsenic toxicity and the regulatory role of KP-10 in steroidogenesis, testicular tissues from both *in vivo* and *in vitro* experiments were evaluated for expression of mRNAs for Caspase 3, StAR and Cyp11a1 through RT-PCR. The obtained results were in agreement with previously reported findings as significantly increased Caspase 3 mRNA expression was observed upon 35-days exposure to $NaAsO_2$ alone via drinking water and 2 or 24 h incubation with media containing $NaAsO_2$ alone doses when compared to treatment free controls. Whereas, StAR and Cyp11a1 mRNA exhibited significant decline in these experimental groups. However, supplementation with both KP-10 and NAC alone equally prevented this change and significantly decreased the Caspase 3 mRNA expression while increased the StAR and Cyp11a1 expression as compared to $NaAsO_2$ alone treatment in both experimental regimens. In addition, the synergistic effect of combined supplementation with these therapeutics was also evident at molecular level as significantly greater downregulation of Caspase 3 and upregulation of StAR/Cyp11a1 was observed when compared to alone KP-10 and NAC supplementation.

Current observations are well justified considering the outcomes of earlier investigations. Such as, steroidogenic effect of NAC has been reported in rat testes where it affectively attenuated the dimethoate- (mentioned earlier in chapter 5) induced suppression of StAR and other steroidogenic genes (Jallouli et al., 2016). Moreover, NAC has also been found to elevate the Cyp11a1 mRNA expression in porcine trophoblast cells *in vitro* (Ding et al., 2021).

In the context of anti-apoptotic effect of KP-10, a recent study has demonstrated that KP-54 bears the potential to decrease the protein levels of apoptotic biomarkers including BAX and cleaved caspase 3 (CC3) in brains of rats suffered from

subarachnoid hemorrhage (SAH) which results in an increased oxidative stress leading to neuronal apoptosis (Huang et al., 2021). In addition, the antioxidant properties of different isoforms of KP have been previously reported in rat liver (Ayedin et al., 2010), uterus and ovary (Aslan et al., 2017).

As mentioned earlier in chapter 5, previous findings have revealed the steroidogenic role of KP-10 in Leydig cells (Hsu et al., 2020). In addition, involvement of paracrine signaling pathway in regulating steroidogenesis has also been proposed as expression of Kiss1R on spermatocytes has also been discovered (Sharma et al., 2020). Additionally, indirect effect of KP-10 supplementation on steroidogenic genes in *in vivo* experiment can be explained as activation of HPG axis by peripherally administered KP leads to elevation of circulating LH and testosterone levels (Dhillo et al., 2005).

Therefore, RT-PCR results obtained from current *in vivo* and *in vitro* experiments, suggest attenuating role of KP-10 supplementation against $NaAsO_2$-induced suppression of steroidogenesis and activation of apoptosis. These effects can be explained either indirectly through regulation of HPG axis or directly by involvement of intra-testicular signaling pathways. Moreover, NAC supplementation has been found to mediate its antioxidant effect against arsenite-induced oxidative damage at genetic level by suppressing caspase 3 mRNA expression. Steroidogenic effect of NAC has also been verified through current observations.

Although, current study aimed to comprehensively evaluate the biochemical, histological, hormonal and molecular parameters however, a few limitations were also encountered which need future investigation. Such as, use of KP-antagonist along with testing other isoforms of KP will further verify the current observations. Moreover, prolonged incubation periods along with pre and post treatment assessment are also necessary for direct evaluation of both KP-10 and NAC supplementation in testicular cultures. Additionally, mRNA expression of other oxidative stress related and steroidogenic genes along with their protein estimation will provide the better insight into complete assessment of underlying intra-signalling pathways. Lastly, current study was mainly focused on testicular toxicity in adult mice however further investigations on pre-pubertal mice along with analysing direct effect on spermatozoa and other accessory sex organs will further support the research hypothesis.

## General Conclusion

Considering the outcomes of current experiments presented in this thesis, it can be concluded that KP-10 bears the potential to be considered as an endogenous treatment option either alone or in combination with another established therapeutic like NAC against $NaAsO_2$-induced deleterious effects on male reproductive system. Both direct and indirect role of KP-10 in protecting testicular physiology against arsenite-induced toxicity is verified through *in vitro* and *in vivo* experiments. Additionally, current study also highlights the cytotoxic and endocrine disrupting effects of sodium arsenite on male reproductive system.

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# Intraperitoneal kisspeptin-10 administration ameliorates sodium arsenite-induced reproductive toxicity in adult male mice

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## Abstract

The current study investigated the protective ameliorative effect of intraperitoneally administered kisspeptin-10 (50 nmol/day) against reproductive toxicity in adult male mice challenged with 35 days of exposure to sodium arsenite in drinking water. Mice were divided into tap water control, sodium arsenite-alone (4 ppm and 10 ppm), kisspeptin-alone (intermittent and continuous) and combined (sodium arsenite +kisspeptin-10 intermittent and continuous) treatment groups. Results revealed protective effect of both intermittent and continuous kisspeptin doses on reproductive organs against sodium arsenite-induced toxicity. This was indicated by an increase ($p < 0.001$) in the activity of antioxidant enzymes and a decrease ($p < 0.001$) in the levels of oxidative stress biomarkers. Concomitant significant increase was noticeable in the relative organ weight ($p < 0.01$), and serum testosterone and seminal fructose ($p < 0.001$), and a significant improvement in sperm parameters was also observed. A significant downregulation of lactate dehydrogenase concentration demonstrated further the protective effect of kisspeptin against tissue damage. Histologically, both treatment regimens of kisspeptin combined with sodium arsenite exposure prevented massive germ cell loss and tissue damage, a condition prominent in sodium arsenite-alone-treated mice. The study demonstrates for the first time kisspeptin's potential to mitigate the biochemical and histotoxic effects of arsenic on male reproductive system.

**KEYWORDS**
kisspeptin, laboratory mice, reproductive toxicity, sodium arsenite

## 1 | INTRODUCTION

Chronic exposure to heavy metals may indeed be one of the underlying causes of rapidly increasing male infertility (Ma et al., 2019). Heavy metals are those environmental contaminants that are known to cause reproductive dysfunction in males. A negative correlation has been found between progressive motility of mature spermatozoa and levels of cadmium (Cd) and arsenic (As) in humans (He et al., 2020; Li et al., 2018). Oxidative damage caused by the generation of free radicals (reactive oxygen or nitrogen species, ROS/RNS) is considered to be the underlying cause of heavy metal poisoning (Souza et al., 2016). In this context, the presence of high content of unsaturated fatty acids in the cell membranes of spermatozoa makes them more susceptible to oxidative damage (Tremellen, 2008), a major contributing factor towards male factor infertility (Bisht et al., 2017).

  

Being well-known toxic metalloid and having ubiquitous distribution, arsenic is considered a serious health hazard. Exposure to arsenic has been linked with diseases such as cancer, diseases of the gastrointestinal tract (GIT), diabetes and neurological disorders. Inorganic arsenic is also known to cause impairment of the andrological functions through lowering the sperm count and decreasing the weights of gonadal and accessory sex glands (Kim & Kim, 2015). Trivalent arsenic is more toxic than its pentavalent counterparts. This is so because it readily blocks several enzymatic pathways (Brachowsky et al., 1999). Deleterious effects of sodium arsenite ($NaAsO_2$) on testicular and epididymal organ cultures in mice have been recently documented. These include increased ROS levels, decreased antioxidant enzyme levels, decreased blood testosterone concentration and excessive sperm DNA damage, particularly at slightly high concentrations (50 and 100 μM) (Anwar & Qureshi, 2019). In addition, dose-dependent induction of apoptosis and germ cell loss were also reported in postnatal day 5 CD1 mouse testes exposed to arsenic (Anwar et al., 2020).

To counter the arsenic-induced oxidative stress, which is the major cause of testicular damage (Kim et al., 2011), a biochemical agent with strong antioxidant properties is required as a candidate countering agent. Kisspeptin (KP-10), which is an endogenous reproduction-related small neuropeptide, appears to bear such potential. It was initially isolated from melanoma cell lines and was characterised as a 54 amino acid protein encoded by an oncosuppressor gene '*KISS1*' (Lee et al., 1996). Later, it was established that kisspeptide binds as a ligand to a G protein-coupled receptor called GPR54 (Ohtaki et al., 2001). KISS1 gene products include several isoforms comprising 54, 14, 13 and 10 amino acids. All these isoforms are in general termed kisspeptins. The decapeptide KP-10 is the main peptide since this sequence of amino acids, which is common to all isoforms, is the sequence required for complete stimulation of GPR54 (Pasquier et al., 2014). KP-10 is although mainly expressed in the hypothalamic area of the central nervous system (CNS), but its expression has also been reported in several peripheral tissues including the ovaries, testes, liver, pancreas (Richard et al., 2008). KP has long been considered a puberty-onset protein (Tena-Sempere, 2006). It stimulates the release of gonadotrophin-releasing hormone (GnRH) from the hypothalamus, which then regulates the release of anterior pituitary gonadotrophins, the luteinising hormone (LH) and follicle-stimulating hormone (FSH) (Mikkelsen & Simonneaux, 2009).

A relationship between oxidative stress and gonadotrophins has already been demonstrated by previous studies (Appasamy et al., 2007; Terasaka et al., 2017). For instance, the production of ROS leads to hypothalamic dysfunction through generation of oxidative stress and subsequent lowering of LH and FSH (Darbandi et al., 2018). Although its role as a binding bridge between sex steroid levels and GnRH has been extensively suggested, literature relating kisspeptin with infertility is still deficient (Trevisan et al., 2018). Considering that KP-10 is an endogenous hormone and safe to use in experimental animals or even humans, its use as a potential therapeutic agent has also been suggested (Abbara et al., 2020; Qureshi & Fatima, 2020). Besides, antioxidant potential of KP-10 has also been reported in a study, which demonstrated that it can protect testicular damage in male rats caused by methotrexate-induced oxidative stress (Güvenç & Aksakal, 2018). Antioxidant activities, including suppression of ROS/RNS, stimulation of antioxidant enzymes and suppression of pro-oxidant enzymes of another neuropeptide hormone, namely melatonin (Hacışevki & Baba, 2018; Maitra & Hasan, 2016), are already on record. KP-10 is a reproduction-related neuropeptide hormone, but similar protective potential of KP-10 against oxidative stress has not been studied. This study explored the potential protective effect of KP-10 against $NaAsO_2$-induced spermatotoxicity and testicular damage in adult male laboratory mice.

## 2 | MATERIALS AND METHODS

### 2.1 | Ethics statement

All experiments were approved by the 'Bioethical Committee of Quaid-i-Azam University' on Care and Use of Animals for Scientific Research (BEC-FBS-QAU/2021-308). Animal handling also strictly conformed with Animals (Scientific Procedures) Act, 1986, and associated guidelines, EU Directive 2010/63/EU for animal experiments.

### 2.2 | Chemicals and reagents

Kisspeptin-10 (KiSS-1; 112–121; H-YNWNSFGLRF-$NH_2$) was obtained as lyophilised powder from Calbiochem. Sodium pentobarbital (Sigma) and heparin (Kota Bharu, Kelantan, Malaysia) were purchased locally. Inorganic sodium arsenite ($NaAsO_2$) was purchased from Sigma-Aldrich.

### 2.3 | Animals

Male Swiss albino mice (35–50 days old) were procured from the National Institute of Health (NIH), Islamabad, and maintained in the Animal House Facility of Quaid-i-Azam University, Islamabad. These were housed in transparent polycarbonate small rodent cages (6 mice per cage) and maintained under standard laboratory conditions (temperature 25 ± 4°C, photoperiod of 12:12-h light: dark hours and 40% humidity). Mice were acclimatised for 1 week. They were given free access to standard rodent chow and fresh drinking water *ad libitum*.

### 2.4 | Dose selection and preparation

Doses of both inorganic arsenic $NaAsO_2$ and KP-10 were selected as per previous investigations (Ayturk et al., 2017; Guvenc et al., 2018). For $NaAsO_2$, 100 ppm stock solution was prepared in distilled water. This was further diluted with drinking tap water to obtain the required concentrations of low (4 ppm) and high (10 ppm) doses. For KP-10, 1 mg lyophilised powder was used to make stock solution in

normal saline, and final injectable dose of 50 nmol/day was worked out in distilled water. $NaAsO_2$ was given orally in drinking water, while KP-10 was administered as intraperitoneal (i.p) injections. Doses were prepared fresh before use.

## 2.5 | Experimental design

Mice ($n = 54$) were selected randomly and allocated to nine groups; each contained six animals. Taking into consideration the length of the spermatogenic cycle, mice were exposed to respective doses of $NaAsO_2$ and KP-10 for 35 days (Reddy et al., 2011). Two regimens of KP-10 doses were devised: intermittent treatment (once a week) and continuous treatment (daily dose for 35 days). Experimental groups were as follows: control having free access to drinking tap water without any treatment; KP alone (intermittent) + drinking tap water; KP alone (continuous) + drinking tap water; $NaAsO_2$ alone 4 ppm in drinking water; $NaAsO_2$ alone 10 ppm in drinking water; $NaAsO_2$ 4 ppm orally in drinking water +KP intermittent; $NaAsO_2$ 4 ppm +KP continuous; $NaAsO_2$ 10 ppm +KP intermittent; and $NaAsO_2$ 10 ppm +KP continuous.

## 2.6 | Necropsy

Towards the end of 35 days of experimental period, mice were fasted overnight, weighed next morning and killed with an overdose of sodium pentobarbital (80 mg/kg b.w). Animals were quickly dissected, venous blood was collected through the heart and centrifuged at 12,204 *g* for 5 min, and serum was collected and stored at −20°C for the determination of testosterone concentration. Testes, epididymides and seminal vesicles were collected and rinsed with saline. One of the testes was fixed in 10% buffered formalin for histology, while the other testis was stored at −20°C to prepare homogenate for the determination of biochemical parameters. Epididymides and seminal vesicles were collected to determine the extent of sperm damage and seminal fructose concentration respectively. Relative organ weight was calculated using the following formula:

$$\text{Relative organ weight: } \text{Weight of the organ (g)} \div \text{Weight of the animal body (g)} \times 100$$

## 2.7 | Sperm analyses

Sperm count, motility and viability were determined by diluting the epididymal fluid with normal saline according to the method of WHO (2010) using an improved Neubauer haemacytometer. Total motile and non-motile spermatozoa were taken into account with motility expressed as percentage of total motile spermatozoa of the total sperm count, whereas sperm viability was determined with 1% trypan blue solution as described by Talbot and Chacon (1981). Unstained spermatozoa were considered viable, and viability was expressed as percentage of unstained spermatozoa of the total count.

## 2.8 | Tissue histology

Fixed testicular tissues were processed for standard histology. Briefly, testicular tissues were dehydrated in ascending ethanolic grades, cleared in xylene and embedded in molten paraffin wax pre-warmed to 56°C. Thick sections (5 μm thickness) were cut on a rotary microtome (Shandon Finesse, Italy). Sections were then dewaxed and rehydrated with descending grades of alcohol. These were stained with the conventional haematoxylin and eosin (H & E) staining, washed in tap water, dehydrated again and coverslipped with DPX (BDH) mountant medium. Sections were photographed on a light microscope (OLYMPUS CX41, Japan) with an attached camera (Canon) at 40x magnification. Photograph plates were prepared using the Adobe Photoshop (ver. 7. 0, Microsoft, Inc.).

## 2.9 | Morphometry

Seminiferous tubular sections ($n = 30$) from each group were used for quantification of morphometric parameters. Sections were randomly selected with preference given to circular seminiferous tubules. Parameters including tubular diameter, lumen diameter and germinal epithelium height were measured at 40× magnification using the software ImageJ (USA). ImageJ was first calibrated to accept the distance of 50 μm (scale bar). Then, by using appropriate tools, the measures of seminiferous tubules were taken to calculate the average distance (μm) between respective components. Tubular diameter was calculated from average distance between the opposing points of tubular boundaries. Similarly, the average distance between tubular and luminal boundaries was taken as epithelial height, whereas the distance between opposing points at the inner layer of epithelial membrane was considered as luminal diameter.

### 2.9.1 | Homogenate preparation

Testicular tissues were homogenised in 1 ml of phosphate-buffered saline (PBS) and centrifuged at 12,204 *g* for 10 min. The supernatants were collected and stored at −20°C to conduct later the biochemical assays.

### 2.9.2 | Oxidative stress biomarkers, antioxidant enzymes and non-enzymatic GSH

Testicular homogenates were prepared according to the methods as described (Anwar & Qureshi, 2019), for the assessment of reactive oxygen species (ROS) and lipid peroxidation (TBARS), levels of reduced glutathione (GSH) and antioxidant enzymes, the superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT).

#### 2.9.3 | Lactate dehydrogenase (LDH)

Testicular LDH levels were determined from stored supernatants using a commercial LDH kit (AMP, diagnostics) and following the protocol given therein by the manufacturer.

#### 2.9.4 | Seminal fructose

For seminal vesicle homogenates, tissue was mixed 1 ml distilled water and centrifuged at 12,204 *g* for 10 min. The supernatants were then collected and stored at −20°C for determination of fructose concentration (Ramzan et al., 2013). Seminal vesicle fructose concentration was then determined using a commercial fructose kit (FertiPro NV). Assay was carried out according to manufacturer's instructions, and fructose levels were determined on a spectrophotometer (Agilent 8453, USA). The absorbance of control and samples was measured at 505 nm.

#### 2.9.5 | Determination of testosterone concentration

Serum testosterone levels were determined through enzyme-linked immunosorbent assay (ELISA) using the commercial kit (Bioactive, Germany). Inter- and intra-assay coefficients of variation were, respectively, 6.4% and 3.8%. The absorbance of control and samples was read at 450 nm.

#### 2.9.6 | Statistical analysis

Data were analysed statistically using the software GraphPad Prism (version 5.0). One-way analysis of variance followed by Tukey's multiple comparison test was applied to compare the difference between the means of control and treatment groups. The level of significance was set at $p < 0.05$, $p < 0.01$ and $p < 0.001$. Data are expressed as mean ±standard error of mean (s.e.).

## 3 | RESULTS

### 3.1 | Body weight and relative organ weight

No significant alteration occurred in the body weights in any of the experimental groups. Relative weights of testes ($p < 0.05$), epididymides ($p < 0.05$; $p < 0.01$) and seminal vesicles ($p < 0.01$) were, however, found significantly lowered in mice exposed to 4 ppm and 10 ppm doses of $NaAsO_2$, while no such change was observed in mice where a treatment of intermittent or continuous KP-10 doses was given along with exposure to $NaAsO_2$ (Table 1).

### 3.2 | Sperm count, motility and viability assay

Epididymal sperm count, progressive sperm motility and percentage of viable spermatozoa decreased significantly ($p < 0.001$) upon treatment with 4 ppm and 10 ppm doses of $NaAsO_2$. In contrast, co-administration of KP-10 as intermittent or continuous doses along with respective doses of $NaAsO_2$ prevented the decline in the above parameters (Table 2).

### 3.3 | Biochemical analysis

#### 3.3.1 | Testicular lactate dehydrogenase (LDH)

Significantly increased ($p < 0.001$) LDH levels in mice exposed to $NaAsO_2$ alone (4 ppm and 10 ppm) indicated testicular damage, which was restored with KP-10 treatment to near control level (Figure 1a).

**TABLE 1** Body weight and relative organ weights (W/W%) of mice following 35 days of exposure to 4 ppm and 10 ppm doses of sodium arsenite ($NaAsO_2$) alone and/or with 50 nmol/day KP-10

| Treatments | Bodyweight (g) | Testes | Epididymides | Seminal vesicles |
|---|---|---|---|---|
| Tap water(control) | $38.33^a \pm 0.81$ | $0.70^a \pm 0.02$ | $0.51^a \pm 0.01$ | $0.73^a \pm 0.02$ |
| KP alone (intermittent) | $35.00^a \pm 0.82$ (−8.68) | $0.73^a \pm 0.04$ (4.28) | $0.49^a \pm 0.04$ (−3.92) | $0.69^a \pm 0.02$ (−5.47) |
| KP alone (continuous) | $34.92^a \pm 0.55$ (8.89) | $0.74^a \pm 0.04$ (5.71) | $0.46^a \pm 0.01$ (−9.80) | $0.71^a \pm 0.02$ (−2.73) |
| $NaAsO_2$ (4 ppm) | $37.92^a \pm 1.52$ (−1.06) | $0.52^b \pm 0.03^*$ (−25.71) | $0.39^b \pm 0.02^*$ (−23.52) | $0.51^b \pm 0.03^{**}$ (−30.13) |
| $NaAsO_2$ (4 ppm)+KP intermittent | $34.75^a \pm 1.08$ (−9.33) | $0.62^a \pm 0.05$ (−11.42) | $0.41^a \pm 0.03$ (−19.60) | $0.68^a \pm 0.04$ (−6.84) |
| $NaAsO_2$ (4 ppm)+KP continuous | $35.42^a \pm 0.88$ (−7.59) | $0.64^a \pm 0.02$ (−8.57) | $0.48^a \pm 0.04$ (−5.88) | $0.71^a \pm 0.03$ (−2.73) |
| $NaAsO_2$ (10 ppm) | $34.58^a \pm 1.26$ (−9.78) | $0.50^c \pm 0.03^*$ (−28.57) | $0.29^c \pm 0.02^{**}$ (−43.13) | $0.47^c \pm 0.02^{**}$ (−35.61) |
| $NaAsO_2$ (10 ppm)+KP intermittent | $38.50^a \pm 1.20$ (0.44) | $0.63^a \pm 0.03$ (−10) | $0.50^a \pm 0.02$ (−1.96) | $0.67^a \pm 0.02$ (−8.21) |
| $NaAsO_2$ (10 ppm)+KP continuous | $36.75^a \pm 1.60$ (−4.12) | $0.66^a \pm 0.02$ (−5.71) | $0.45^a \pm 0.04$ (−11.76) | $0.70^a \pm 0.03$ (−4.1) |

*Note:* Values are mean ±s.e.

Different superscripts in a column show significant difference at $^*p < 0.05$ and $^{**}p < 0.01$. Values in parentheses represent % change from tap water control ($n = 6$/group).

**TABLE 2** Sperm count, motility and viability parameters in mice following 35 days of exposure to 4 ppm and 10 ppm doses of sodium arsenite ($NaAsO_2$) alone and/or with 50 nmol/day KP-10

| Treatments | Sperm count ($10^6$ $mL^{-1}$) | Sperm motility % | Sperm viability% |
|---|---|---|---|
| Tap water (control) | $67.66^a \pm 1.14$ | $67.00^a \pm 2.59$ | $64.00^a \pm 1.93$ |
| KP alone (intermittent) | $71.66^a \pm 1.66$ | $62.66^a \pm 2.37$ | $67.33^a \pm 1.85$ |
| KP alone (continuous) | $74.16^a \pm 2.18$ | $68.66^a \pm 2.04$ | $68.33^a \pm 2.02$ |
| $NaAsO_2$ (4 ppm) | $46.54^b \pm 2.39^{***}$ | $43.16^b \pm 2.03^{***}$ | $47.83^b \pm 2.67^{***}$ |
| $NaAsO_2$ (4 ppm)+KP intermittent | $65.33^a \pm 1.68$ | $68.16^a \pm 2.27$ | $68.66^a \pm 2.06$ |
| $NaAsO_2$ (4 ppm)+KP continuous | $67.00^a \pm 2.29$ | $67.16^a \pm 2.25$ | $67.00^a \pm 2.46$ |
| $NaAsO_2$ (10 ppm) | $31.52^c \pm 1.94^{***}$ | $33.83^c \pm 1.90^{***}$ | $33.33^c \pm 1.94^{***}$ |
| $NaAsO_2$ (10 ppm)+KP intermittent | $67.50^a \pm 2.09$ | $66.00^a \pm 1.36$ | $69.16^a \pm 2.22$ |
| $NaAsO_2$ (10 ppm)+KP continuous | $64.83^a \pm 2.85$ | $67.33^a \pm 2.15$ | $68.66^a \pm 2.55$ |

*Note:* Values are mean ±s.e.

Different superscripts in a column show significant difference at $^{***}p < 0.001$ ($n = 6$/group).

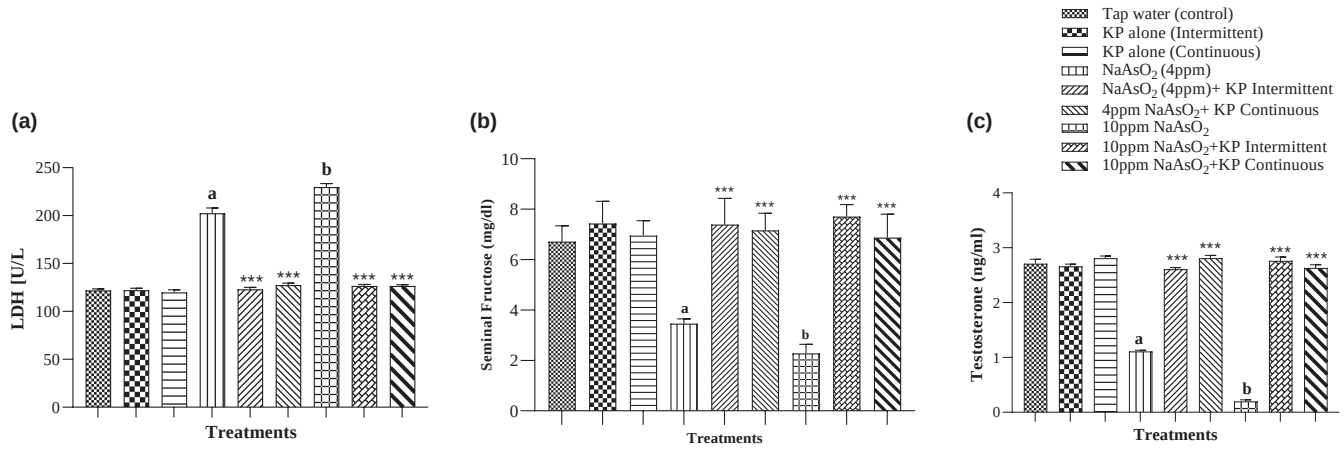


**FIGURE 1** Mean testicular LDH level (U/L), seminal fructose concentration (mg/dl) and serum testosterone concentration (ng/ml) of adult male mice ($n = 6$/group) upon 35 days of exposure to 4 ppm and 10 ppm doses of $NaAsO_2$ and KP-10 (50 nmol/day). Letters a & b show significant difference of 4 ppm and 10 ppm doses of $NaAsO_2$ alone, respectively, from control. *** $p < 0.001$ = significantly decreased testicular LDH levels in KP+$NaAsO_2$-treated groups as compared to $NaAsO_2$-alone (4 and 10 ppm) treatment group (a); and significantly increased seminal fructose levels (b) and serum testosterone concentration (c) in KP-10 +$NaAsO_2$-treated groups as compared to $NaAsO_2$-alone treatment group

### 3.3.2 | Seminal fructose level

Exposure to $NaAsO_2$ (4 ppm and 10 ppm) alone led to significant decline in seminal fructose concentrations ($p < 0.001$). This decline was prevented by intermittent or continuous KP-10 treatment in the combined $NaAsO_2$ and KP-10 groups of mice (Figure 1b).

### 3.3.3 | Oxidative stress biomarkers and antioxidant enzymes

Testicular homogenates procured from mice exposed to $NaAsO_2$-alone doses revealed significantly increased levels of ROS and TBARS ($p < 0.01$; $p < 0.001$) with a concomitant significant decrease in the levels of antioxidant enzymes, SOD, POD and CAT ($p < 0.001$). In contrast, KP-10 treatment combined with $NaAsO_2$ doses led to a significant reduction in the ROS and TBARS levels while improving the activity of antioxidant enzymes (Table 3).

### 3.3.4 | Reduced glutathione (GSH)

Testicular GSH levels were found significantly decreased ($p < 0.001$) in $NaAsO_2$ (4 ppm and 10 ppm) treatment groups, but exposure to $NaAsO_2$ along with KP-10 treatment revealed prevention of GSH decline in the testes (Table 3).

## 3.4 | Serum testosterone

Mice exposed to 4 ppm and 10 ppm doses of $NaAsO_2$ demonstrated significant decrease ($p < 0.001$) in serum testosterone, whereas

**TABLE 3** Oxidative stress parameters in testicular homogenates of mice following 35 days of exposure to 4 ppm and 10 ppm doses of sodium arsenite ($NaAsO_2$) alone and/or with 50 nmol/day KP-10

| Groups | ROS (abs) | TBARS (nM $min^{-1}$ mg $protein^{-1}$) | SOD Unit $mg^{-1}$ | POD Unit $min^{-1}$ | CAT Unit $min^{-1}$ | GSH μM/g |
|---|---|---|---|---|---|---|
| Tap water control | $1.17^{a} \pm 0.03$ | $0.81^{a} \pm 0.04$ | $16.04^{a} \pm 1.28$ | $7.35^{a} \pm 0.75$ | $6.31^{a} \pm 0.33$ | $8.06^{a} \pm 0.57$ |
| KP alone (intermittent) | $1.15^{a} \pm 0.02$ | $0.79^{a} \pm 0.10$ | $13.28^{a} \pm 1.08$ | $6.41^{a} \pm 0.55$ | $5.10^{a} \pm 0.39$ | $7.95^{a} \pm 0.51$ |
| KP alone (continuous) | $1.15^{a} \pm 0.02$ | $0.89^{a} \pm 0.13$ | $12.68^{a} \pm 1.83$ | $6.41^{a} \pm 0.69$ | $5.54^{a} \pm 0.27$ | $8.76^{a} \pm 0.36$ |
| $NaAsO_2$ (4 ppm) | $1.48^{b} \pm 0.04^{**}$ | $1.62^{b} \pm 0.06^{***}$ | $5.27^{b} \pm 0.05^{*}$ | $2.29^{b} \pm 0.46^{***}$ | $3.68^{b} \pm 0.26^{***}$ | $3.98^{b} \pm 0.52^{***}$ |
| $NaAsO_2$ (4 ppm)+KP intermittent | $1.22^{a} \pm 0.02$ | $0.89^{a} \pm 0.09$ | $12.93^{a} \pm 0.87$ | $6.09^{a} \pm 0.55$ | $5.47^{a} \pm 0.33$ | $7.78^{a} \pm 0.66$ |
| $NaAsO_2$ (4 ppm)+ KP continuous | $1.22^{a} \pm 0.03$ | $0.81^{a} \pm 0.07$ | $15.75^{a} \pm 1.27$ | $6.46^{a} \pm 0.53$ | $6.09^{a} \pm 0.29$ | $8.56^{a} \pm 0.25$ |
| $NaAsO_2$ (10 ppm) | $1.71^{c} \pm 0.14^{***}$ | $1.93^{c} \pm 0.05^{***}$ | $3.29^{b} \pm 1.07^{**}$ | $1.30^{c} \pm 0.21^{***}$ | $0.79^{c} \pm 0.05^{***}$ | $2.19^{c} \pm 0.35^{***}$ |
| $NaAsO_2$ (10 ppm)+KP intermittent | $1.24^{a} \pm 0.03$ | $0.72^{a} \pm 0.11$ | $12.54^{a} \pm 3.63$ | $6.13^{a} \pm 0.47$ | $5.54^{a} \pm 0.33$ | $7.93^{a} \pm 0.39$ |
| $NaAsO_2$ (10 ppm)+KP continuous | $1.21^{a} \pm 0.05$ | $0.69^{a} \pm 0.05$ | $12.40^{a} \pm 2.58$ | $6.76^{a} \pm 0.69$ | $5.52^{a} \pm 0.50$ | $6.61^{a} \pm 0.52$ |

*Note*: Values are mean ±s.e.
Different superscripts in a column show significant difference at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. ($n = 6$/group).

treatment with intermittent or continuous KP-10 doses along with respective doses of $NaAsO_2$, prevented the decline in serum testosterone concentration with no significant difference from control mice (Figure 1c).

### 3.5 | Histology

Control groups of mice (Figure 2a,b,c) demonstrated well-preserved normal histological structure of testes. Treatment with $NaAsO_2$ (4 ppm) (Figure 2d) resulted in expanded lumen with abnormally arranged cells. In contrast, both intermittent (Figure 2e) and continuous (Figure 2f) KP-10 administrations along with $NaAsO_2$ (4 ppm) treatment showed improved distribution of germ cells and normal lumen space in the seminiferous tubules. Likewise, treatment with high dose of $NaAsO_2$ (10 ppm) resulted in severe damage to testicular histology. This was indicated by distorted basement membranes and massive loss of germ cell layers and spermatozoa, distorted seminiferous tubules, damaged Leydig cells, torn basement membranes (BM) along and a disruption of spermatogonia (SG), Sertoli cells, round spermatids (RS) and elongated spermatids (ES) (Figure 2g). However, where exposure to $NaAsO_2$ (10 ppm) was combined with the intermittent (Figure 2h) or continuous (Figure 2i) KP-10 treatment, it led to a restoration of the germinal epithelium, normal lumen, well-formed basement membranes and a marked increase in the germ cell and sperm population across seminiferous tubules.

### 3.6 | Morphometry of seminiferous tubules

Tubular diameters of seminiferous tubules demonstrated no significant change across all experimental groups. However, treatment with $NaAsO_2$ at 4 ppm and 10 ppm doses alone resulted in significantly increased luminal diameter (65.33 ± 1.23 μm and 72.50 ± 1.00 μm respectively from control, $p < 0.001$; Table 4), whereas KP-10 supplementation along with $NaAsO_2$ exposure prevented the expansion of luminal diameter within the seminiferous tubules at both intermittent and continuous dose (50.66 ± 1.00 μm, 50.78 ± 2.12 μm; 50.33 ± 1.00 μm, 48.16 ± 1.04 μm, respectively, at 4 and 10 ppm arsenic doses) (Table 4). Likewise, height of germinal epithelium was significantly decreased ($p < 0.001$) due to massive germ cell loss in $NaAsO_2$-alone-treated groups (Table 4), while both intermittent and continuous KP-10 treatments maintained the epithelial height equivalent to control. Moreover, KP-10 treatment either intermittent or alone showed no significant change in morphometric parameters as compared to the control group (Table 4).

## 4 | DISCUSSION

The present study investigated the protective potential of KP-10 (kisspeptin, a hypothalamic reproduction-related neuropeptide)

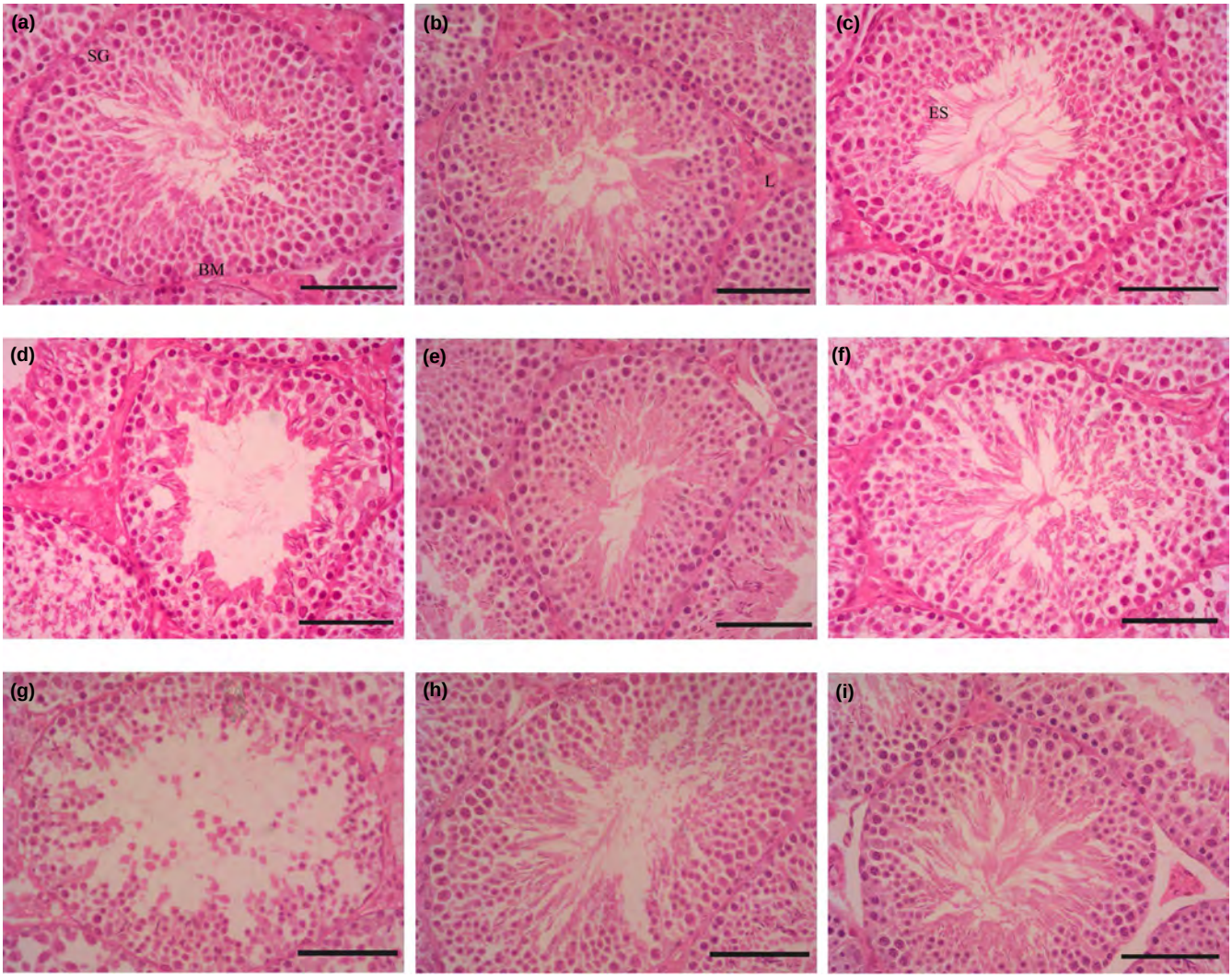


**FIGURE 2** Photomicrographs of testicular sections of adult male mice following 35 days of exposure to tap water control (a), 50 nmol/day KP-10 alone either intermittent (b) or continuous (c) shows normally arranged spermatogonia (SG), elongated spermatids (ES) and basement membrane (BM); 4 ppm $NaAsO_2$ alone (d) shows abnormally expanded luminal space and prominent germ cell loss; $NaAsO_2$ (4 ppm) combined with KP-10 intermittent (e) and KP-10 continuous supplementation (f) shows normal luminal space, and improved germ cell distribution and spermatozoa; exposure to 10 ppm $NaAsO_2$ alone (g) shows massive germ cell loss and abnormal testicular epithelium; $NaAsO_2$ combined with KP-10 intermittent (h) and KP-10 continuous supplementation (i) restored germinal epithelium, normal lumen, basement membranes and spermatozoa. Scale bar =50μm

against heavy metal-induced reproductive toxicity. Laboratory mice were exposed orally to two different doses of $NaAsO_2$ (4 ppm and 10 ppm) in drinking water for 35 days, while separate groups of mice were given equivalent exposure to $NaAsO_2$ but supplemented along with intermittent or continuous KP-10 doses. Results demonstrated lowering of ROS and TBARS, increase in the activity of antioxidant enzymes the SOD, POD and CAT, and restoration of circulating testosterone, seminal fructose and testicular LDH levels to within control concentrations upon supplementation with KP-10 in mice exposed to $NaAsO_2$. Concomitant significant improvement occurred in the epididymal sperm count, and percentage of motile and viable spermatozoa. Apparently, it was the KP-10 that facilitated the recovery of germ cell layers within the seminiferous tubules and a restoration of reproductive dysfunction. The toxic effects of $NaAsO_2$ included decreased tissue indices, and increased levels of oxidative stress biomarkers and lactate dehydrogenase along with decreased activities of antioxidant enzymes, serum testosterone, seminal fructose and massive germ cell loss.

Presently, no significant difference was observed in the body weights of control or treatment groups. A slight decrease was, however, observed in the body weight of mice upon KP-10 treatment as depicted by per cent weight change given in Table 1. This might be due to the reason that KP-10 is found to interfere with food intake and gastrointestinal hormones in mice, thus acting as an appetite suppressor (Dong et al., 2020). However, the observed change was not significant enough to compete with the toxic effects of arsenic exposure that quite possibly led to significantly decreased relative weights of testes and accessory sex organs.

**TABLE 4** Morphometry of mice seminiferous tubules following 35 days of exposure to 4 ppm and 10 ppm doses of sodium arsenite ($NaAsO_2$) alone and/or with 50 nmol/day KP-10

| Treatments | Tubular diameter (μm) | Lumen diameter (μm) | Epithelial height (μm) |
|---|---|---|---|
| Tap water(control) | $201.16^{a} \pm 3.85$ | $48.00^{a} \pm 1.00$ | $82.00^{a} \pm 1.06$ |
| KP alone (intermittent) | $205.66^{a} \pm 2.56$ | $48.16^{a} \pm 1.22$ | $81.66^{a} \pm 1.00$ |
| KP alone (continuous) | $203.33^{a} \pm 1.08$ | $48.00^{a} \pm 1.46$ | $82.00^{a} \pm 1.03$ |
| $NaAsO_2$ (4 ppm) | $206.00^{a} \pm 2.30$ | $65.33^{b} \pm 1.23^{***}$ | $54.33^{b} \pm 1.40^{***}$ |
| $NaAsO_2$ (4 ppm)+KP intermittent | $208.66^{a} \pm 2.13$ | $50.66^{a} \pm 1.00$ | $80.50^{a} \pm 1.01$ |
| NaAsO2 (4 ppm)+KP continuous | $204.50^{a} \pm 2.02$ | $50.78^{a} \pm 2.12$ | $80.50^{a} \pm 1.05$ |
| NaAsO2 (10 ppm) | $208.16^{a} \pm 2.25$ | $72.50^{c} \pm 1.00^{***}$ | $32.83^{c} \pm 1.32^{***}$ |
| $NaAsO_2$ (10 ppm)+KP intermittent | $206.67^{a} \pm 2.89$ | $50.33^{a} \pm 1.00$ | $81.66^{a} \pm 1.14$ |
| $NaAsO_2$ (10 ppm)+KP continuous | $205.66^{a} \pm 1.56$ | $48.16^{a} \pm 1.04$ | $82.00^{a} \pm 1.25$ |

*Note:* Values are mean ±s.e.

Different superscripts in a column show significant difference at $^{***}p < 0.001$. ($n = 30$ sections/group).

As regards the $NaAsO_2$-induced reproductive toxicity, outcomes of the current experimentation are in agreement with previous studies, which have frequently reported similar observations. For instance, Reddy et al. (2011) have demonstrated similar decrease in testicular weights, sperm parameters, serum testosterone and antioxidant enzyme activities in mice upon exposure to 4 ppm dose of $NaAsO_2$ in drinking water. Likewise, Souza et al. (2016) also noticed decreased reproductive performance in rats chronically exposed to both 0.01 ppm (0.01 mg/L) and 10 ppm (10 mg/L) doses of $NaAsO_2$. Of note, tissue toxicity caused due to the activation of oxidative stress mechanisms has also been comprehensively studied in the pituitary gland of rats during in vitro and in vivo investigations (Ronchetti et al., 2016).

Besides direct assessment of oxidative stress through testicular oxidant and antioxidant system, we also analysed tissue damage through the determination of testicular LDH activity. LDH levels are known to elevate in several diseases due to which its activity is used as a biochemical biomarker of disease (Klein et al., 2020). Increased tissue LDH indicates enhanced lipid peroxidation caused by oxidative stress (Jovanovic et al., 2010). Current results also revealed significantly increased LDH levels upon exposure to $NaAsO_2$ alone. However, testicular LDH levels remained significantly lower in mice exposed to $NaAsO_2$ but supplemented with intermittent or continuous KP-10 supplementation. These results suggest that KP-10 mitigated the oxidative damage and preserved cellular integrity by decreasing the lipid peroxidation as indicated by a decrease in the levels of ROS and TBARS (Table 3).

As regards the KP-10-alone treatment, no significant change was observed in biochemical, spermatological, hormonal or histological evaluation. For example, levels of oxidative stress biomarkers and antioxidant enzyme activities remained nearly equal to tap water control group. Similarly, testicular LDH and seminal fructose levels also showed no significant change. Tissue histology and morphometry also revealed no significant change in the arrangement of germ cells within seminiferous tubules, tubular diameter, luminal space or epithelial height. Similarly, serum testosterone levels also showed no significant increase upon treatment with either intermittent or continuous KP-10 alone. Although KP-10 treatment is well known to stimulate the HPG axis, thereby increasing the circulating LH and testosterone levels in experimental animals (Saito et al., 2012), in the present study, KP-10 was continuously administered for 35 days, which have somehow desensitised its receptors, thus depicting no such increase in circulating testosterone levels.

Keeping in view the past literature on kisspeptins, it is reiterated therefore that KP-10, being an endocrine regulator and an antioxidant both (mentioned elsewhere), can counter the toxic effects of arsenic heavy metal, which is a strong pro-oxidant and a known endocrine disruptor. The reason for selecting KP-10 as an intrinsic antioxidant was that it is an endogenous neuropeptide that regulates puberty onset and reproduction and that this sequence of amino acids is common to all isoforms and is required for receptor activation (Tena-Sempere, 2006). Presently, KP-10 treatment was found to be very effective as both intermittent and continuous supplementation. KP-10 equally prevented the $NaAsO_2$-induced oxidative damage and also maintained the serum testosterone levels to near-normal concentration at 50 nmol dose. Investigators have although tested variable doses (low and high) of KP-10 to study reproduction in several animal species (Ramaswamy et al., 2007; Tavor et al., 2006). However, it is pertinent to mention that our present study demonstrates that KP-10 can prevent the toxic effects of arsenic on reproduction at 50 nmol dose. Thus, unlike $NaAsO_2$-alone treatment, relative weights of testes, epididymides and seminal vesicles did not show significant decrease upon KP-10 supplementation. Histological examination also revealed well-preserved seminiferous tubules depicting normal germ cell layers. These observations are justifiable since the proper growth and functioning of testes and accessory glands requires continuous stimulation by androgens (Dohle et al., 2003).

In the present study, two experimental regimens, intermittent and continuous, were selected for supplementation with KP-10. The reasons were firstly, to compare the potential efficacy of these treatments for the first time; secondly, to circumvent the risk that if continuously administered, instead of mitigating the toxic effect of $NaAsO_2$, it might promote testicular degeneration due to

desensitisation of its receptor; and lastly, to minimise the risk of getting erroneous results due to the stress imposed from continuous handling and dosing for such a long duration.

Although Ramzan and Qureshi (2011) have previously reported dose-dependent testicular degeneration, this was demonstrated in pre-pubertal rats. Moreover, Thompson et al. (2006) compared the different isoforms of KP peptide, KP-10, KP-14 and KP-54 for their efficacy to activate the HPG axis. They found that chronic subcutaneous administration of kisspeptin-54 in adult male rats resulted in decreased LH and testosterone levels, which resulted in testicular degeneration. Current findings, however, did not present any deteriorating effect of KP-10 on adult male mouse reproductive system; instead, it occurred to be a protective agent against the toxic effects of $NaAsO_2$. In the present study, we also carried out kisspeptin-alone treatment as intermittent or continuous doses. The results revealed that KP-10 supplementation maintained ROS and TBARS to control values, and kept antioxidant enzymes, SOD, POD and CAT, LDH, testosterone and seminal fructose to normal levels. Moreover, KP-10 maintained the testicular histology and spermatozoa in healthy and viable state. If the present study is compared with the previous ones, the reason for contrasting results might be that aforementioned studies have specifically reported the dose-dependent damaging effect of KP in pre-pubertal and adult male rats without any further intervention, which could have disrupted the HPG axis. Therefore, it is likely that in the absence of any endocrine disruptor, continuous exposure to KP-10 doses could have desensitised its receptor. Conversely, in the present study, mice were exposed to $NaAsO_2$ that readily affects the HPG axis resulting in downregulation of testosterone secretion. Presumably then, exogenous supplementation with the KP-10 not only restored serum testosterone but also prevented testicular degeneration, which would otherwise have been caused by arsenic.

The mechanism through which KP-10 mediates its antioxidant effects necessitates further investigations because literature is still in its infancy as regards the role of kisspeptin during chemical or metal-induced toxicity. However, existing knowledge related to the regulatory role of KP-10 in puberty onset and reproduction can provide the most logical explanation. Hamden et al. (2008) have previously reported that sex steroids, specifically oestrogen, play an antioxidant role against free radical damage. Moreover, testosterone is converted into oestradiol by the activity of aromatase enzyme, thus suggesting its indirect effect as a potential antioxidant (Lephart, 1996), since KP-10 is currently found to prevent the significant decline in testosterone concentration, which was observed upon $NaAsO_2$-alone exposure. Therefore, it is proposed that the ameliorative effect of KP-10 against arsenic toxicity might have been mediated through the pathway mentioned above. Furthermore, previous literature also supports the current outcomes such as Güvenç and Aksakal (2018) who observed improvement in spermatological parameters upon KP-10 administration in rats treated with methotrexate (an anticancer drug). Moreover, oxidative stress was also decreased as indicated by increased activities of antioxidant enzymes. Similarly, Aydin et al. (2010) had also reported the antioxidant potential of KP in rat liver. Considering the outcomes of interventional studies on human subjects, therapeutic potential of KP in fertility disorders has been suggested (Clarke et al., 2015). These authors presume that deep down mechanism of KP-10 in the restoration of altered reproduction-related parameters at biochemical and cellular levels upon exposure to arsenic can be attributed to activation of the free radical scavenging system of the cell.

Localisation of KP-10 and its receptor in testes and germ cells is well established (Sharma et al., 2020). In addition, KP-10 has been shown to increase the intracellular calcium concentration in spermatozoa (Hsu et al., 2014). Knowingly, calcium is essential in regulating the motility and capacitation of spermatozoa (Costello et al., 2009). Considering these studies, current findings also suggest the direct effect of KP-10 through sperm-activating proteins. Further in vivo and in vitro studies are definitively required to explore the protective effect of KP-10 on spermatozoa challenged with heavy metal exposure. In the present case, sperm count, motility and viability were significantly improved upon KP-10 supplementation. For instance, sperm motility elevated on average to 67% with KP-10 as compared to NaAsO2 alone (around 38% on average), which is almost the same as our control demonstrating an improvement in the sperm motility. However, the 67% sperm motility in the control mice appears although low, but previous reports indicate that even 60.87% motility is considered normal (Reddy et al., 2011).

Presently, intraperitoneal administration of KP-10 might have interfered with water intake, thereby decreasing arsenic intake. However, this would have been better justified if arsenic concentration in testes and accessory sex organs was measured and compared between respective experimental groups. Less water intake due to KP-10 administration might be one of the reasons; however, it was ensured during the experiments that the drinking water be replaced with exactly the same dose of $NaAsO_2$ daily while replacing with fresh water, and easily accessible to each animal in the cage. The drinking bottles with stainless steel nozzles were tightly fixed in the cages and were filled with 250 ml water on a daily basis. Each morning the leftover water level was measured in the bottles to determine the water intake. This was divided over six animals per cage to get an approximation of how much quantity of water was consumed and to estimate $NaAsO_2$ intake by each animal per day for 35 days. Since the animals remained in good physiological condition until the end of experiments and did not show any untoward signs of lethargy or dehydration and were not in a position to waste the water because of tightly fixed bottles and nozzles, we presume that water intake was adequate as per metabolic need of each animal. Thus, we cannot presume that the KP-10, acting directly on water intake neurons or via the HPG axis, suppressed the drinking centre through inhibition of the drinking reflex. Laboratory mouse is although a good and handy model of studying hormones and toxicity; however, a prominent limitation is high metabolic rates and that small rodents get into stress very quickly. The factor of stress was, however, tried to be minimised as much as possible by keeping few animals in a cage; the investigators themselves handled all animals during whole of the experiments, and regularly monitored them for any abnormal signs.

Keeping in view these findings and our current observations, treatment with KP-10 appears to be a promising countering agent in mitigating the oxidative damage caused by free radicals generated by $NaAsO_2$. This is the first report as regards mitigating effects of KP-10 on metal toxicity. However, it warrants further research to comprehensively evaluate the differential effect of various forms of KP peptide along with administration of antagonist to kisspeptin receptor. Moreover, evaluation of expression level of oxidative stress-related genes to study the signalling mechanism involved will provide a better understanding about the role of KP-10 in testicular oxidant–antioxidant system. Finally, the current study was conducted using murine animal model, but to determine the clinical significance of KP-10 in fertility treatment, comparable results in a primate animal model would be more appropriate.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest of academic or financial nature with any individual or organization.

## DATA AVAILABILITY STATEMENT

Data supporting current findings are available from the corresponding author upon reasonable request.

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