Riboflavin recovery of spermatogenic dysfunction via a dual inhibition of oxidative changes and regulation of the PINK1-mediated pathway in arsenic-injured rat model

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Short title: Recovery of spermatogenic dysfunction via riboflavin

Abstract

Objective: Arsenic trioxide (As_2O_3) poisoning and associated potential lesions are of a global concern. Inversely, riboflavin (vitamin B2, VB2) as a component of flavoproteins could play a vital role in the spermatogenic enzymatic reactions. Thus, this research aimed to explore potential beneficial roles of VB2 during As_2O_3-injured-toxicity.

Methods: Rats were randomly allocated into 4 groups (n=8/group) and challenged as follows (for 30 days continuously): Group 1 received normal saline; Group 2 was treated with 3 mg As_2O_3/L; Group 3 received 40 mg VB2/L; Group 4 received 3 mg As_2O_3/L + 40 mg VB2/L. Both As_2O_3 and VB2 were dissolved in deionized water. Malondialdehyde (MDA), Glutathione Peroxidase (GSH-Px), Superoxide dismutase (SOD), and Catalase (CAT) were assessed for the oxidative profile, while TAS (Total Antioxidative Status) levels were evaluated for the antioxidant system, in both serum and testicular tissue. $P<0.05$ was considered statistically significant.

Results: The results show that As_2O_3 significantly decreased the body weight, testicular weight and testis volume, semen quality and testicular cell count ($p<0.05$). Furthermore, MDA content in the testicular tissue of the As_2O_3 group rats was significantly higher in comparison to the vehicle group ($p<0.05$). Likewise, TAS and the activities of GSH-Px, CAT and SOD were reduced ($p<0.05$) when compared to the control. As_2O_3 induced testicular damage and seminiferous tubular atrophy. Monodansylcadaverine assays mirrored the histopathology observations. Meanwhile, As_2O_3 upregulated the expression of mitophagy-related genes including PINK1, Parkin, USP8, LC3-I, Fis1 and Mfn2. The p38 gene, responsible to stress

stimuli, was also upregulated by As_2O_3 administration. Meanwhile, exposure to VB2 led to a significant decrease of the expression levels of mitophagy related genes.

Conclusions: Our study revealed that VB2 supplementation protected testicular structures against As_2O_3-induced injury via a dual inhibition of oxidative changes and a regulation of the PINK1-mediated pathway.

Key words: Oxidative stress, Arsenic trioxide, p38, PINK1 pathway, Riboflavin, Spermatogenesis

Introduction

Arsenic trioxide (As_2O_3) is a common environmental contaminant that is widely distributed in Pakistan, China, India, Bangladesh, and other Asian countries [Zheng et al. 2017]. In the substance priority list revised and published by the Agency for Toxic Substances and Disease Registry 2019, As_2O_3 occupies the 1st rank in terms of its toxicity (https://www.atsdr.cdc.gov/spl/#2019spl). Hence, there is an increasing interest in this chemical, due to its associations with an ever-increasing rise of health issues in industrialized countries as well as its globalized effects. The traditional As_2O_3 solution has numerous side effects, such as hyperleukocytosis, liver and kidney dysfunction, as well as effusion [Unnikrishnan et al. 2004].

The world's population continues to grow older rapidly, mostly because of two reasons: an overall fertility decline, and an increasing worldwide longevity [Barbier et al. 2010]. Meanwhile, health sciences have been facing major challenges in the management of reproductive disorders. As reported, As_2O_3 administration has led to a variety of impaired organ functions, particularly in the case of the male reproductive system [Souza et al. 2016]. Current evidence indicates that exposure to As_2O_3 decreases the number, viability, and motility of spermatozoa [Bourguignon et al. 2017], causes damage to spermatogonia and spermatocytes [Huang et al. 2016], and leads to sperm malformations [da Silva et al. 2017]. Besides, As_2O_3 toxicity may be accompanied with a decreased secretion of gonadotropins, testosterone synthesis and an impaired steroidogenesis that could further affect a proper spermatogenic process [Chiou et al. 2008; Alamda et al. 2017].

Mitochondria are a fundamental source of adenosine triphosphate for cellular health and function, but when damaged, they may generate a plethora of stress signals, which may result in cellular dysfunction and ultimately programmed cell death. Thus, a major component of maintaining cellular homeostasis lies in the recognition and removal of aberrant mitochondria through autophagy-mediated degradation, i.e., mitophagy [Hamacher-Brady and Brady, 2016].

Mitophagy, the selective autophagic elimination of dysfunctional mitochondria, is necessary for the maintenance of mitochondrial health and is predominantly regulated by the PINK1-mediated pathway [Koentjoro et al. 2017]. Currently, there is no definite evidence if and/or how As_2O_3 could induce mitophagy in male reproductive organs.

Despite the food sources of riboflavin or vitamin B2 (VB2; as an effective antioxidant in the nutrition) are well-known (e.g., milk, breads, fortified cereals), only a few nutritional studies have been published to elucidate potential effects of VB2 on the recovery of spermatogenesis. VB2 serves as a critical coenzyme for an array of dehydrogenases and oxidases responsible for a normal cell growth and function [Mantheya et al. 2006]. A previous study has revealed that VB2 exhibits anti-inflammatory and anti-stress activities in eukaryotic cells [Rivlin, 2001]. As reported earlier, B vitamin family has been used as a therapeutic agent for the treatment of male infertility and maintenance of a normal sperm function [Beltrame and Sasso-Cerri, 2017]. However, little is known about the effects of VB2 in the recovery of spermatogenesis.

Thus, our study examined the hypothesis that VB2 could protect the testicular structures affected by As_2O_3 toxicity via a dual inhibition oxidative changes and regulation of the expression of mitophagy-related genes.

Materials and Methods

Chemicals and Ethics

Unless otherwise indicated, all reagents were obtained from Merck (Darmstadt, Germany). The kits to evaluate reactive oxygen species (ROS), and selected oxidative stress markers (including Total Antioxidative Status (TAS)), malondialdehyde (MDA), glutathione peroxidase (GSH), superoxide dismutase (SOD), and catalase (CAT)) were purchased from Nanjing Jiancheng Bioengineering Institute (China). The Animal Care and Use Committee of the Islamic Azad University Kermanshah approved all experimental procedures of the study that were performed according to international guidelines (IAUK.REC.98-02-32-52385).

Animal Selection

Male adult Wistar rats (weighing 215-225 g; 8-weeks-old) obtained from the Razi Vaccine and Serum Research Institute of Iran (Tehran, Iran) were housed under temperature and light-controlled conditions. Body weight as well as food and water consumption per animal were recorded weekly. No significant differences were found in these measurements between animals in any of the experimental groups during the course of the experiment.

Experimental Design

Rats were randomly allocated into 4 groups (n=8/group). The animals were challenged as follows (for 30 days continuously): Group 1 received normal saline; Group 2 was treated with 3 mg As_2O_3/L; Group 3 received 40 mg VB2/L; Group 4 received 3 mg As_2O_3/L + 40 mg VB2/L. As_2O_3 and VB2 were dissolved in deionized water. Daily doses of As_2O_3 and VB2 were gradually and slowly dissolved in deionized water for 5 min. 24 hrs after the last treatment, the animals were euthanized by anesthesia with 20 mg/kg ketamine and 0.64 mg/kg xylazine (Alfasan, Woerden, the Netherlands) and weighed.

The testes were processed for: weigh and volume, semen quality, oxidative stress indices including TAS, MDA, GSH-Px, CAT and SOD, histopathology and mRNA expression levels of mitophagy related genes (Figure 1). Right testes were removed quickly, frozen in liquid nitrogen and stored at –80 °C for total RNA extraction. One part of the left testis was fixed in Bouin's solution for histopathological experiments. The other part was kept for electron microscopy observations.

Testis Measurements

The testis weight and volume were measured based on the protocol described in our previous study [Olfati et al. 2019]. The absolute total volume (V_{ref}) of each testis was measured by the following formula [Howard and Reed, 2005]:

$$V = t\,(a/p)\,\sum P$$

t = mean interval distance between slices.

a/p = area/test points ratio.

$\sum P$ = total number of points counted in the slice.

Sperm Analysis

Following removal, left epididymides were cut into 3–4 pieces, and dipped into a cell-culture dish containing 1 ml normal saline solution preheated to 37 °C and incubated for a few minutes (5% CO_2) in order to allow sperm to swim out the epididymal tubules. An aliquot of sperm suspension was diluted 1:20 with the Ham's F10 medium and transferred into a Neubauer's hemocytometer. Spermatozoa were counted under a light microscope at ×400 and expressed as million/mL of suspension (World Health Organization, Department of Reproductive Health and Research, 2010). Sperm motility was determined by placing a drop of 10 µL of the sperm suspension into a 37 °C pre-warmed slide and covered with a coverslip. At least 10 fields were assessed for each sample using a bright-field microscope with a closed diaphragm and the percentage of motile spermatozoa was estimated subjectively [Sakhaee et al. 2012; Tabarraei et al. 2019]. Sperm viability was analyzed by the eosin-nigrosin staining in 500 spermatozoa.

A drop of stained sperm suspension was put on a clean slide and a thin smear was made and allowed for drying. This slide was examined under a light microscope at ×1000 and spermatozoa with white and pink heads were considered as alive or dead, respectively [Olfati et al. 2018].

Histopathological Procedures

The collected testicular tissue was fixed in Bouin's solution for 24 hrs, and subsequently dehydrated in a series of graded ethanol. A different portion of the tissue was fixed in 10% buffered neutral formaldehyde for 72 hrs, cut into 5 µm sections by the Leica slicer (Leica, Inc., Germany), stained with the hematoxylin and eosin kit (H&E) according to the manufacturer's instructions and examined by light microscopy (Olympus, Tokyo, Japan, BX60).

Number of Germinal Cells

The relative number of the cells was estimated by the optical dissector principle [Mayhew and Gundersen, 1996]. Dissectors were generated as successive focal planes inside a thick section of the testicular tissue. A high numerical aperture oil immersion lens was applied. Five thick (in µm) visual fields from each paraffin block per each group were analyzed histologically to evaluate the tissue architecture. Twenty to 25 thick (in µm) visual fields were selected from each block for the counting of the spermatogenic cells (spermatogonia, spermatocytes and spermatids), Sertoli and Leydig cells. The counts were carried out by a cell counter in x fields. The number of cells was counted assuming their nuclei as the counting unit. Only the cells within the unbiased counting dissector frame and satisfying the Sterio rule were counted. Finally, the number of the cells was estimated using the optical dissector method and following the provided formula [Kaplan et al. 2012]:

$$N_{v(\text{cell/ref})} := \frac{\sum Q^{-}}{\sum A \times h}$$

Determination of Oxidative Stress Markers

The method used to evaluate the oxidative stress level in this experiment was performed as previously described by Jiang et al. [2019]. Briefly, the the ROS level was assessed using a fluorescence spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA, USA) and 2,7-dichlorofluorescin dictate using assay kits and based on the manufacturer's instructions. Appropriate amounts of the right testis (200 mg) were pre-incubated (70 min) with DCFH-DA (10 µM) at 37 °C to allow for the DCFH-DA to be incorporated into all membrane-

bound vesicles. The conversion of DCFH (λ excitation=485 nm) to DCF (green fluorescence, λ emission=525 nm) was evaluated using a fluorescence spectrophotometer. The levels of MDA, TAS, GSH-Px, the enzymatic activities SOD, and reduced CAT were measured using commercial kits. Absorbance of each parameter was monitored at 532, 520, 420, 550 and 405 nm, respectively.

MDC Staining for the Detection of Autophagic Vacuoles

Autophagy was evaluated by the monodansylcadaverine (**MDC**) staining. Left testes were stored in 4% paraformaldehyde, sectioned, dehydrated and cleared in dimethyl-benzene (xylene), dehydrated in graded ethanol solutions and allowed to air dry completely in the laboratory. Subsequently, the samples were stained with 50 mM/L MDC dye for 45 min at 37 °C in the dark. The stained samples were washed with phosphate-buffered saline 5 times (5 min each) in the dark, and then allowed to dry at laboratory temperature. HistoChoice® clearing agent was used to inhibit fading of the fluorescence in the darkness. Finally, the optical intensity of the autophagic vacuoles was examined under a fluorescent microscope (Olympus, Tokyo, Japan, BX60).

QPCR method

Total RNA was isolated from right testis weighing 25-30 mg using the Trizol reagent (Life Technologies, Carlsbad, CA, USA). Two % agarose gel electrophoresis was used to assess the integrity of total RNA and the A260/280 ratio in the range of 1.8–2.0 was evaluated by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). RNA was reverse transcribed using the PrimeScript™ RT Master Mix kit (PINK1, Parkin, USP8, USP30, LC3-I, Rab7, Fis1, Mfn2, and p38). QRT-PCR was carried out using the QuantStudio 7 Flex qRT-PCR system (Stratagene, USA) and SYBR@ Premix Ex Taq™ II kit. Specific primers were designed by Invitrogen, USA (Table 1). β-actin (reference gene) was used to normalize the expression level of target genes. Duplicated Ct values were measured for each sample, and the comparative Ct method was used to determine the relative expression level of the target genes [McBride and Coward, 2016].

Statistical Analysis

Statistical analysis was performed using the SPSS 13.0 software. The data were processed with one-way analysis of variance (ANOVA) followed by Dunnett's new multiple range test. Values of $p<0.05$ were considered as statistically significant. The results are shown as mean±SEM, unless indicated otherwise.

Results

Results presented in Table 2 reveal a significant decrease in the body weight, testis weight, and testis volume in As_2O_3-treated animals ($p<0.05$) when compared to the control group. These parameters were preserved in case of VB2 administration (Table 2; $p<0.05$).

In comparison to the control group, As_2O_3-injured animals exhibited a significantly lower semen quality (including the sperm number, viability and motility; $p<0.05$). Concurrently, exposure to VB2 led to a significant increase in the spermatogenic rate (Table 3; $p<0.05$).

Figure 2 (sections 1 - 4) shows representative sections of histopathology of the testicular tissue of rats. Control animals exhibited normal histological structures with a regular morphology (sections 1 and 3). Meanwhile, the administration of As_2O_3 caused a widespread damage to the testicular cells and tissues accompanied by a seminiferous tubular atrophy (section 2), presenting with induced seminiferous tubular deformities, an increased interstitial tissue, vascular hyperemia, congested blood vessels and a shrinkage of the basal lamina. On the other hand, VB2 administration enabled the repair (proportionally) of the testicular cells, leading to the occurrence of an organized germinal epithelium (section 4).

Table 4 shows that exposure to As_2O_3 lead to a significant decrease in the number of testicular cells (all types including spermatogonia, spermatocytes, spermatids, Sertoli and Leydig cells) in comparison to the control ($p<0.05$), while a significant increase in the quantity of testicular cells was observed in both VB2 groups ($p<0.05$), reflecting the histopathological observations.

The assessment of oxidative stress markers (Table 4) reveals significant ($p<0.05$) changes in their contents in rats exposed to As_2O_3 when compared to the control at the end of the experiment. The MDA concentration in the As_2O_3 group was significantly higher when compared to the control group ($p<0.05$). Likewise, our results indicate that the average TAS and the activities of GSH-Px, SOD and CAT were significantly decreased ($p<0.05$) in the animals treated with As_2O_3. All observed prooxidant changes were reversed by VB2 administration.

According to the fluorescence microscopy, MDC-labeled autophagic vacuoles appeared as distinct, dot-like structures (Figure 3, sections 1-4). An increase in the amount of MDC-labeled autophagic vacuoles was observed in the group exposed to As_2O_3 (sections 2 and 4). All pathological alterations were reversed proportionally by the VB2 treatment (section 4).

mRNA expression levels of mitophagy genes such as PINK1, Parkin, USP8, LC3-I, Rab7, Fis1, Mfn2 and p38 were examined by QRT-PCR (Figure 4). All mitophagy markers (except of Rab7) were significantly up regulated in the As_2O_3 treated groups when compared to the control group ($p<0.05$). However, p38 was also upregulated by As_2O_3 administration.

Meanwhile, exposure to VB2 led to a significant decrease of the expression levels of mitophagy related genes ($p<0.05$).

Discussion

In the era of a rising environmental contamination, research in andrology needs to be more aware of the potential detrimental effects of environmental pollutants on the spermatogenic process. As the body of evidence grows in support of restorative therapies for the recovery of spermatogenesis, it is important to be familiar with emerging treatment options, as well as the timeframe of their administration [McBride and Coward, 2016]. Vitamin supplementation in animal models for the promotion of health has become an increasingly more common scientific approach. As such, this study is the first to report the potential beneficial health effects of VB2 on As_2O_3-exposed rats presenting with a spermatogenic dysfunction, and subsequently explored its possible mechanisms of action.

As observed in this study, As_2O_3 significantly affected male reproductive cells and tissues [Souza et al. 2016]. Specifically, a significantly diminished testicular weight and the quantity of testicular cells were recorded post As_2O_3 exposure, partly due to an increased oxidative stress. This phenomenon is in line with earlier reports using mice [Sarkar et al. 2008; Rao et al. 2013] and rat [Huang et al. 2016] models, which have postulated that changes in the testicular weight may occur due to tissue-specific toxicity of As_2O_3. It is known that the regulation of the cell number is extremely important for the maintenance of the size, weight and function of tissue and organ structures [Beltrame and Sasso-Cerri, 2017].

Inversely, the semen quality including the sperm count, viability and motility improved in rats administered with VB2. Our results of the semen analysis reveal a rapid recovery with medical therapy (VB2; 30 days continuously) aimed to stimulate the endogenous testicular functions. This fast improvement could be associated with antioxidant properties of VB2 translated into its ability to prevent lipid peroxidation and to protect germ cells from oxidative damage by scavenging free radicals. Lipid peroxidation is an important factor contributing to spermatogenic dysfunction [Hana et al. 2019]. Multiple studies have demonstrated that VB2 attenuated lipid peroxidation, proposing that its mechanism of action could lie in flavin adenine dinucleotide (**FAD**) replenishment [Angelini et al. 2016] or demethylation of key enzymes playing important roles in the phospholipid metabolism [Wang et al. 2018]. In addition, similar results were reported from an oral antioxidant study in which treatment with the B vitamin family was found to improve the sperm vitality, motility, and DNA integrity [Abad et al. 2013]. Furthermore, systematic reviews suggest positive effects of the B vitamin family on the semen

quality: first, by increasing the sperm count, enhancing the sperm motility and reducing sperm DNA damage [Banihani, 2017]; and second, by stimulating DNA synthesis and thus contributing to the cell division [Oh and Brown, 2003].

In this study, typical feature pathological and histological changes (atrophy and deformities) were observed in the testicular morphology of the groups exposed to As_2O_3, leading to the assumption that As_2O_3 exerts its primary toxicity in the testicular tissue (Figure 2). Accordingly, latest studies have revealed morphometrical and morphological changes in the testis of laboratory animals caused by chronic As_2O_3 administration [Huang et al. 2016; Guvvala et al. 2016; de Araujo Ramos et al. 2017].

Sub-chronic exposure to As_2O_3 is known to be associated with an induced oxidative stress in reproductive structures, which may lead to a decreased spermatogenic efficiency by an increased occurrence of oxidative insults [Huang et al. 2016]. Our study showed that As_2O_3 promotes excessive generation of ROS, which could disrupt the oxidative milieu in testicular tissues. Subsequently, enzymatic and non-enzymatic antioxidants fail to inhibit the over generation of lipid peroxides, especially MDA (the end-product of lipid peroxidation). In As_2O_3 administered groups, the testicular damage was associated with elevated MDA levels. Similarly, previous studies stated that As_2O_3 exposure enhanced the MDA production in brain [Sun et al. 2018] and reproductive tissues [Shao et al. 2018] of chicken, which indirectly reflected on the severity of ROS attacks. Moreover, most of the recent studies have claimed that excess ROS will break the oxidative homeostasis, reflected in an increased MDA content, a decreased ability to resist hydroxyl radicals and an inhibition of numerous anti-oxidative enzymatic activities [Reddy et al. 2017; Sun et al. 2018]. Lower levels of ROS and MDA in the VB2 group might be responsible for the withdrawal of the inhibitory effects of As_2O_3 toxicity on the testicular antioxidant system as well as a diminution of free radical generation. All observed changes in the oxidative markers were proportionally abolished by VB2 as a candidate therapy.

Autophagy begins with the formation of autophagosomes, which envelop a portion of the cytoplasm and deliver cytoplasmic components to the degradative organelle (lysosome/vacuole) for further breakdown and recycling [Schneider and Cuervo, 2014; Marshall and Vierstra, 2018]. Our experiments suggest that As_2O_3 exposure induces the formation of autophagosomes in male reproductive cells or tissues, indicating that As_2O_3 could enhance testicular autophagy. As_2O_3-associated alterations to the autophagic flux could be considered a form of embodiment of As_2O_3-induced toxicity. In addition, the dysregulation of autophagy may occur due to the impact of As_2O_3 on the autophagosomal-lysosomal function.

Apart from the de novo formation of autophagosomes, another reason for their accumulation may be associated with the blockage of autophagosomal degradation due to the aggregation of p38 (the gene responsive to stress stimuli). Inversely, As_2O_3-induced testicular cell death was blocked by VB2 as a candidate therapy. In this case, a balance between autophagy and cell proliferation might have occurred in the groups supplemented with VB2 since the tissue was kept in homeostasis.

As shown in Figure 3, upon administration of VB2, the diffused distribution of MDC in the cytoplasm was converted to a dot-like appearance. Concurrently, based on the histopathology and MDC results, VB2 could promote the activity of the mitophagy pathway to counteract the stress caused by environmental pollutants and to facilitate the cell proliferation by suppressing stress responses (ROS production) as well as by promoting metabolism and survival. Collectively, our findings indicate that VB2 exposure changes the expression patterns of mitophagy-related genes, which could affect the degradation of autophagosomes. Hence, it is recommended that future experiments should be focused more on the exact relationships among specific signaling pathways involved in the process of mitophagy in the male reproductive system.

Our data revealed that As_2O_3 as an extracellular stimulus, could have an impact on the PINK1-mediated pathway in the male reproductive system, which may ultimately act either as a death safeguard. PINK1 protein is known as a sensor of mitochondrial damage and mitophagy [Horibe et al. 2019]. In addition, Parkin protein acts in the facilitation of the mitochondrial translocation [Zhoua et al. 2019]. In injured animals, increased ROS levels can activate the PINK1-mediated signaling pathway. It has been previously reported that the PINK1/Parkin pathway plays a pivotal role as a mitophagy mediator in mammals [Chen et al. 2013; Eiyama and Okamoto, 2015], which is mainly involved in the elimination of damaged mitochondria. Thus, PINK1-mediated pathway is important for the regulation in the expression patterns of mitophagy, which is essential to maintain the integrity of testicular cells or tissues.

The results of mRNA expression levels of mitophagy markers reveal that As_2O_3 could induce a mitochondrial impairment in the testicular cells or tissues, which may represent one of the potential mechanisms of As_2O_3-associated reproductive toxicity. Thus, studies on the expression patterns of mitophagy makers in the male reproductive organs may be advantageous to understand whether exposure to As_2O_3 induces a mitophagy response against reproductive toxicity. The present findings suggest that VB2 improved the spermatogenic homeostasis via PINK1-mediated pathway. It is known that VB2 is mainly metabolized in the liver and becomes FAD to regulate metabolism [Kumar et al. 2002; Barile et al. 2016]. Taken together, these

results demonstrate that VB2 exerts a vital protective effect on the spermatogenic dysfunction in a rat model, which could be attributed to its direct protective effects on damaged testis via inhibiting oxidative changes as well as regulating the expression of mitophagy-related genes. Besides, our results reveal a potential involvement of the PINK1-mediated signaling pathway in the fate of spermatogenesis, however more evidence is needed to further demonstrate its effects on the testicular function in As_2O_3-injured animal or human models.

Latest studies state that the expression levels of p38 probably play an important role in ROS-induced damage to the blood-testis barrier (**BTB**) [Chen et al. 2018]. Our present results reveal a marked down-regulated expression of p38 (key kinases for mitochondrial adaptation) [Takahashi et al. 2019] following VB2 administration when compared to the As_2O_3 exposed groups. Thus, VB2, transferred from the blood to the male reproductive organs could inhibit As_2O_3-induced disruption of the BTB by regulating oxidative stress-mediated p38 pathways, which could provide an explanation to the improvement of the spermatogenic dysfunction. While these protective effects of VB2 may be partly contributed by co-treatment with p38 pathways. Further studies focusing on the specific effect of VB2 on the BTB functions would clarify this speculation.

Till now, there was no report available describing the effect of p38 expression patterns on the spermatogenic dysfunction, while our study is the first to reveal a hint that p38 overexpression could lead to an enhanced testicular mitophagy. Hence, it is recommended that future experiments should focus more on the exact relationships among signaling pathways triggering mitophagy in the male reproductive system.

Conclusions

In conclusion, the results of the present study indicate a beneficial relationship between VB2 therapy and regeneration of the spermatogenic function. Likewise, the data presented suggest that VB2 therapy could be a potentially effective strategy to modify the detrimental effects of As_2O_3 in an animal model by inhibiting oxidative changes and by regulating the PINK1-mediated pathway.It should be noted that similarly to previous studies in this era, there are some limitations. In this case, the limitations involve the type and design of the studies in which the cause and effective relationship between the variables cannot be determined since most of these studies are observational and cross-sectional. They use different cut off points to determine the normal levels of VB2, as well as other cut off points to test other biomarkers with Western blotting. Also the use of VB2 supplementation was not controlled in the investigations. These limitations build the foundation for interventional studies with adequate samples and follow up periods to clarify the roles of VB2 in PINK1-mediated pathway that

351 could be applied in the prevention and treatment of male reproductive dysfunction in the future.

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355 **Conflict of interests**

356 None.

357 **Authors' contribution**

358 The corresponding author designed the study. All co-authors contributed to this work and

359 reviewed the final manuscript.

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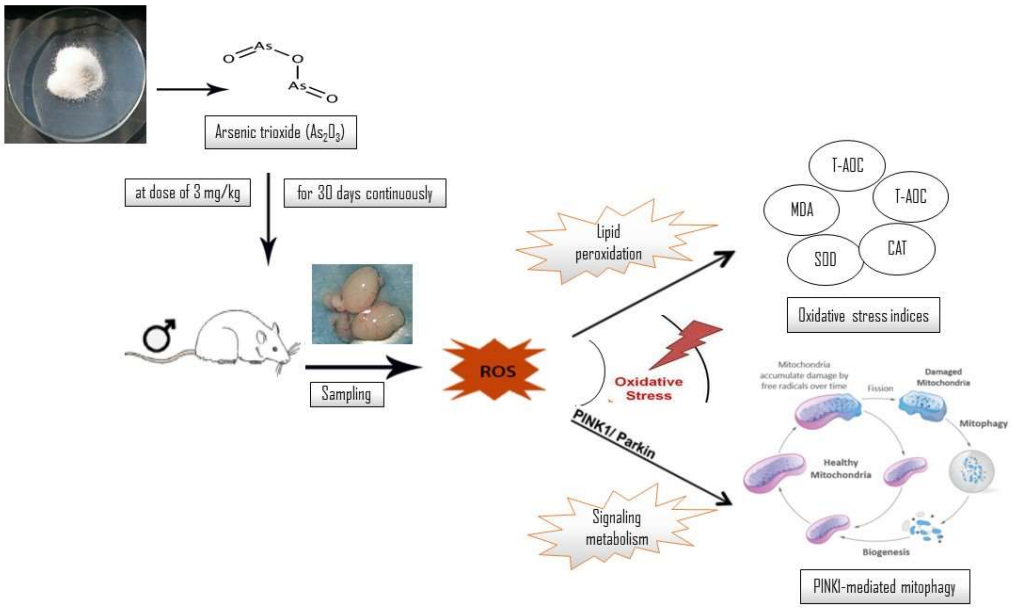


Figure 1. Experimental design. Healthy rats were treated with As_2O_3 (3 mg/kg) and 40 mg VB2/L daily for 30 days continuously. Following treatment, testicular structure and function were evaluated with a special focus on the dual mitophagy pathway and oxidative stress markers.

492 **Table 1**

493 Primers used for QRT-PCR: sequence and product size

Target gene	Gene Bank accession no.	PCR fragment length (bp)	Sequences (5′–3′)
PINK1	NM_026880.2	172	Forward: ctgcagatgctgtttctggc
			Reverse: agccaccttcttcagccttc
Parkin	NM_001317726.1	177	Forward: cctgcaaacaagcaaccctc
			Reverse: tcaaagctaccgacgtgtcc
USP8	NM_001252580.1	167	Forward: agagaacaacgagcacctgg
			Reverse: acatgaaggcctcgaaggtg
USP30	NM_001033202.3	224	Forward: agctgtgtcatctgcacctc
			Reverse: ttgctccacttctgctcagg
LC3-I	NM_025735.3	196	Forward: atcatcgagcgctacaaggg
			Reverse: agatgtcagcgatgggtgtg
Rab7	NM_001364358.1	203	Forward: tttgccctaagcaggtctgg
			Reverse: agaaacagctctccagtcgc
Fis1	NM_001163243.1	245	Forward: agaacaaccaggccaaggag
			Reverse: aaagggaaggcgatggtgag
Mfn2	NM_001285920.1	186	Forward: aacaaggactggacagctcg
			Reverse: tgtgctcaggctggagaaag
p38	NM_001168508.1	231	Forward: tgtgtttgcatgctgtgctc

Reverse: agatggcaagggttcaggtg

Table 2

Effect of riboflavin on the body weight, testicular weight, and testicular volume in an arsenic-injured rat model

Groups (n=8)	Body weight (g)	Testis weight (g)	Testis volume (cm^3)
Vehicle	258.75±8.65 [a]	1.51±0.072 [a]	1.41±0.03 [a]
As_2O_3 3 mg/kg	196.00±3.17 [b]	0.92±0.192 [b]	0.66±0.10 [b]
VB2 40 mg/kg	255.00±4.38 [a]	1.46±0.042 [a]	1.39±0.19 [a]
As_2O_3+VB2	251.50±5.22 [a]	1.39±0.058 [a]	1.33±0.03 [a]

As_2O_3: arsenic trioxide; VB2: vitamin B2 or riboflavin.

Values are given as means±S.D. The same superscripts (a-b) are not significantly different from each other in each column ($p<0.05$).

Total volume (V) of each testis: $V = t\,(a/p)\,\sum P$

Where "t" was the mean interval distance between slices, (a/p) represented the area related with each test point and "$\sum P$" was the total number of points counted in all slices.

503 **Table 3**

504 The effect of riboflavin on the semen quality in an arsenic-injured rat model

Groups (n=8)	Spermatozoa ($\times 10^6$)	Viability (%)	Motility (%)
Vehicle	36.88±0.69 [a]	73.26±0.12 [a]	72.73±0.53 [a]
As_2O_3 3 mg/kg	19.65±0.48 [b]	34.14±0.56 [b]	39.30±0.12 [b]
VB2 40 mg/kg	39.23±1.19 [a]	78.70±0.92 [a]	74.25±0.92 [a]
As_2O_3+VB2	33.01±0.88 [a]	71.90±0.77 [a]	70.24±0.18 [a]

505 As_2O_3: arsenic trioxide; VB2: vitamin B2 or riboflavin.

506 Values are given as means±S.D. The same superscripts (a-b) are not significantly different from each other in
507 each column ($p<0.05$).

Table 4

The effect of riboflavin on the number of spermatogenic cells in an arsenic-injured rat model ($\times 10^8$)

Groups (n=8)	Spermatogonia	Spermatocyte	Spermatid	Sertoli	Leydig
Vehicle	89.80±2.25 [a]	124.50±6.24 [a]	211.00±10.29 [a]	24.20±3.65	22.59±2.06 [a]
As_2O_3 3 mg/kg	55.40±3.63 [b]	85.31±9.82 [b]	136.44±7.16 [b]	19.35±1.76	14.12±1.05 [d]
VB2 40 mg/kg	90.03±3.23 [a]	130.00±11.22 [a]	220.80±8.32 [a]	24.09±1.31	23.91±1.43 [a]
As_2O_3+VB2	86.20±4.01 [a]	119.10±9.49 [a]	205.80±6.69 [a]	20.41±1.25	21.48±1.17 [a]

As_2O_3: arsenic trioxide; VB2: vitamin B2 or riboflavin.

Values are given as means±S.D. The same superscripts (a-b) are not significantly different from each other in each column ($p<0.05$).

515 **Table 5**

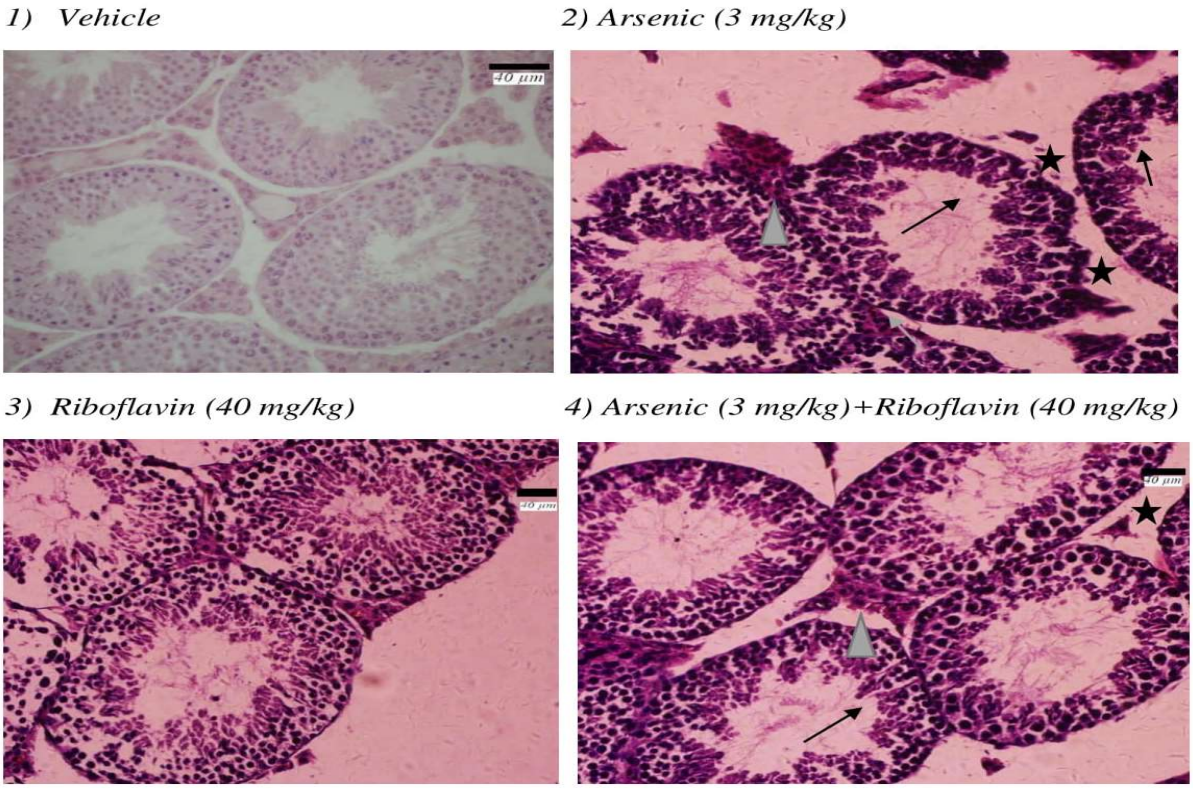
516 The effect of riboflavin on the oxidative stress markers in an arsenic-injured rat model

Groups (n=8)	ROS ($\times 10^5$)	TAS	MDA	GSH-Px	SOD	CAT
Vehicle	1.11±0.55 [b]	95±9.04 [a]	2.3±0.08 [b]	755±38.6 [a]	28±2.08 [a]	255±18.2 [a]
As_2O_3 3 mg/kg	2.09±0.93 [a]	41±5.22 [b]	11±1.07 [a]	330±28.9 [b]	12±1.32 [b]	131±11.9 [b]
VB2 40 mg/kg	1.01±0.48 [b]	98±8.19 [a]	2.2±0.33 [b]	770±41.2 [a]	29±2.18 [a]	261±17.3 [a]
As_2O_3+VB2	1.34±0.19 [b]	81±7.62 [a]	1.9±0.11 [b]	680±40.1 [a]	25±2.02 [a]	238±16.7 [a]

517 As_2O_3: arsenic trioxide; VB2: vitamin B2 or riboflavin.

518 Values are given as means±S.D. The same superscripts (a-b) are not significantly different from each other in
519 each column ($p<0.05$).

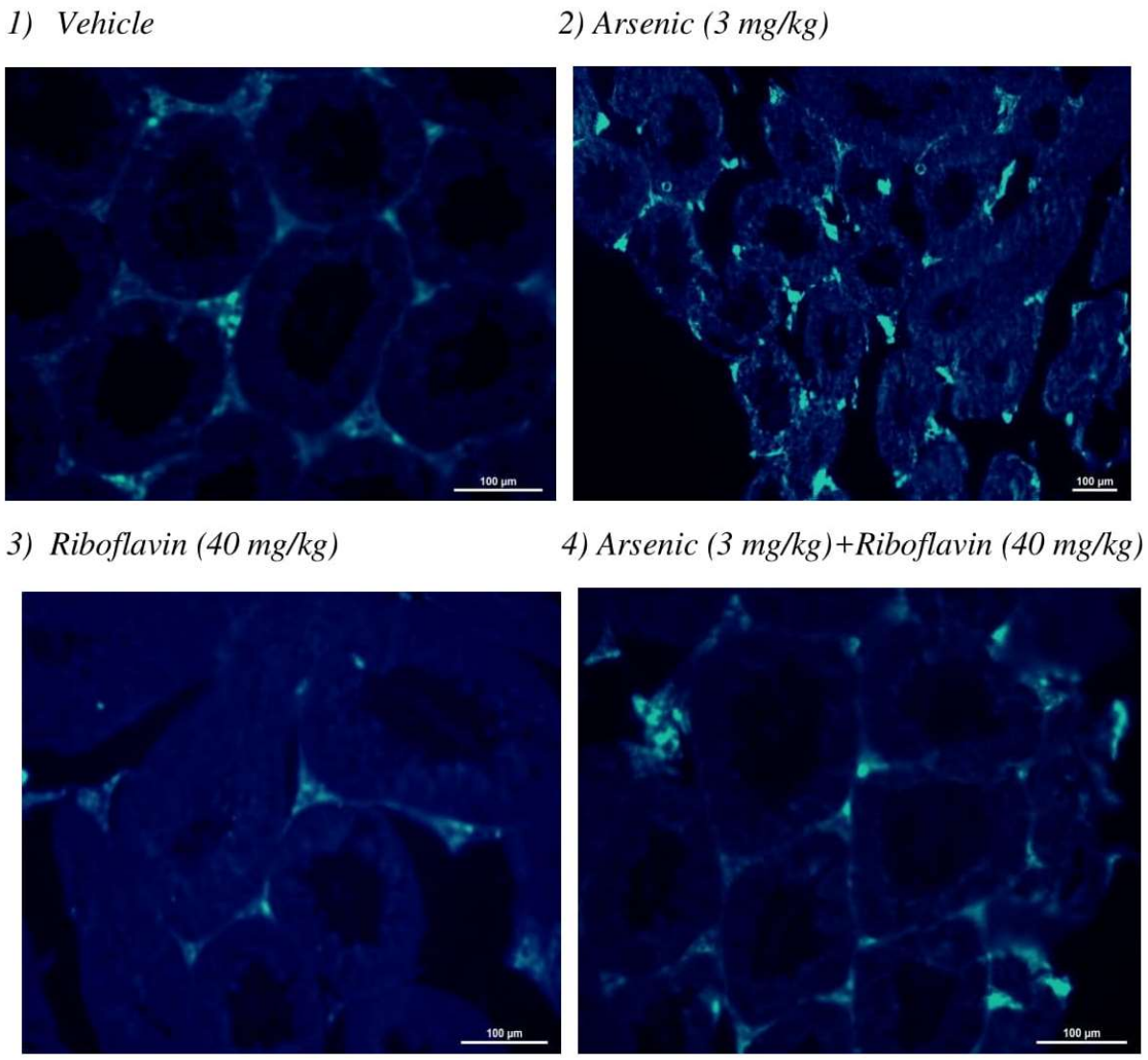
520 ROS (DCF Fluorescence Intensity): reactive oxygen species; TAS (% of control): total antioxidant status; MDA
521 (nmol/mg protein): malondialdehyde; GSH-Px (μg/g tissue): glutathione peroxidase, SOD (U/mg protein):
522 superoxide dismutase; CAT (mU/mg protein): catalase



523

524 **Figure 2.** Histopathological changes in the testicular tissue of rats (H&E staining). The
525 magnification is ×40.

Symbols	Definition
Black arrows	Extensive atrophy in the seminiferous tubules and destruction of the germinal epithelium
Gray arrows	Shrinkage of the basal lamina
Asterisks ★	Increased interstitial space
Hallow arrow head	Congested blood vessels and vascular hyperemia



526

527 **Figure 3.** Qualitive monodansylcadaverine (MDC)-labeled autophagic vacuoles in rat testis.
528 As_2O_3-induced autophagy was detectable by autofluorescence emitted by MDC staining
529 (400×magnification, Bar=10 µm).

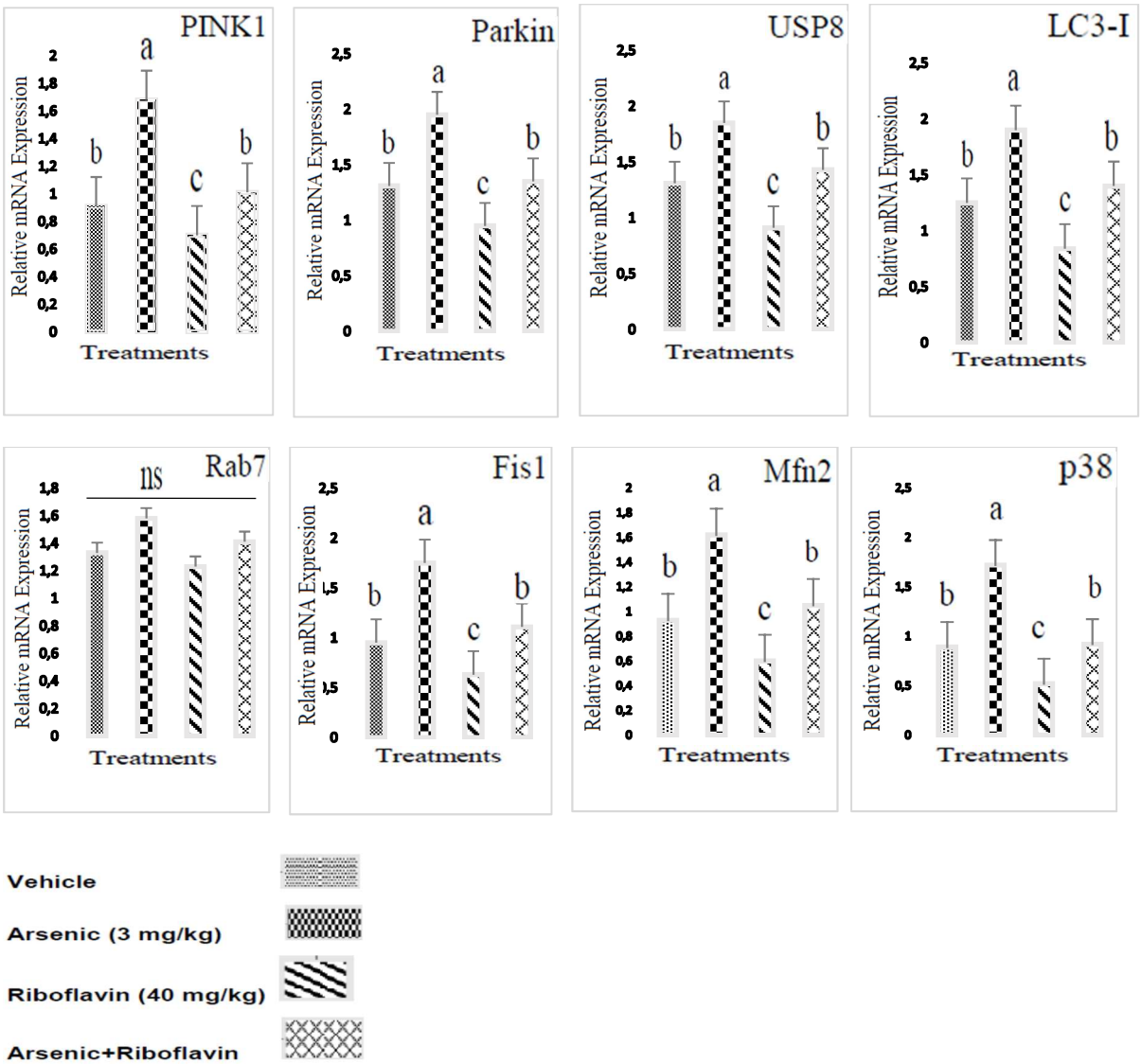


Figure 4. Changes in the expressions of mitophagy key markers in mRNA induced by As_2O_3; mRNA expressions levels of PINK1, Parkin, USP8, LC3-I, Rab7, Fis1, Mfn2 and p38 as detected by qPCR. All data were expressed as relative values against their respective control group. β-actin was used as an internal control. The values are presented as mean±SEM (n=5). The same superscripts (a-c) are not significantly different from each other ($p<0.05$).