

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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Received February 14, 2021

Accepted May 6, 2021

Epub Ahead of Print June 2, 2021

Summary

Arsenic trioxide (As₂O₃) poisoning and associated potential lesions are of a global concern. Inversely, riboflavin (vitamin B2) 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 vitamin B2 during As₂O₃-injured-toxicity. 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/l As₂O₃, Group 3 received 40 mg/l vitamin B2, Group 4 received 3 mg/l As₂O₃ + 40 mg/l vitamin B2. Both As₂O₃ and vitamin B2 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. The results show that As₂O₃ 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 As₂O₃ 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. As₂O₃ induced testicular damage and seminiferous tubular atrophy. Monodansylcadaverine assays mirrored the histopathology observations. Meanwhile, As₂O₃ 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 As₂O₃ administration. Meanwhile, exposure to Vitamin B2 led to a significant decrease of the expression

levels of mitophagy related genes. Our study revealed that vitamin B2 supplementation protected testicular structures against As₂O₃-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

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Introduction

Arsenic trioxide (As₂O₃) 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, As₂O₃ 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 As₂O₃ 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, As₂O₃ 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 As₂O₃ 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, As₂O₃ 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 (Saha *et al.* 2019). 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 As₂O₃ could induce mitophagy in male reproductive organs.

Despite the food sources of riboflavin or vitamin B2 (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 vitamin B2 on the recovery of spermatogenesis. Vitamin B2 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 vitamin B2 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 vitamin B2 in the recovery of spermatogenesis.

Thus, our study examined the hypothesis that vitamin B2 could protect the testicular structures affected by As₂O₃ 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/l As₂O₃, Group 3 received 40 mg/l vitamin B2, Group 4 received 3 mg/l As₂O₃ + 40 mg/l vitamin B2. As₂O₃ and vitamin B2 were dissolved in deionized water. Daily doses of As₂O₃ and vitamin B2 were gradually and slowly dissolved in deionized water for 5 min. 24 h 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 (Fig. 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 ($5\% \text{CO}_2$) 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 $\times 400$ 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 $10 \mu\text{l}$ 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 $\times 1000$ 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 h, and subsequently dehydrated in a series of graded ethanol. A different portion of the tissue was fixed in 10 % buffered neutral formaldehyde for 72 h, cut into $5 \mu\text{m}$ 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).

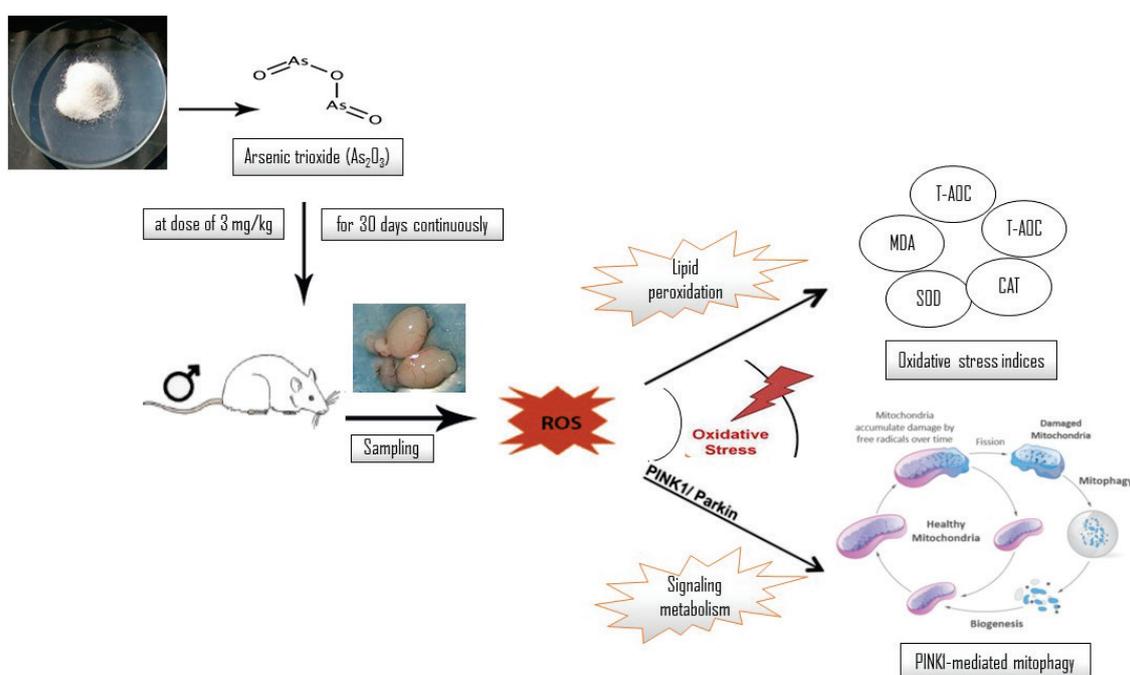


Fig. 1. Experimental design. Healthy rats were treated with As_2O_3 (3 mg/kg) and 40 mg/l vitamin B2 daily for 30 days continuously. Following treatment, testicular structure and function were evaluated with a special focus on the dual mitophagy pathway and oxidative stress markers.

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):

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

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 ROS level was assessed using a fluorescence spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA) and 2,7-dichlorofluorescein diacetate 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 \pm 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 vitamin B2 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 vitamin B2 led to a significant increase in the spermatogenic rate (Table 3, $p < 0.05$).

Table 1. Primers used for QRT-PCR: sequence and product size

Target gene	Gene Bank accession no.	PCR fragment length (bp)	Sequences (5'-3')
<i>PINK1</i>	NM_026880.2	172	Forward: ctgcagatgctgtttctggc Reverse: agccaccttcttcagccttc
<i>Parkin</i>	NM_001317726.1	177	Forward: cctgcaacaagaaccctc Reverse: tcaaagctaccgacgtgtcc
<i>USP8</i>	NM_001252580.1	167	Forward: agagaacaacgagcactgg Reverse: acatgaaggcctcgaagggtg
<i>USP30</i>	NM_001033202.3	224	Forward: agctgtgtcatctgcacctc Reverse: ttgctccacttctgctcagg
<i>LC3-I</i>	NM_025735.3	196	Forward: atcatcgagcgtacaagg Reverse: agatgtcagcgatgggtgtg
<i>Rab7</i>	NM_001364358.1	203	Forward: ttgccctaagcaggctctgg Reverse: agaacagctctccagtcgc
<i>Fis1</i>	NM_001163243.1	245	Forward: agaacaaccaggccaaggag Reverse: aaaggaaggcgtatgggtgag
<i>Mfn2</i>	NM_001285920.1	186	Forward: aacaaggactggacagctcg Reverse: tgtgtcaggctggagaaag
<i>p38</i>	NM_001168508.1	231	Forward: tgtgtttgcatgtgtgctc Reverse: agatggcaagggttcagggtg

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)
<i>Vehicle</i>	258.75±8.65 ^a	1.51±0.072 ^a	1.41±0.03 ^a
<i>As₂O₃ 3 mg/kg</i>	196.00±3.17 ^b	0.92±0.192 ^b	0.66±0.10 ^b
<i>Vitamin B2 40 mg/kg</i>	255.00±4.38 ^a	1.46±0.042 ^a	1.39±0.19 ^a
<i>As₂O₃+vitamin B2</i>	251.50±5.22 ^a	1.39±0.058 ^a	1.33±0.03 ^a

As₂O₃: arsenic trioxide. 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: $V = t(a/p) \Sigma P$, where "t" was the mean interval distance between slices, (a/p) represented the area related with each test point and "ΣP" was the total number of points counted in all slices.

Table 3. The effect of riboflavin on the semen quality in an arsenic-injured rat model

Groups (n=8)	Spermatozoa (×10 ⁶)	Viability (%)	Motility (%)
<i>Vehicle</i>	36.88±0.69 ^a	73.26±0.12 ^a	72.73±0.53 ^a
<i>As₂O₃ 3 mg/kg</i>	19.65±0.48 ^b	34.14±0.56 ^b	39.30±0.12 ^b
<i>Vitamin B2 40 mg/kg</i>	39.23±1.19 ^a	78.70±0.92 ^a	74.25±0.92 ^a
<i>As₂O₃+vitamin B2</i>	33.01±0.88 ^a	71.90±0.77 ^a	70.24±0.18 ^a

As₂O₃: arsenic trioxide. 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).

Fig. 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 As₂O₃ caused a widespread damage to the testicular cells and tissues accompanied by a seminiferous tubular atrophy (section 2), presenting with induced seminiferous tubular

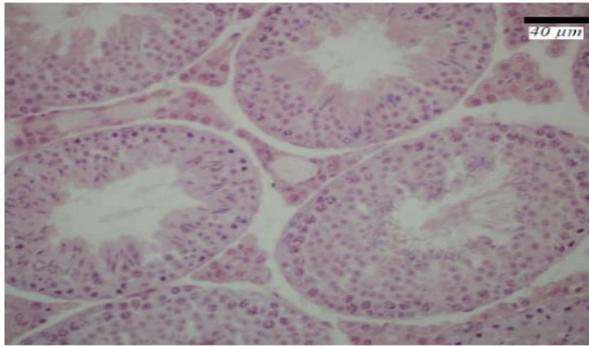
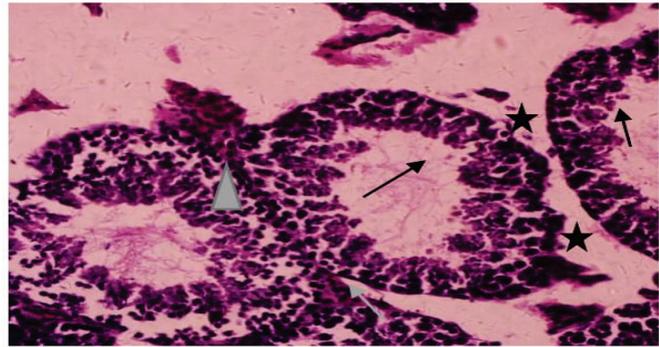
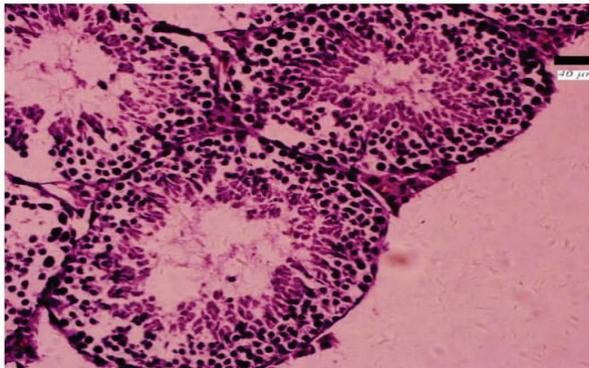
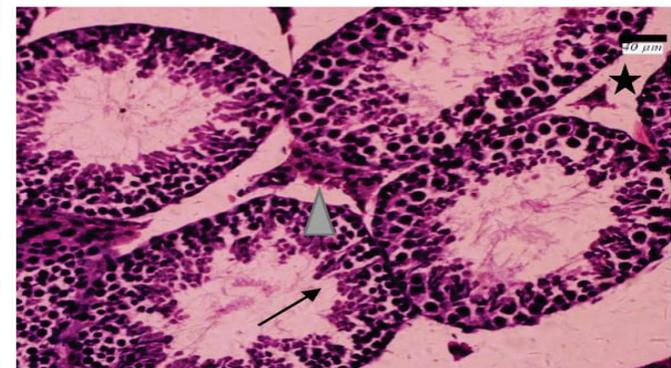
1) *Vehicle*2) *Arsenic (3 mg/kg)*3) *Riboflavin (40 mg/kg)*4) *Arsenic (3 mg/kg)+Riboflavin (40 mg/kg)*

Fig. 2. Histopathological changes in the testicular tissue of rats (H&E staining). The magnification is $\times 40$. Symbols: 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

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
<i>Vehicle</i>	89.80 \pm 2.25 ^a	124.50 \pm 6.24 ^a	211.00 \pm 10.29 ^a	24.20 \pm 3.65	22.59 \pm 2.06 ^a
<i>As₂O₃ 3 mg/kg</i>	55.40 \pm 3.63 ^b	85.31 \pm 9.82 ^b	136.44 \pm 7.16 ^b	19.35 \pm 1.76	14.12 \pm 1.05 ^d
<i>Vitamin B2 40 mg/kg</i>	90.03 \pm 3.23 ^a	130.00 \pm 11.22 ^a	220.80 \pm 8.32 ^a	24.09 \pm 1.31	23.91 \pm 1.43 ^a
<i>As₂O₃+vitamin B2</i>	86.20 \pm 4.01 ^a	119.10 \pm 9.49 ^a	205.80 \pm 6.69 ^a	20.41 \pm 1.25	21.48 \pm 1.17 ^a

As₂O₃: arsenic trioxide. Values are given as means \pm S.D. The same superscripts (a-b) are not significantly different from each other in each column ($p < 0.05$).

Table 5. 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
<i>Vehicle</i>	1.11 \pm 0.55 ^b	95 \pm 9.04 ^a	2.3 \pm 0.08 ^b	755 \pm 38.6 ^a	28 \pm 2.08 ^a	255 \pm 18.2 ^a
<i>As₂O₃ 3 mg/kg</i>	2.09 \pm 0.93 ^a	41 \pm 5.22 ^b	11 \pm 1.07 ^a	330 \pm 28.9 ^b	12 \pm 1.32 ^b	131 \pm 11.9 ^b
<i>Vitamin B2 40 mg/kg</i>	1.01 \pm 0.48 ^b	98 \pm 8.19 ^a	2.2 \pm 0.33 ^b	770 \pm 41.2 ^a	29 \pm 2.18 ^a	261 \pm 17.3 ^a
<i>As₂O₃+vitamin B2</i>	1.34 \pm 0.19 ^b	81 \pm 7.62 ^a	1.9 \pm 0.11 ^b	680 \pm 40.1 ^a	25 \pm 2.02 ^a	238 \pm 16.7 ^a

As₂O₃: arsenic trioxide. Values are given as means \pm S.D. The same superscripts (a-b) are not significantly different from each other in each column ($p < 0.05$). ROS (DCF Fluorescence Intensity): reactive oxygen species; TAS (% of control): total antioxidant status; MDA (nmol/mg protein): malondialdehyde; GSH-Px (μ g/g tissue): glutathione peroxidase, SOD (U/mg protein): superoxide dismutase; CAT (mU/mg protein): catalase

deformities, an increased interstitial tissue, vascular hyperemia, congested blood vessels and a shrinkage of the basal lamina. On the other hand, vitamin B2 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 vitamin B2 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 vitamin B2 administration.

According to the fluorescence microscopy, MDC-labeled autophagic vacuoles appeared as distinct, dot-like structures (Fig. 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 vitamin B2 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 (Fig. 4). All mitophagy markers (except of Rab7) were significantly up regulated in the As_2O_3 treated groups when

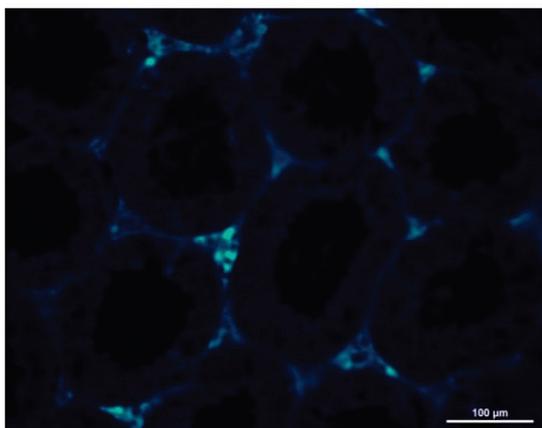
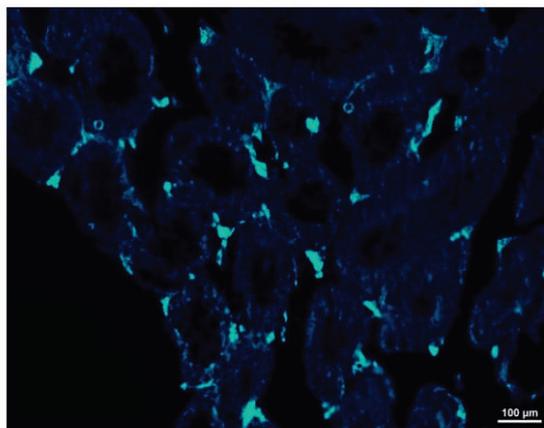
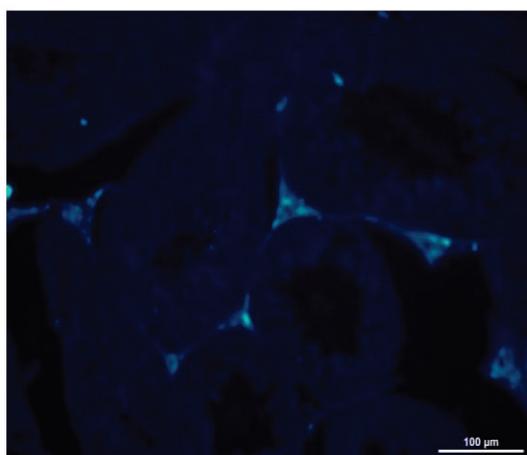
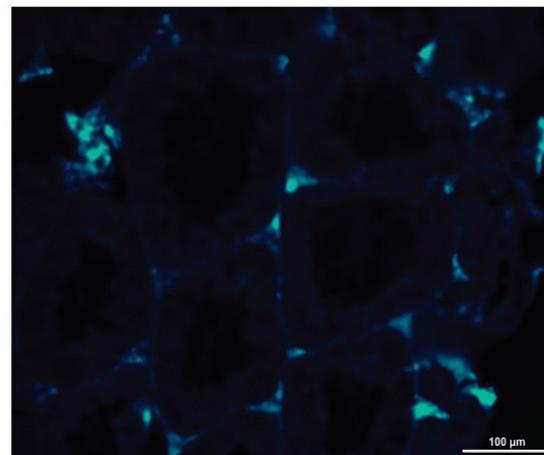
1) *Vehicle*2) *Arsenic (3 mg/kg)*3) *Riboflavin (40 mg/kg)*4) *Arsenic (3 mg/kg)+Riboflavin (40 mg/kg)*

Fig. 3. Qualitative monodansylcadaverine (MDC)-labeled autophagic vacuoles in rat testis. As_2O_3 -induced autophagy was detectable by autofluorescence emitted by MDC staining (400×magnification, Bar=10 µm).

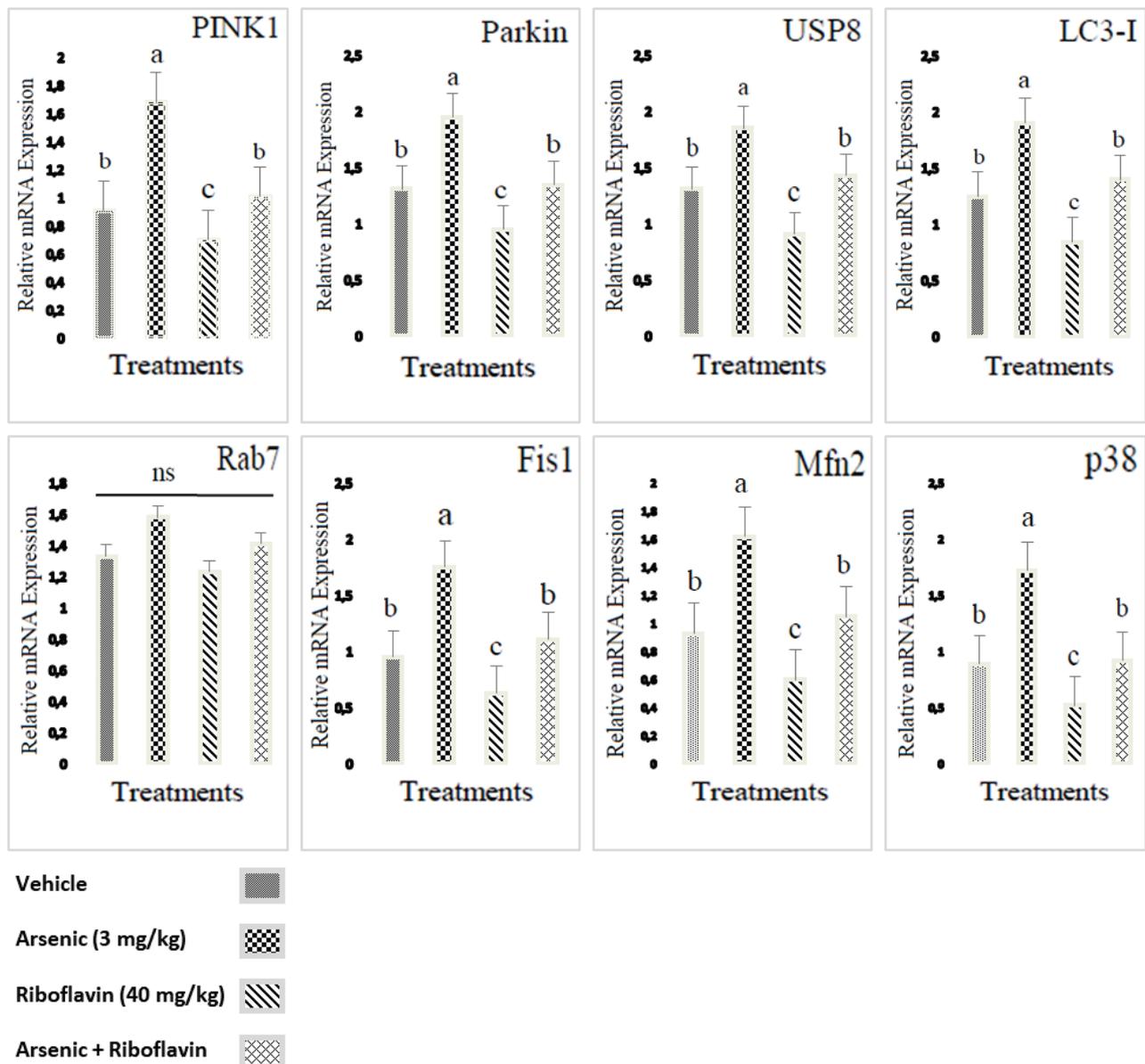


Fig. 4. Changes in the expressions of mitophagy key markers in mRNA induced by As_2O_3 , mRNA expressions levels of PINK1, Parkin, USP8, LC3-I, Rab7, Fis1, Mfn2 and p38 as detected by qPCR. All data were expressed as relative values against their respective control group. β -actin was used as an internal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

compared to the control group ($p<0.05$). However, p38 was also upregulated by As_2O_3 administration. Meanwhile, exposure to vitamin B2 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 vitamin B2 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 As₂O₃ 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 As₂O₃. 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 vitamin B2. Our results of the semen analysis reveal a rapid recovery with medical therapy (vitamin B2, 30 days continuously) aimed to stimulate the endogenous testicular functions. This fast improvement could be associated with antioxidant properties of vitamin B2 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 vitamin B2 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 As₂O₃, leading to the assumption that As₂O₃ exerts its primary toxicity in the testicular tissue (Fig. 2). Accordingly, latest studies have revealed morphometrical and morphological changes in the testis of laboratory animals caused by chronic As₂O₃ administration (Huang *et al.* 2016, Guvvala *et al.* 2016, de Araujo

Ramos *et al.* 2017).

Sub-chronic exposure to As₂O₃ 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 As₂O₃ 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 As₂O₃ administered groups, the testicular damage was associated with elevated MDA levels. Similarly, previous studies stated that As₂O₃ 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 vitamin B2 group might be responsible for the withdrawal of the inhibitory effects of As₂O₃ 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 vitamin B2 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 As₂O₃ exposure induces the formation of autophagosomes in male reproductive cells or tissues, indicating that As₂O₃ could enhance testicular autophagy. As₂O₃-associated alterations to the autophagic flux could be considered a form of embodiment of As₂O₃-induced toxicity. In addition, the dysregulation of autophagy may occur due to the impact of As₂O₃ 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, As₂O₃-induced testicular cell death was blocked by vitamin B2 as a candidate therapy. In this case, a balance between

autophagy and cell proliferation might have occurred in the groups supplemented with vitamin B2 since the tissue was kept in homeostasis.

As shown in Figure 3, upon administration of vitamin B2, the diffused distribution of MDC in the cytoplasm was converted to a dot-like appearance. Concurrently, based on the histopathology and MDC results, vitamin B2 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 vitamin B2 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 (Zhou *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 vitamin B2 improved the spermatogenic homeostasis via PINK1-mediated pathway. It is known that vitamin B2 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 vitamin B2 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 vitamin B2 administration when compared to the As_2O_3 exposed groups. Thus, vitamin B2, 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 vitamin B2 may be partly contributed by co-treatment with p38 pathways. Further studies focusing on the specific effect of vitamin B2 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 vitamin B2 therapy and regeneration of the spermatogenic function. Likewise, the data presented suggest that vitamin B2 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 vitamin B2, as well as other cut off points to test other biomarkers with Western blotting. Also the use of vitamin B2 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

vitamin B2 in PINK1-mediated pathway that could be applied in the prevention and treatment of male reproductive dysfunction in the future.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This study was supported by the APVV 15-0544 and KEGA 008SPU-4/2021 projects. The authors thank Prof. Dr. Mehrdad Payandeh for providing drugs and facilities.

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