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The Effects of Maternal Exposure Riboflavin to Prevent Uterus Arsenic Damage in Offspring Rats

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ABSTRACT

Background: In this study, the efficacy of riboflavin (VB2) in preventing uterus As_2O_3 damage was investigated for the first time in the literature.
Methods: The rats received 40 μg LHRHa for estrus synchronization. 48 pregnant Wistar rats were included in the study. Four groups were formed with 7 rats in each group: Sham, 1.5 mg arsenic trioxide (As_2O_3/L) alone or in combination with VB2 (20 and 40 mg/L) in drinking water (for 21 days continuously). Moreover, similar to maternal generation treatment the F1-female generation was arranged (for 35 days continuously until puberty).
Results: Based on the results, As_2O_3 reduced body weight and feed intake ($P < 0.05$). Furthermore, the serum malondialdehyde levels in the As_2O_3 group were significantly higher than that of the control group ($P < 0.05$). At the same time, the total antioxidative status and the activities of glutathione peroxidase, superoxide dismutase, and catalase were reduced ($P < 0.05$). Meanwhile, As_2O_3 remarkably increased the inflammatory markers production [interleukin 6 and C-reactive protein] ($P < 0.05$). As_2O_3 administration induced uterus apoptosis-related genes by upregulating caspase-3, iNOS, and Bax genes and downregulating Bcl-2 gene of pubertal F1-female rats ($P < 0.05$).
Conclusion: Our observation indicated that VB2 therapy is potentially an effective strategy to modify the detrimental effects of As_2O_3 in pubertal F1-female rats via suppresses oxidative damages.

1. Introduction

Arsenic trioxide is a common environmental contaminant that is widely distributed all around the world. In the substance priority list issued by the Agency for Toxic Substances and Disease Registry 2019, arsenic occupies the 1st rank in terms of its toxicity. Hence, over the past decade researchers have paid more attention to arsenic and its globalized effects. Several studies exist in the literature on the arsenic-impaired organ systems in human and experimental animals such as brain [1], cardiovascular [2], respiratory [3], reproductive [4], and renal [5] particularly the maternal-fetal interface.

The maternal-fetal interface is the critical target organ of chronic toxic insults. Latest studies have shown that maternal exposure to environmental agents affect the succeeding generation [6,7,8]. Meanwhile, health science has faced major challenges for managing maternal transfer disorders and common therapeutic methods often lead to unsatisfactory results.

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Riboflavin, formerly known as vitamin B2 (VB2)with molecular formula $C_{17}H_20N_4O_6$, and molecular weight 376.36 g/mol serve as the main precursor used by animal cells to form both flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) co-enzymes [9], in which VB2 plays important roles in the enzymatic reactions. Both FMN and FAD co-enzymes in association with their client flavoproteins participate in critical cellular processes that include energy and lipid metabolism, redox signaling, programmed cell death, growth regulation, and biologic rhythms regulation [10, 11]. Despite the VB2 food sources (as an effective antioxidant in the nutrition) such as milk, breads, and fortified cereals only a few nutritional studies have been published about the blunted effect of VB2 on oxidative stress recovery. The evidence accrued suggests that VB2 is an antioxidant nutrient that may prevent lipid peroxidation and reperfusion oxidative injury [12]. VB2 was known to be associated with various exert protective effects types like steroidogenesis, hepatoprotective, hyperglycemia, and neuroprotective [9, 13, 14,15].

Based on our knowledge until now no studies are available that examine the effect of VB2 on the maternal-fetal interface in the As_2O_3-treated rat model. Therefore, the present study tested the hypothesis that VB2 protects pubertal F1-female rats from As_2O_3 toxicity via suppressing oxidative damage and cell apoptosis. Thus, this study provides a novel mechanistic approach concerning As_2O_3 toxicity.

2. Materials and Methods

2.1. Chemicals and ethics

All the reagents were obtained from Merck (Darmstadt, Germany). The kits were purchased from Nanjing Jiancheng Bioengineering Institute, (Nanjing, China) to evaluate reactive oxygen species (ROS), various oxidative stress indices including total antioxidative status (TAS), malondialdehyde (MDA), glutathione peroxidase (GSH), superoxide dismutase (SOD), catalase (CAT), and inflammatory markers including interleukin 6 (IL-6) and C-reactive protein (CRP). The study protocol was carried out in compliance with the guidelines for care and handling of laboratory animals, which was approved by an ethics committee at Islamic Azad University, Kermanshah, Iran (98-02-32-51985).

The current study was conducted using 48 female Wistar rats weighing between 230-280 g obtained from the Pasteur Institute of Iran (Karaj, Iran). During the study, all the animals were kept under standard laboratory conditions (12 h of daylight, 12 h of darkness, ventilation, constant temperature, free access to water and food). The rats received 40 μg LHRHa (Sigma, St Louis, MO) for estrus synchronization. Pregnant rats were placed in an individual birth plastic cages and all the facilities were prepared for their parturition.

Four groups were formed with 7 rats in each group (for 21 days continuously: a complete gestation cycle in rat): group 1 served as vehicle group which received normal saline, group 2 was treated with 1.5 mg As_2O_3/L with molecular formula As_2O_3 and molecular weight 197.841 g/mol, group 3 received 1.5 mg As_2O_3/L + 20 mg VB2/L [7,8-dimethyl-[N-10-ribityl] isoalloxazine], and group 4 received 1.5 mg As_2O_3/L + 40 mg VB2/L. As_2O_3 and VB2 dissolved in deionized water. At birth, the weights of offspring and litter size (pups number/mother) were recorded. Similar to maternal generation treatment, the F1-female generation was also arranged into four groups (n =7 in each group) as follows: 1.5 mg As_2O_3/L alone or in combination with VB2 (20 and 40 mg/L/day) for 35 days continuously (until puberty). Mean body weight (BW) and feed intake in each group were recorded weekly.

2.2. Sampling

Intracardiac blood samples were collected in gel biochemistry tubes, centrifuged for 10 min at 3000xg, aliquoted, and stored at -80 °C. The rats were administered intraperitoneal (IP) anesthesia (ketamine 20 mg/kg and xylazine 0.64 mg/kg) before the surgical procedure. Tissue samples obtained for biochemical assessment were stored under laboratory conditions at -80 °C (Figure 1).

2.3. Oxidative stress indices determination

The method used to detect the oxidative stress indices level in this study was performed as previously described by Jiang et al. (16). Briefly, the ROS level was assayed using a fluorescence spectrophotometer and 2, 7-dichlorofluorescein dictate based on the assay kits and the manufacturer's instructions. Appropriate amounts of uterus homogenate (200 mg) were pre-incubated for 70 min with DCFH-DA (10 μM) at 37 °C to allow the DCFH-DA to be incorporated into any membrane-bound vesicles. The conversion of DCFH to DCF (green fluorescence) was evaluated using a fluorescence spectrophotometer (λ excitation = 485 nm and λ emission = 525 nm). Obtained tissue samples were tested for levels of MDA, T-AOC, GSH, SOD, and CAT were measured by commercial kits. Supernatant absorbance was measured at 532, 520, 420, 550, and 405 nm, respectively.

The serum IL-6 and CRP concentrations were measured using ELISA (Blue Gene, Shanghai, China: code: IL-6: #HS600B, and CRP: #1000). Standard commercial kits were used for analysis and the procedures were adopted as recommended by the manufacturer of the kits. All the samples were analyzed in duplicates and the mean of the duplicate was used for the statistical analysis.

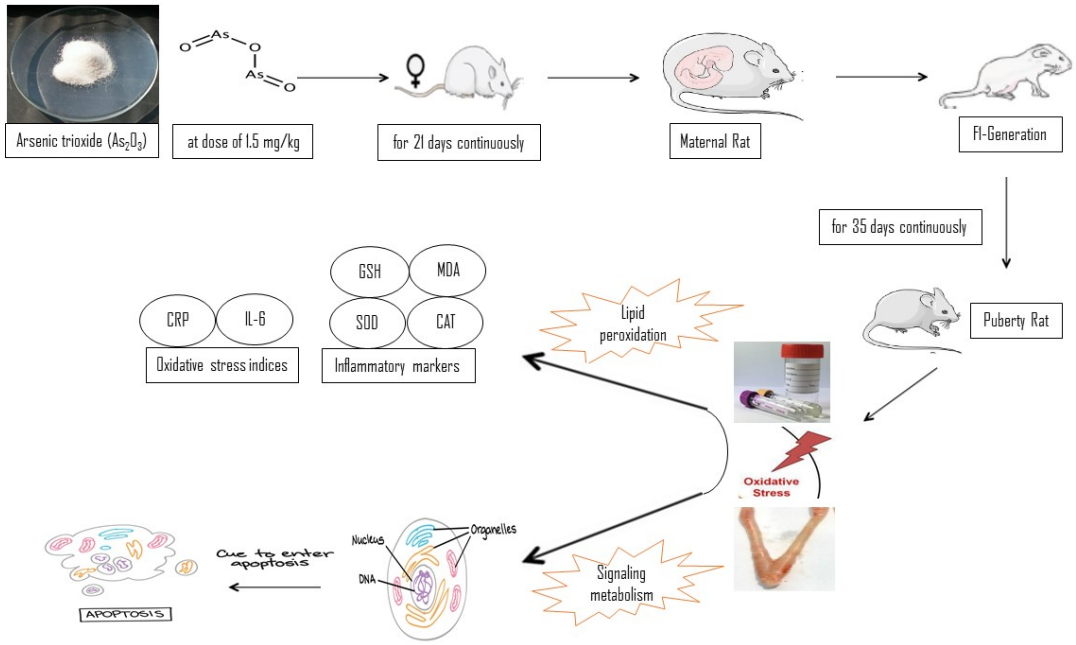

Figure 1: Experimental design. Pregnant healthy Sprague Dawley rats were administrated with As_2O_3 [1.5 mg As_2O_3/L] daily for 21 days continuously [a complete gestation cycle in rat]. At birth, blood samples were evaluated via inflammatory and oxidative indices. Then, the uterus samples were removed quickly for total RNA extraction analysis

2.4. Quantitative real-time PCR

Total RNA was isolated from the right testis weighing 25-30 mg using Trizol reagent (Life Technologies, Carlsbad, CA, USA). The 2% agarose gel electrophoresis was used to assess the integrity of the total RNA and the A260/280 ratio was in the range of 1.8–2.0 evaluated by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). RNA was reverse transcribed using a PrimeScriptTM RT Master Mix kit. qRT-PCR was carried out using the QuantStudio 7 Flex qRT-PCR system (Stratagene, USA) and SYBR@ Premix Ex TaqTM 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.

2.5. Statistical analysis

Statistical analysis was performed using SPSS 13.0 software. The results were expressed as the means and standard deviations (mean ± SD) and performed with one-way analysis of variance (ANOVA) followed by Dunnett's new multiple range test and values of $P < 0.05$ were considered as statistically significant.

Table 1: Primers used for qRT-PCR, sequence, and product size

Gene name	Product size [bp]	Primer sequence
Caspase 3	214	Forward:-TCTTCATTCAGGCCTGCCG
		Reverse:-TGCGCGTACAGTTTCAGCATGG
iNOS	177	Forward:-AATGCAGGAGATGGTCCGCAAG
		Reverse:-ATGCGCACATCGCCACAAAC
Bax	111	Forward:-AGGCGAATTGGCGATGAACTGG
		Reverse:-AAACATGTCAGCTGCCACACGG
Bcl-2	240	Forward:-TGGTGGACAACATCGCTCTGTG
		Reverse:-TTTGTTTGGGGCAGGTCTGCTG

Primer sets designed using free online software Primer3Plus [v. 0.4.0] http://primer3plus.cgi.

3. Results and Discussion

Effects of As_2O_3 and VB2 treatments on the BW and feed intake of animals are presented in Table 2. Results regarding BW and feed intake show a significant decrease in the rats fed a diet supplemented with As_2O_3 as compared to the control group at the end of the experiment ($P < 0.05$). These weights and feed intake recovered with the application of the VB2 as a candidate therapy.

Results shown in Table 3 revealed a significant effect of dietary supplementation of As_2O_3 on oxidative stress biomarkers of the rats.

Table 2: Riboflavin effect on body weight and feed intake in arsenic-treated of female rat offspring

Group [mg/kg/day]	Body weight [g]	Feed intake [g]
Vehicle [control]	21.03±1.11 [a]	2.98±0.67 [a]
As_2O_3 1.5	13.81±0.98 [b]	1.87±0.32 [b]
As_2O_3+VB2 20	15.19±0.78 [b]	2.08±0.58 [b]
As_2O_3+VB2 40	15.69±1.03 [b]	2.11±0.60 [b]

As_2O_3: arsenic trioxide; VB2: vitamin B2 or riboflavin
Values are given as means ± SD. The same superscripts (a-b) are not significantly different from each other in each column ($P < 0.05$).

The MDA contents of the As_2O_3 group were significantly higher than that of the control group ($P < 0.05$). At the same time, results of fluorescence spectrophotometer assay showed that the TAS and the activities of GSH, SOD, and CAT were reduced in As_2O_3-treated animals ($P < 0.05$). Meanwhile, As_2O_3 increased the production of inflammatory markers including IL-6 and CRP levels. All these changes were recovered by VB2 as a candidate therapy.

mRNA expression levels of apoptosis-related genes including caspase-3, iNOS, Bax, and Bcl-2 were examined by qRT-PCR (Figure 2). Compared with the control group, mRNA expression levels of all apoptosis-related genes were significantly up-regulated in 1.5 mg As_2O_3/L exposed ($P < 0.05$), and Bcl-2 mRNA expression significantly decreased in As_2O_3 exposure groups ($P < 0.05$). The qRT-PCR analysis in the tissue samples (uterus) shows that VB2 as a candidate therapy markedly reduced the toxicity of As_2O_3 via down-regulation of the expressions of apoptosis-related genes.

In the era of rising environmental contaminants, researchers need to be more aware of the detrimental effects of these agents on the maternal-fetal system. Supplementing vitamins in the animal model for promoting health is an increasingly more common management tool, is increasing. Likewise, this experiment first reported the potential beneficial health effect of VB2 on As_2O_3-induced rats with female reproductive dysfunction and explored the possible mechanisms.

Millions of people are exposed to arsenic via drinking water, contaminated soil, air, fish, and other sea organisms rich in methylated arsenic species. Occupational exposure was observed especially in the USA, Bangladesh, Pakistan, India, China, and other Asian countries [17]. Further understanding of environmental contaminants including arsenic and their effects on both mother and her fetus can help improving offspring disorders. To the best of our knowledge, this is the first comprehensive research exploring the mechanistic basis for the functional effects of VB2 in F1 female generation by using As_2O_3-induced laboratory animal models.

In the present study, the difference in BW, feed intake, inflammatory, and oxidative indices between the control group and treated As_2O_3 animals is likely attributed to differences in the daily content of drinking water. It can be concluded that As_2O_3 increases lipolysis through increased production of free radicals, ROS formation, and decreased feed intake. On the other hand, MDA (an end product of lipid peroxidation) is an index for the level of ROS-induced biological damage [18]. Results also showed that As_2O_3 increased serum MDA concentration of pubertal F1-female rats. In agreement with the results of the current study, previous studies have reported that arsenic enhances free radicals production and ROS formation, thus it increases lipid peroxidation and subsequently MDA levels in blood and tissues [1, 4]. Meanwhile, in VB2 groups the decreased serum ROS and MDA concentrations confirm the antioxidant activity of VB2 administration. This phenomenon can be explained by the antioxidant theory. The antioxidant theory states that increased antioxidant vitamins decrease lipid peroxidation. The VB2 is a vital co-enzymes in lipid metabolism. It is known that VB2 is mainly metabolized in the liver and becomes FAD to regulate metabolism [19, 20]. Our study proved that VB2, as an effective antioxidant, could prevent lipid oxidation under the As_2O_3 toxicity in female rat offspring. Broadly, fatty acids oxidation depends on adequate concentrations of the B-vitamins family-like riboflavin [21].

Basically, B vitamins have beneficial effect on inflammation and oxidative stress indices. Other researchers that indicated that VB2 prevent inflammatory and oxidative changes produce by ROS production and inhibit the lipoproteins oxidation [12,15,22] confirmed this idea. Considering the major metabolic pathways that flavoenzymes influence, nutritional deficiencies of VB2 impact primarily on lipid metabolism [9] Several studies have demonstrated VB2 attenuated lipid peroxidation, the mechanism was merely focused on FAD replenishment of VB2 and demethylation of key enzymes of phospholipid metabolism [23,24]. It is possible that VB2 reduces lipid peroxidation by decreasing corticosterone concentration because corticosterone increases lipid peroxidation. Hence, it is recommended that future trials should focus more on exact relations between vitamin therapy and glucocorticoid secretion in animal models.

Maternal nutrition is becoming increasingly recognized as a determinant of chronic disease in offspring [25]. Based on our laboratory results, dietary inclusion of VB2 at two dosages, 20 and 40 mg/kg/day, reduced the serum concentration of antioxidant machinery including GSH, SOD, and CAT. Meanwhile, blood studies have demonstrated that IL-6 and CRP as a predictor of toxic substances damage risk after adjusting potential baseline blood factors. In the present study, As_2O_3 treatments attenuated the host system by altering the levels of inflammatory markers in the serum of pubertal F1-female animals. On contrary, in VB2 groups the increased VB2 levels in drinking water are paralleled with decreased serum inflammatory markers levels.

Based on the reports of the Institute of Medicine Feed and Nutrition Board, the body absorbs little VB2 and stores only small amounts of it in the liver, heart, and kidneys [26]. On the other hand, the demand for some vitamins increases

Table 3: Effects of riboflavin on inflammatory and oxidative stress indices in arsenic-treated of female rat offspring

Group [mg/kg/day]	Vehicle [control]	As_2O_3 1.5	As_2O_3 + VB2 20	As_2O_3 + VB2 40
ROS	1.18 ± 0.63 [b]	2.32±0.82 [a]	1.52 ± 0.61 [b]	1.43 ± 0.55 [b]
MDA	1.73 ± 0.31 [b]	3.33±0.87 [a]	2.12 ± 0.65 [b]	2.09 ± 0.71 [b]
GSH	70.3 ± 5.33 [a]	35.1±4.02 [b]	65.2 ± 1.96 [a]	66.8 ± 2.16 [a]
SOD	70.3 ± 5.33 [a]	73.2±6.01 [b]	84.5 ± 6.28 [a]	88.3 ± 7.12 [a]
CAT	532.5 ± 78.9 [a]	440.1±56.8 [b]	508 ± 61.7 [a]	520 ± 70.5 [a]
IL-6	72.2 ± 13.6 [b]	88.9±18.5 [a]	79.1 ± 16.0 [b]	75.9 ± 15.1 [b]
CRP	6.36 ± 1.48 [b]	7.55±0.82 [a]	6.83 ± 0.93 [b]	6.71 ± 1.12 [b]

As_2O_3: arsenic trioxide; VB2: riboflavin.
Values are given as means ± SD [n=7]. The same superscripts (a-e) are not significantly different from each other in each row [$P < 0.05$].
ROS [DCF Fluorescence Intensity]: reactive oxygen species; T-AOC [% of control]: total antioxidant capacity; MDA [mmol/mg protein]: malondialdehyde; GSH [µmol/ml]: glutathione peroxidase; SOD [U/ml]: superoxide dismutase; CAT [U/ml]: catalase; IL-6 [mg/l]: interleukin 6; CRP [mg/l]: C-reactive protein.

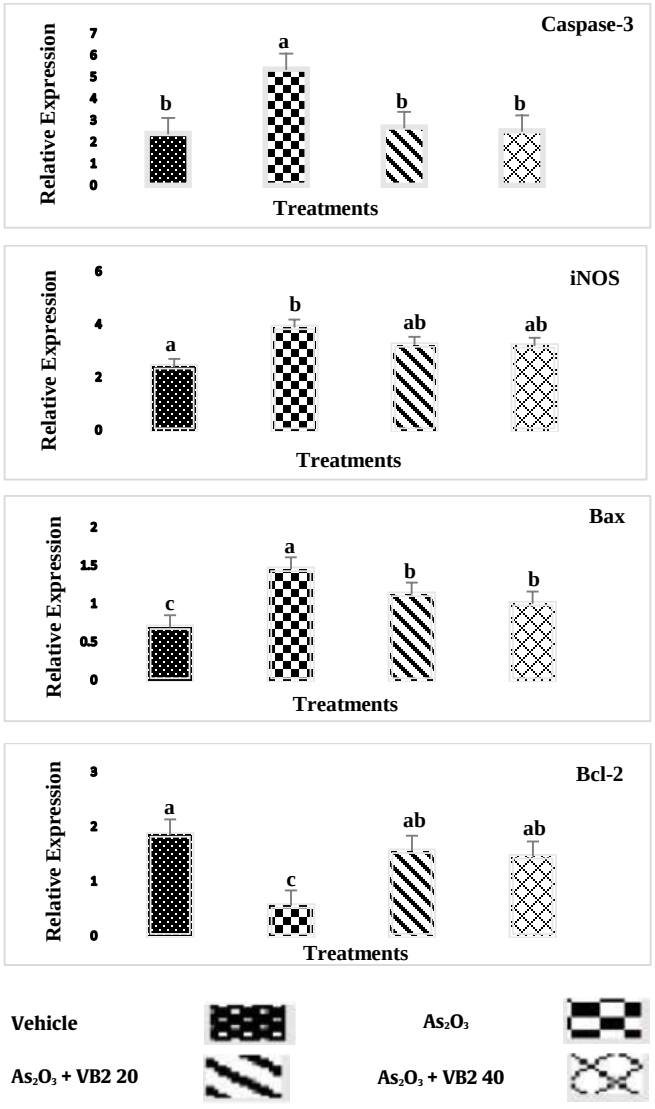

Figure 2: Changes in the expressions of apoptosis key markers in mRNA induced by As_2O_3; mRNA expressions levels of *caspase-3*, *iNOS*, *Bax*, and *Bcl-2* as detected by qRT-PCR.
All the data were expressed as relative values against their respective control group. The values are presented as mean ± SD (n = 5). β-actin was used as an internal control. [a-c] mean values with common letter [s] above bars do not differ significantly [$P < 0.05$].

During gestation. The obtained results revealed that maternal use of VB2 during the sensitive period of fetus development reduced the oxidative stress defects in particular. It can be concluded that As_2O_3 is linked as a risk factor in a number of both mother and her fetus damages. Generally, it was observed that As_2O_3 exposure could significantly inhibit the growth and development of rodents. As reported, after treatment with As_2O_3, $HepG_2$ cells and hippocampus cells underwent significant apoptosis [27, 28].

As_2O_3-induced uterus cells death was blocked by VB2 as a candidate therapy. In this case, a balance between apoptosis and cell proliferation might have occurred in supplemented groups with VB2 since the tissue was in homeostasis. VB2 exerts protective effects through its antioxidant, anti-fibrosis, and anti-inflammatory properties that consequently restore the structure and functionality of the target organs [29].

In this study, the mRNA expression levels of an anti-apoptotic gene like Bcl-2 were significantly down-regulated and the mRNA expression levels of pro-apoptotic genes including caspase 3 and Bax were significantly up-regulated in uterus tissue. Overexpression of the Bcl-2 gene could increase cell viability and prevent apoptosis in adverse tissue circumstances [30,31,32]. As_2O_3 administered generates inflammatory–oxidative stress that induces apoptosis of uterus tissue by activating caspase 3 and iNOS gene expressions occurs together with Bax/Bcl-2 imbalance. Our findings indicated that VB2 as a candidate therapy could change the expression of apoptosis-related genes and contribution with genes related to survivalists, which has been an effective strategy to overcome the drawbacks of As_2O_3 toxicity.

4. Conclusion

In summary, the obtained data demonstrated that VB2 therapy is potentially an effective strategy for correcting the detrimental effects of As_2O_3 in pubertal F1-female rats via inhibiting oxidative changes and cell apoptosis; however, a full understanding of the mechanism by which these vitamin function is still lacking.

Little work has been done about the effect of B vitamin supplements family on the reproductive system. Thus, the present information obtained in the rat model can pave the way for investigating VB2 function, not only about its effects on female reproduction but also as a potential antioxidant with systemic activity. This can help to develop novel treatments and biotechnological applications both in human medicine and animal production.

Authors' Contributions

Parichehr Nouri: Formal analysis; Methodology; Writing – review and Editing. **Ali Olfati**: Conceptualization; Data curation, Investigation; Writing – original draft. All the authors read and approved the final manuscript.

Conflicts of Interest

The Authors declare that there is no conflict of interest.

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Abbreviations

As_2O_3: arsenic trioxide; VB2: riboflavin; FMN: flavin mononucleotide; FAD: flavin adenine dinucleotide; ROS: reactive oxygen species; TAS: total antioxidative status; MDA: malondialdehyde, GSH: glutathione peroxidase; SOD: superoxide dismutase; CAT: catalase; IL-6: interleukin 6; CRP: C-reactive protein; BW: body weight

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