Acta Veterinaria-Beograd 2024, 74 (1), 60-70
UDK: 599.32:591.2;
591.2:[615.9:546.19
DOI: 10.2478/acve-2024-0005

Research article

IMPACT OF ARSENIC ON MOUSE OVARIES OVER THREE GENERATIONS

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(Received 18 November 2023, Accepted 23 February 2024)

This study aimed to measure the total arsenic content deposited in the ovaries of three consecutive generations of mice. The animals were treated with two different concentrations to determine whether histological changes were caused in the ovaries. The control group of mice received tap water, whereas the experimental groups were given different concentrations of dissolved arsenic (III)-oxide. The arsenic content in the ovaries in both experimental groups increased with each generation. The highest content was recorded in the third generation of the second experimental group. Between the two experimental groups in each generation, significant differences in the average number of *corpora lutea* and ovarian follicles were identified. Arsenic caused structural changes in the ovaries in both experimental groups in all three consecutive generations.

Keywords: arsenic (III)-oxide, ovary, morphometry, total arsenic content, mice.

INTRODUCTION

Arsenic (As), an important heavy metal, causes health problems in humans and animals [1]. In recent years, the increasing danger of As exposure has become a subject of great concern because of rapid urbanization and industrialization [2]. Humans can be exposed to As by eating As-contaminated food, crops, and drinking water [3,4].

Vojvodina, a northern region of Serbia, belongs to the Pannonian Basin, whose aquifers contain high concentrations of arsenic.

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The WHO has set a temporary threshold value of 10 μg/L of water, but the aim is for the called value to equal zero. The Vojvodina Region administratively consists of 45 municipalities. The National Monitoring Programme of Drinking Water Quality in Serbia shows that public water supply systems in 20 municipalities (44.4% of all municipalities in Vojvodina) contain high amounts of arsenic. The arsenic content in drinking water in some parts of Vojvodina (primarily Zrenjanin) is higher than that in other geologically independent parts of the world. The highest measured value of arsenic concentration in the water supply network of Vojvodina (measured in the settlement of Taraš, municipality Zrenjanin) was 0.859 mg/L [5]. Water is prohibited for humans by law, but in those places, the water supply is accessible to animals without any restriction.

Arsenic has been a known water pollutant in Vojvodina for more than 40 years, but public water supply systems lack the technical and financial resources for its removal. Because arsenic does not change the color or taste of water, exposed populations as well as domestic animals continue to use the contaminated water for drinking, cooking, and soil/garden/plant watering throughout their lives, thus ingesting large amounts of arsenic [5].

Arsenic can accumulate in the tissues of animals and has a cumulative effect, which may lead to humans being exposed to arsenic in the food chain.

In the literature, there are no data on the cumulative effect of arsenic on the structure of the ovaries exposed to arsenic through three consecutive generations. They have no data on histological changes in the structure of the ovaries of mice treated with arsenic (III)-oxide, and morphometric analysis of the ovaries structure has not been performed. This study examined the impact of arsenic on reproductive function.

MATERIALS AND METHODS

Animals and housing conditions

The experiment was approved by the Ethics Council – Veterinary Administration, Ministry of Agriculture, Forestry and Water Management of Serbia (number: 323-07-00615/019–05 dated 29/01/2020). Male and female mice (Mus musculus, strain: NMRI) aged 0-6 months were used in this experiment. They were housed in the vivarium of the Institute for Rabies Control, Pasteur Institute, Novi Sad. Throughout the experiment, the animals were housed in group cages at 20-22 °C, 50 ± 5% relative humidity, on a 12-h light–dark cycle, and had free access to water and food.

Chemical and dosing

For the experiment, arsenic (III)-oxide ("Centrohem"StaraPazova) was dissolved in distilled water. Two different concentrations of arsenic (III)-oxide were applied. One experimental group of animals was treated with a dose of 10.6 mg/L (A) and the

other with 106 mg/L (B). The animals in the control group (C) received water from the Novi Sad Water Supply Network. The concentration of arsenic (III)-oxide of 10.6 mg/L represents the highest concentration measured in the water supply network of Zrenjanin, 0.859 mg/L (measured in Taraš settlement, Serbia) [5] by converting the values of arsenic concentration from human to animal model (mice) according to the protocol [6] human equivalent dose calculation based on body surface area. To convert the human dose in mg/kg to the animal equivalent dose (AED), multiply or divide the human dose by the Km ratio for mice (Multiply human dose by Km =12.3; Dividing human dose by Km =0.081). In our case, 0.859 mg/L represents the value for the human model, whereas the equivalent value for the animal model (mouse) is 10.6 mg/L.

The concentration of arsenic (III)-oxide of 106 mg/L was applied as the maximum tolerated dose according to the recommendation of the toxicologist to develop the effects of arsenic on the organism, which will appear faster compared with the dose of 10.6 mg/L, which the animals should be exposed to much longer to manifest the effects.

Formation of the generation of experimental groups

For the tests of arsenic (III)-oxide administration across generations, male and female mice of the same parental origin were divided into two groups. The progeny produced was designated as the first (G1) generation in each group. After continued administration of the above treatment, the second (G2) and third (G3) generations of mice were obtained.

To obtain the G1 generation of experimental animals, the offspring (males and females, 3 weeks old) had to be separated from each other by random selection. Twelve females and six males were distributed among three cages in the first-generation experimental group. Four females and two unrelated males were placed in each cage. In both the second experiment and the control group, the same number and arrangement of animals were placed in cages to form the first generation. Each experimental group was continuously exposed to a specific dose of arsenic (III)-oxide from the time the mice were placed in the cages to the next generation.

After weaning, the 3-week-olds were taken to the "nursery". There they remained until they were fully grown (about 8 to 10 weeks). Male and female juvenile mice (eight to ten weeks old) were randomly selected from the nursery to form the first generation of animals. To avoid inbreeding, mice were divided among different nurseries. After female copulation and gestation, males were removed from their cages, and one-day-old live young were counted. The young were raised in cages with their mothers for up to three weeks to form the next generation. They were then moved to the nursery until sexual maturity. To form the next generation, male and female juveniles were selected from the nursery according to the above schedule and the number of animals in the cage. The female animals were divided into separate cages after lactation according to

the experimental group and the generation to which they belonged. Organ samples were collected to determine the amount of total arsenic deposited and to analyze histological changes in the ovaries.

Measurement of total arsenic content deposited in ovaries in three consecutive generations of mice

The samples for measurement were prepared by the microwave digestion method in the Ethos, Microwave Labstation, and Milestone systems. The samples were digested with 8ml of diluted HNO_3 (2:1) and 2ml of H_2O_2 (30%) at Tmax=180^0C. The given programme of microwave digestion lasted 30 min. with max. with a power of 1000W. Arsenic concentration was measured using the coupled plasma technique with mass detection on an Agilent ICP-MS 7700x instrument using the 75As isotope. The determination was performed in the helium mode with an integration time of 1 s per point (He-M, IT 1 s/P). The calibration curve was created using certified AccuTraceTM Reference Standard (USA) concentration 1000μg/ml. To determine the effectiveness of microwave digestion, the samples were spiked with a known concentration of standard solutions of the investigated element. The experimentally determined limit of quantification (LoQ) for arsenic by the method used is <0.001mg/kg. The arsenic content of the native samples was calculated on the basis of the measured number of pulses (counts), the calibration function, and the dilution using the MassHunter Workstation software programme.

Histological processing of the tissue

Mice were euthanized, and ovaries were fixed in buffered formalin (pH=7.4) dehydrated at ethanol, embedded in paraffin wax, and catting into 5 μm sections, using a LeicaRM2125 RTS rotary microtome. Sections were stained with hematoxylin and eosin (H&E) to observe the ovarian structure under a Leica Dc100 light microscope.

Morphometric analysis

A total of 45 female mice were analyzed. The experiment was designed on the basis of three groups: a control group and two experimental groups into three consecutive generations. Each group consisted of 15 female mice, i.e., 5 female mice per generation. Each specimen was photographed with a Leica Dc100 camera at 100x magnification. Analysis of the obtained images of the midsection of the specimens was performed using ImageJ software. The following characteristics were measured in the two experimental groups and the control group: total number of *corpora lutea*; total number of ovarian follicles at different stages of development (primordial follicles were not counted); total number of antral follicles; and total number of atretic follicles.

Statistical analysis

Statistical data were analyzed by analysis of variance (ANOVA) and Tukey's post hoc test using GraphPad Prism 6 software. The result was considered statistically significant if $p < 0.05$.

RESULTS

Measurement of arsenic content in the ovaries of mice

The total arsenic content in the ovaries of mice was measured over three months and three consecutive generations using an ICP/MS technique. The results are shown in Table 1. In experimental group A, the concentration of deposited arsenic increased in each consecutive generation. The value in G3 was twice that in the first. In the B group, a gradual increase was observed from generation to generation. When comparing the content of deposited arsenic by generation, the value of deposited arsenic in the G1 B group was almost three times higher than that in the G1 A group. In the G2 B and G3 B groups, was almost twice as high as that in the A groups.

Table 1. The total arsenic content in mouse ovaries in three consecutive generations (G1, G2, and G3), in both experimental groups (A and B)—results were based on pools. A pool represents 5 ovaries of mice.

Sample	Control	A group conc. As 10.6 mg/L expressed in As (µg/kg)	B group conc. As 106 mg/L expressed in As (µg/kg)
G1	36.15	429.21	1132.20
G2	36.15	701.49	1280.50
G3	36.15	851.56	1473.27

Morphometric analysis

The results of the morphometric analysis showed that the average number of *corpora lutea* in the group did not decrease in the G1 generation. In contrast, there was a significant decrease in G2 and G3 compared with the controls. In the B group, the results showed a decrease in the number of corpora lutea, but unlike the A group, the decrease was uniform, and there were no statistical differences in the decrease in the number of corpora lutea between generations (Fig.1).

The average number of follicles in G1 and G3 (A group) decreased compared with that in the control group, whereas the number of follicles in generation G2 decreased significantly. The average number of follicles in the B group showed a gradual decrease from generation to generation. However, the number of follicles in the G1 generation was significantly lower than that in the G1 generation of the A group (Fig.2).

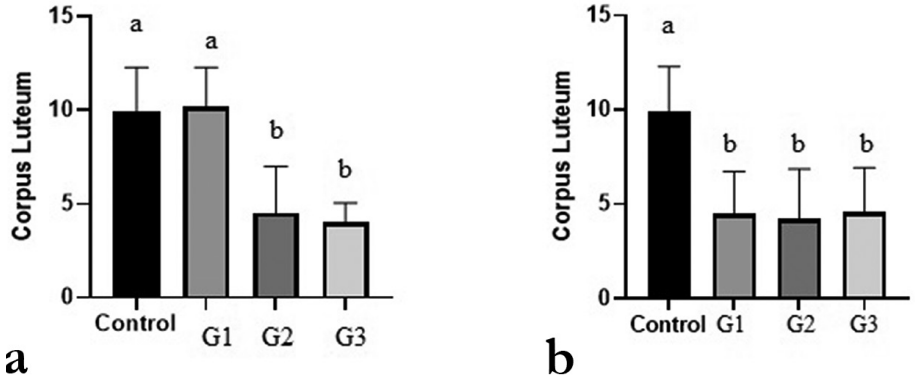

Fig.1.The number of corpora lutea over three consecutive generations (G1, G2, G3) treated with As_2O_3 concentrations of 10.6 mg/L (A-group) and 106 mg/L (B-group). Columns represent means ± SEM of the number of corpora lute based on 5 female mice per group. Different superscript letters (a,b) indicate significant differences ($p<0.05$) between the corresponding controls (a) and arsenic-treated mice (b).

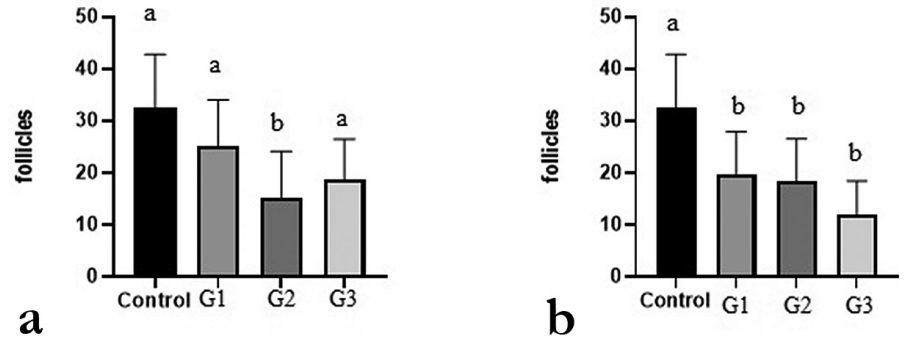

Fig.2. The average number of ovarian follicles throughout three consecutive generations (I, II, III) treated with As_2O_3 concentrations of 10.6 mg/L (A-group) and 106 mg/L (B-group). Columns represent means ± SEM of the number of ovarian follicles based on 5 female mouse ovaries per group. Different superscript letters (a,b) indicate significant differences ($p<0.05$) between the corresponding controls (a) and arsenic-treated mice (b).

The average number of antral follicles in the A group showed a decrease in all three generations compared with the control, which was particularly marked in the G2 generation. The average number of antral follicles in the B group gradually decreased from generation to generation, and the number of antral follicles in the G3 generation was significantly lower than that in the G3 of the A group (Fig. 3).

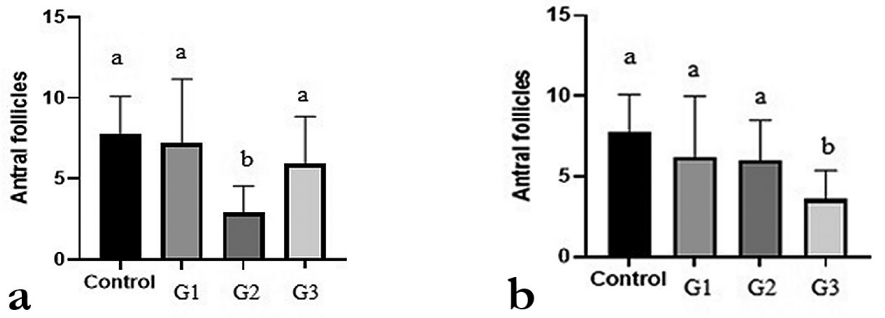

Fig.3. The mean number of ovarian antral follicles throughout three consecutive generations (I, II, III) treated with As_2O_3 concentrations of 10.6 mg/L (A-group) and 106 mg/L (B-group). Columns represent the means ± SEM of the number of ovarian antral follicles based on 5 female mouse ovaries per group. Different superscript letters (a,b) indicate significant differences ($p<0.05$) between the corresponding controls (a) and arsenic-treated mice (b).

The average number of atretic follicles in the group had the highest number in the G3 generation, whereas the lowest number was recorded in the G2 generation. In the B group, the number of atretic follicles was highest in the G2 generation and lowest in the G3 generation. (Fig.4).

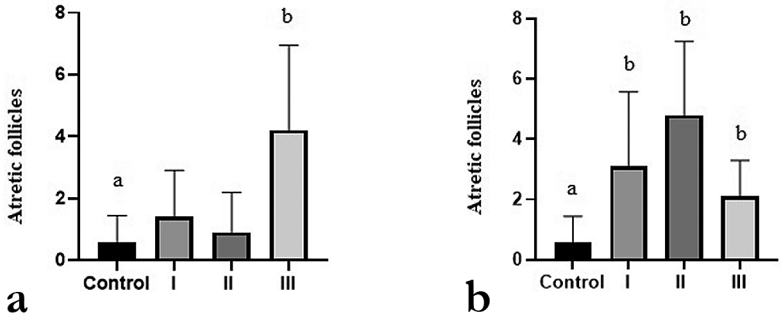

Fig.4. The mean number of atretic follicles throughout three consecutive generations (I, II, III) treated with As_2O_3 concentrations of 10.6 mg/L (A-group) and 106 mg/L (B-group). Columns show the means ± SEM of the number of ovarian follicles based on 5 female mouse ovaries per group. Different superscript letters (a,b) indicate significant differences ($p<0.05$) between the corresponding controls (a) and arsenic-treated mice (b).

The results of the morphometric analysis showed changes in the histological structure. Between the two experimental groups in each generation, significant differences in the average number of *corpora lutea* and ovarian follicles were identified. The average number of *corpora lutea* decreased significantly in the G2 and G3 generations. A similar result was found in the B group, but unlike the A group, the decrease was uniform and did not significantly differ between generations. The average number of follicles in the G2 generation decreased significantly in the A group, whereas a gradual decrease from generation to generation was observed in the B group. In the A group, the average number of antral follicles decreased significantly in the G2 generation. From

generation to generation, the average number of antral follicles gradually decreased in the B group. The number of antral follicles in the G3 generation was significantly lower in the B group than in the G3 generation of the A group.

The G3 generation of the group had the highest average number of atretic follicles, whereas the G2 generation had the lowest. In the B group, the number of atretic follicles was highest in the G2 generation and lowest in the G3 generation. Morphometric measurements showed that arsenic causes changes in certain ovarian structures. Thus, in the A group, there was no regularity between generations, whereas in the B group, a gradual decrease in the average number of certain structures was observed from generation to generation. An exception was atretic follicles, where no intergenerational regularity was observed in any of the experimental groups.

Histological analysis of the ovaries

Histological analysis revealed that the hematoxylin–eosin (H&E) staining results of the control group showed normal tissue development, normal size, and number of follicles at different maturation stages.

The results of the analysis of the ovaries showed that arsenic in the applied concentrations in both experimental groups led to changes in the histological structure. A greater number of follicles with degeneration were observed. In the follicles, degenerating oocytes and apoptotic granulosa cells are registered. The changes were most pronounced in the granulosa cells of the antral follicles (Fig. 5).

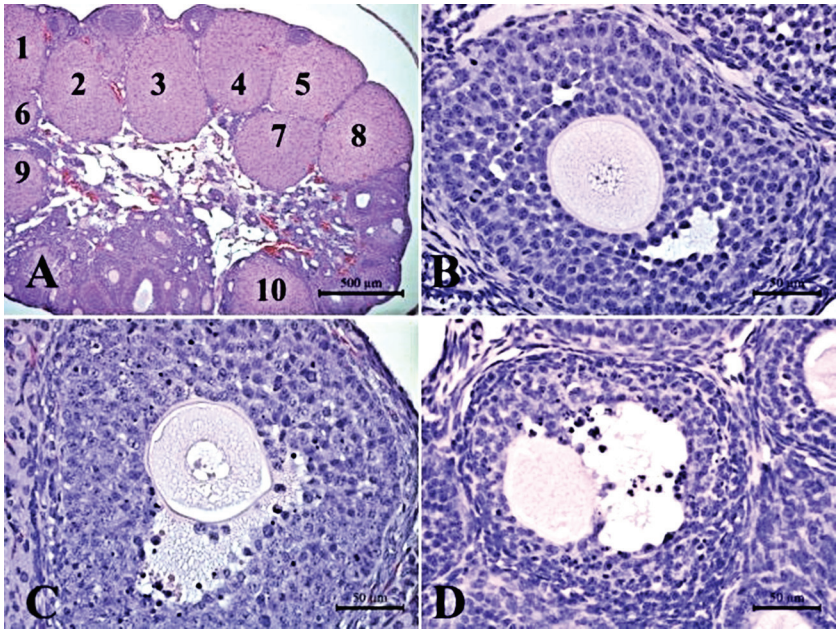

Figure 5. Mice ovaries in group B: (A) G1, Corpora lutea; (B) preserved early antral follicle; (C, D) degenerated oocytes in follicles; B, C, D – G3 — hematoxylin-eosin stain.

The structure of the stromal cells was slightly damaged, whereas the structure of the *corpora lutea* was unclear.

DISCUSSION

Based on the available literature, this is the first study to measure different concentrations of As_2O_3 in the ovaries of mice over three consecutive generations. This study aimed to determine possible structural changes in the ovaries.

Although there are no signs of illness, the animals are active, fed, drink water regularly, and reproduce. Chronic arsenic poisoning likely has harmful effects because arsenic can be deposited in the organs and has a cumulative effect. The total content of deposited arsenic increased from generation to generation in both experimental groups. The levels of arsenic deposited in the B experimental group of all generations were significantly higher than those in the first experimental group. The highest levels were measured in the third generation, confirming that arsenic has a cumulative effect. According to a similar study [7], cadmium chloride is a highly toxic chemical to the ovarian parenchyma of mice, as indicated by the finding that ovarian changes increased with increasing cadmium chloride concentration. Cadmium, lead, or mercury toxicity to the ovaries manifests as decreased follicular growth, follicular atresia, *corpora lutea* degeneration, and changes in the reproductive process [8].

Morphometric and histological analyses of the ovaries treated with the applied concentration of arsenic showed changes in the histological structure, leading to the conclusion that the cumulative effect of arsenic has a deleterious effect on the structure of the ovary, but tumor changes were not observed. In Khalaf et al., 2019, and An et al., 2022 [9,10], oxidative stress caused apoptosis of ovarian granulosa cells in rats, and zinc oxide-induced apoptotic changes in ovaries were observed in female rats [11]. Similar results for different histological staining of ovarian tissue were observed in [11]. Oxidative stress is involved in ovarian toxicity caused by diverse stimuli, including environmental toxicants. There is strong evidence that ROS are involved in the initiation of apoptosis in antral follicles caused by several chemical and physical agents [12].

CONCLUSIONS

Arsenic in the applied concentration caused significant morphometric and histological changes in mouse ovaries. The arsenic content in the ovaries in both experimental groups increased through three consecutive generations. The highest content was recorded in the third generation of the second experimental group. Between the two experimental groups in each generation, significant differences in the average number of *corpora lutea* and ovarian follicles were identified. We conclude that arsenic significantly affects reproduction. Prolonged exposure to arsenic contamination in

drinking water or water supplies measured in Zrenjanin could harm the reproductive capacity of humans and animals (including domesticated animal species).

Authors' contributions

AB carried out the experimental animal studies, performed the statistical analysis, and drafted the manuscript. DL carried out the experimental design. ŽM participated in the toxicology analysis. KPM participated in the review and editing of the manuscript. AR, RG and MM conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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UTICAJ ARSENA NA JAJNIKE MIŠEVA PRAĆENO KROZ TRI UZASTOPNE GENERACIJE

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Cilj istraživanja je određivanje količine deponovanog ukupnog arsena u jajnicima tokom tri uzastopne generacije miševa. Životinje su tretirane sa dve različite koncentracije arsena sa ciljem da se utvrde eventualne histološke promene u jajnicima. Kontrolna grupa miševa je dobijala vodu iz novosadske vodovodne mreže. Eksperimentalnim grupama su primenjene različite koncentracije rastvorenog arsen (III)-oksida. Količina deponovanog arsena u jajnicima obe eksperimentalne grupe se povećavala u svakoj narednoj generaciji. Najveća količina deponovanog arsena je zabeležena u trećoj generaciji druge eksperimentalne grupe. Kod obe eksperimentalne grupe u svakoj generaciji su zabeležene značajne razlike u broju žutih tela i folikula jajnika. Arsen je prouzrokovao strukturne promene u jajnicima obe eksperimentalne grupe u sve tri uzastopne generacije.