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The Effect and Activity of Free Radical Enzymes Due to Arsenic Exposure Through the Vulva and Vagina

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Abstract

BACKGROUND: Geogenic arsenic is ubiquitous, found in water and soil that is used daily, can be exposed to the female body through the genital organs. The vulva and vagina are open channels that allow toxic agents to enter the internal genitalia and distributed throughout the body.

AIM: This study investigated the effects of vaginal arsenic exposure through vulvar immersion and vaginal douching in *Rattus norvegicus* on the damage of uterus and ovaries through oxidative mechanisms (malondialdehyde [MDA], superoxide dismutase [SOD], and H_2O_2).

METHODOLOGY: The experimental animals were divided into three treatment groups, that is, K0 (control group), K1 (group treated with vulvar immersion in 0.8 mg/L arsenic solution), and K2 (group treated with vaginal douching using 0.5 mL of 0.8 mg/L arsenic solution). For each group, the treatment was repeated 6 times and carried out for 14 days. Before the study, a 7-day acclimatization period was conducted for adaptation purposes. The experimental animals were euthanized using ketamine xylazine. The uterus and ovaries were collected for MDA, SOD, and H_2O analysis, as well as histopathology examination.

RESULTS: The vaginal douching group had the highest MDA level both on the uterus (210.66 ± 4.92 μM) and the ovaries (214.67 ± 2.50 μM). The immersion group also experienced an increase in malondialdehyde (MDA) in the uterus (198.66 ± 3.33 μM) and ovaries (206.33 ± 0.21 μM). However, a higher level of MDA was found in the ovaries. The highest H_2O_2 level was also found in the uterine and ovarian organs in the douching group. In contrast, the lowest SOD levels of uterine and ovarian were identified in the vaginal douching group. Arsenic exposure through vaginal immersion and douching affected the uterine MDA, SOD, and H_2O_2 levels ($p < 0.05$). Arsenic exposure through vaginal douching also affected the ovarian MDA, SOD, and H_2O_2 levels ($p < 0.05$). There was a significant difference in the mean of inflammatory cells (infiltrated neutrophils, macrophages, and lymphocytes) in the uterus and ovaries in the control, immersion, and vaginal douching groups ($p < 0.05$).

CONCLUSION: Exposure to 0.8 mg/L arsenic solution through vulvar immersion and vaginal douching can cause oxidative stress and trigger inflammation of the uterine and ovarian tissue.

Introduction

Aceh Province is reported to have several volcano systems such as Jaboi, Seulawah, and Geureudong [1], [2], [3], [4]. The volcano activity causes to appear the hot spring manifestation which becomes one of surface water sources. Ie Seu'um is one of hot spring manifestations in Seulawah Agam volcano system which has been used as ecotourism. The challenge of the geothermal water utilize is the high heavy metal content containing inside the water which is dangerous to the organism's body [5], [6], [7]. Recent study reported the present of arsenic in the geothermal water of Ie Seu'um. The arsenic presence is caused by anthropogenic and geochemical activities [8]. This arsenic has contaminated the resident well water. The river water also contaminated because the geothermal water flows and merges with river water. Arsenic levels in river sediments reached 6.86 mg/kg, while in residents' well water, it reached 800 g/L or 0.8 mg/L [9]. This level is already above the safe threshold for water use because the allowable level of arsenic is only 10 g/L for drinking water and 50 g/L for clean water [5], [10].

Residents lived around the geothermal area of Ie Seu'um have been used the geothermal water and the river water since long time ago for various daily needs, such as agriculture, drinking water, tourist areas, and personal hygiene activities. Besides, most women lived in the surrounding of the estuary work as oysters seekers. The work is done by sitting or squatting at the bottom of the river. Estuary water and sediment with high arsenic concentrations will wet and soak the genital organs during their work. They work without

personal protection 2–3 h/day, every day for the rest of his life. Tourists also use the geothermal hot springs for bathing and evaporation. Visitors believe hot springs can cure various diseases.

The threat of arsenic poison for women exposed to arsenic through the genital organs has not been widely explored. The female genital organs can be contacted with arsenic when washing, bathing, swimming, bathing, or working. Female oysters' seekers at the estuary in Aceh, Indonesia, are exposed to arsenic from the estuary water and sediments during working time. They douche the genital organs using well water in settlements that are also contaminated with arsenic to clean their genital organs from the estuary mud. Exposure to estuary water in females seeking oysters has worsened Pap smear test results [6].

In addition to the above contaminated sources, the female genital organs can also be contaminated by chemical substance from the use of feminine hygiene and diapers in infants and adults. Almost all women around the world use diapers and feminine hygiene products. Some of these products contain heavy metals, such as Cd, Co, Cr, Cu, Ni, and Pb [7], [11]. Various feminine care products include tampons, pads, panty liners, feminine sprays, douches, anti-itch cream, feminine wipes, and feminine powder. These products are applied by sticking them to the vulva or smeared and douching through the vagina. These products' harmful active substances enter and are absorbed by the vagina when the vulva is submerged for a long time or through the vaginal douching process [12].

The vagina is a potential channel for delivering systemic macromolecules and peptides in various therapies. However, the ability of vaginal absorption is influenced by the ability of the epithelium in the vaginal tissue. The vaginal and vulvar mucosa can absorb various chemicals without going through a metabolic process. The vaginal mucosa, including the vulva, clitoris, labia minora, and urethra, can secret and absorb fluids more rapidly than the skin [7], [13]. Long-term exposure to arsenic in the body can increase the production of reactive oxygen species (ROS), disturbing the balance between oxidative stress and antioxidants. This balance disorder causes disturbances in women's reproductive system at childbearing age and even up to menopause [14].

In general, hazardous chemicals enter the body through oral, dermal, pulmonary, and parenteral routes [15]. Another exposure channel to be aware of in women is the genital organs. Studies on the oxidative effects of female reproductive organs due to arsenic exposure that enters the body through oral, ingestion, inhalation, dermal, and parenteral routes have been widely conducted [16]. However, studies on oxidative effects due to arsenic exposure through the vaginal are limited.

This study aims to examine the damage to the uterus and ovaries in experimental animals (*Rattus norvegicus* female). Uterine damage was observed through the oxidative channel exposed to a fixed concentration of the arsenic solution. Arsenic levels refer to the level obtained from the measurements in community wells around the Ie Seu'um geothermal area of Aceh, Indonesia. Exposure was done vaginally by immersing the vulva and vaginal douching. Oxidative effects were assessed through the levels of MDA, superoxide dismutase (SOD), and hydrogen peroxide (H_2O_2) in the uterus and ovaries. Damage to the uterus and ovaries was examined by observing the inflammation of the uterine and ovarian tissues through histopathological examination.

Methods

The ethical approval (No. 259/KEPK-FK UNLAM/EC/VII/2020) was issued by the Ethical Committee of Medical Research, Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia. The study was conducted at the Chemistry/Biochemistry Laboratory, Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, South Kalimantan, Indonesia. The experimental animals were obtained from CV. Abadi Jaya farm at Yogyakarta, Indonesia. A 7-day acclimatization period was carried out to 18 healthy, adult female *R. norvegicus* of *Sprague-Dawley* strain, 2–3 months old and weighed 200–300 g. The rats from all study groups were maintained in a controlled temperature environment (25 ± 2°C), with 12 h light/dark cycles. The animals were given c-05 pellet rations and drinking water sourced from the local water company (Perusahaan Daerah Air Minum/PDAM). Food and drink were provided *ad libitum*.

The experimental animals were divided into three groups, with six rats in each group [17]. K1 group treatment: Rats were put in a 39 × 42 × 15 cm plastic container, filled with 0.8 mg/L arsenic solution, and covered with woven iron wire. Each container contained one rat immersed in arsenic solution up to the tail level (the entire vulva was submerged). During the intervention process (immersion), the rats were not fed. Rats were only fed before and after the intervention process. The immersion water temperature referred to the estuary water temperature, 30 ± 2°C. The immersion duration was 2.5 h a day, resembling the duration of female oyster seekers immersed in estuary water. The immersion was done every day for 14 consecutive days. K2 group treatment: Rats were induced with 0.5 mL of 0.8 mg/L arsenic solution through vaginal douching using a 1 mL syringe without a needle. Spraying was carried out every day for 14 consecutive days. The arsenic was obtained commercially from Loba Chemie Laboratory Reagents and Fine Chemicals. The arsenic used is arsenic trioxide, with a purity of 99%. Euthanasia

was performed on the 15th day by injecting ketamine/xylazine [18]. The uterus and ovaries were collected following euthanasia. The collected uterus and left ovary were cut into small pieces. Each piece was fixed in a pH 7 phosphate buffer solution and mashed until a liquid was formed. A 5 mL of the liquid was taken and centrifuged at 3500 rpm for 10 min [19]. The supernatant was collected for MDA, SOD, and H_2O_2 analysis.

Analysis of MDA: A 100 μL of each uterine and ovarian homogenate were put into separate Eppendorf tubes. A 550 μL of distilled water, 100 μL of 10% TCA, 250 μL of 1 N HCl, and 100 μL of 1% NaThio were added to each tube, homogenized, and centrifuged at 500 rpm for 10 min. The supernatant was collected, heated in a 100°C water bath for 30 min, and left at room temperature. The absorbance was measured using a UV–Vis spectrophotometer at λ_{max} of 532 nm [20]. Analysis of SOD: Uterine and ovarian tissues were incubated in a 3 mL solution containing 0.05 M Na_2CO_3 and 0.1 M EDTA pH 10.2. A 100 μL of serum and 100 μL of adrenaline with (3.10^{-4}) BM 189 M were added to each solution. Initial absorption (A_0) was measured using a spectrophotometer at a wavelength of 480 nm. The samples were incubated for 5 min at 30°C and measured for their absorption (A_1) [21]. The SOD enzyme activity was analyzed by measuring the absorbance change over a certain time span. Determination of serum peroxide (H_2O_2): The standard curve was prepared by the following method. Various amounts (10–160 micromol) of H_2O_2 were put into nine different tubes. A 2 ml of dichromate/glacial acetate was added to each tube. After blue precipitate forms, the tube was heated every 10 min in a water bath filled with boiling water to decompose the blue precipitate until a green color of chromic acetate is formed. After cooled to room temperature, H_2O_2 was added to achieve a 3 mL volume for each tube and then transferred to a cuvette. The absorbance was measured using a UV–Vis spectrophotometer at a wavelength of 570 nm [22], [23]. Uterine and ovarian histopathological evaluation: Cell damage was determined by counting inflammatory cells using the hematoxylin and eosin (H and E) staining method. The collected ovary and right uterus were soaked in 10% formalin and cut to a (10–15) mm × (10–15) mm × 5 mm size. Dehydration, clearing, impregnation, and embedding processes were carried out. The samples were cut using a microtome to obtain 3–5 μm thick slices and stained with H and E. The specimen already mounted on slides were soaked in xylol (2 min), xylol (2 min), absolute ethanol (1 min), absolute ethanol (1 min), 95% ethanol (1 min), and ethanol 95% (1 min), respectively. The specimen was rinsed under running water for 10–15 min, dipped 4 times in water, soaked in hyposolution for 3 min, and rinsed under running water for 10 min. Next, the specimen was soaked in Mayer's hematoxylin solution for 5 min, then rinsed under running water for 20 min, followed by eosin 30 s (three dips), alcohol 70, and then dry. The next step was a mounting process to cover the specimen with a coverslip sealed using an adhesive (Entellan brand) [21].

Observation and calculation of the uterine and ovarian inflammatory structure were carried out by counting the number of inflammatory cells. The process was carried out under a binocular microscope using 15 fields of view. Examinations were made by considering the histological structures of the uterine and ovarian zones. Three specimens were mounted on each glass slide. Inflammatory cell count was performed by counting the mean number of macrophage cells, neutrophils, as well as uterine and ovarian lymphocytes [24] using a 40 jar magnification and 40x objective microscope. Inflammatory cells are considered identified if infiltration of inflammatory cells, including lymphocytes and neutrophils, was observed. Inflammatory cell count was carried out by two personnel to reduce research errors.

Data analysis

Statistical analysis is presented as means ± standard deviation (SD) values. Concentrations between groups were compared using the one-way ANOVA with $\alpha = 0.05$ because the experimental unit is homogenous. Homogeneous means that all experimental units were selected and treated in the same way, including the type and strain of experimental animals, age, sex, size, weight, and the same health condition. In addition, the maintenance process was also conducted in the same way: Environment, temperature, feeding, and lighting. All of these variables were made equal because it is suspected that they can affect the treatment. Hence, they are called homogeneous. Statistical test continued Duncan's multiple range test. The parameters were tested in the same way.

Results

The results of the statistical analysis are presented in Table 1. The mean uterine and ovarian MDA, H_2O_2, and SOD levels in the control group are lower than the immersion and douching groups. The highest levels are found in the vaginal douching group.

Table 1: Comparison of posttreatment levels of malondialdehyde, hydrogen peroxide, and superoxide dismutase (mean ± standard deviation)

Variable	Treatment group			*P*
	K0 (Control)	K1 (immersion)	K2 (douching)	
Uterus				
MDA (μM)	195.66 ± 3.07a	198.66 ± 3.33a	210.66 ± 4.92b	<0.001
H_2O_2 (μM)	11.31 ± 0.61a	14.36 ± 1.07b	16.91 ± 0.44c	<0.001
SOD (U/L)	18.00 ± 4.47a	12.00 ± 1.54b	3.83 ± 0.75c	<0.001
Ovary				
MDA (μM)	197.33 ± 0.81a	206.33 ± 1.21b	214.67 ± 2.50c	<0.001
H_2O_2 (μM)	12.26 ± 0.43a	15.05 ± 0.91b	17.12 ± 0.20c	<0.001
SOD (U/L)	18 ± 4.47a	13.33 ± 1.03b	3.33 ± 1.03c	<0.001

Notation difference ^{a,b,c}shows significant effects ($p<0.05$) between the treatment groups. MDA: Malondialdehyde, SOD: Superoxide dismutase, H_2O_2: Hydrogen peroxide.

The ANOVA test result demonstrates that arsenic exposure affected the average uterine MDA, H_2O_2, and SOD levels. However, there is no difference in MDA levels between the immersion and control groups.

As with the uterus, the highest MDA, H_2O_2, and SOD levels in the ovaries are found in the douching group. However, the vulva immersion group also showed a level increase of the three markers. The statistical analysis showed that arsenic exposure affected the average ovarian MDA, H_2O_2, and SOD levels. Although the difference in SD between the control and immersion groups, as shown in Table 1, is minimum, uterine MDA levels have increased due to arsenic exposure through vulvar immersion. This result indicates that oxidative reactions can also happen in the immersion group.

In contrast to vulvar immersion, 0.5 mL of 0.8 mg/L arsenic solution was thoroughly induced into the genital organs when exposed by douching. Arsenic that has entered the vaginal cavity is transferred into the uterus through the uterine cervix, then spreads to both uterine horns and enters both ovaries.

The uterine histological examination showed an increase in the number of inflammatory cells, both in the immersion and douching groups. Inflammatory, lymphocyte, and neutrophil infiltration are visible in the uterine lining. Inflammation was also found in the control group but at a lower degree. The highest number of inflammatory cells was found in the vaginal douching group. The mean number of inflammatory cells in each group is presented in Figure 1.

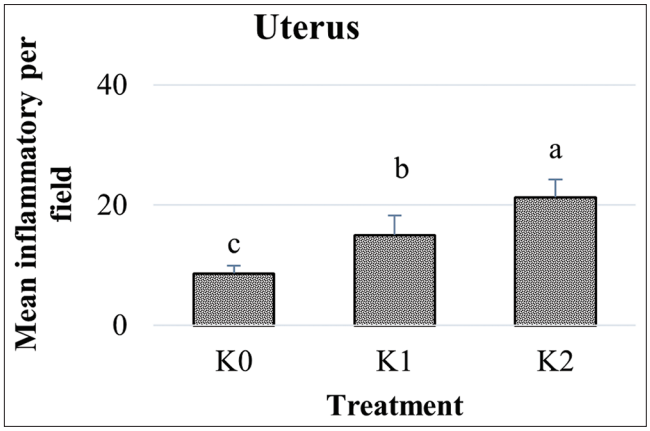

Figure 1: The mean number of inflammatory cells in K0, K1, and K2. Different notations [a,b,c] show significant differences ($p < 0.05$) between treatment groups

The examination demonstrates a significant difference in the level of inflammation between the control and treatment groups. Infiltration of inflammatory cells, including lymphocytes and neutrophils, occurred mostly in the douching group. Inflammation also occurred in the control group but to a lesser extent. The mean number of ovarian inflammatory cells is 6.00 in the control group, 15.83 in the immersion group, and 30.16 in the douching group (Figure 2).

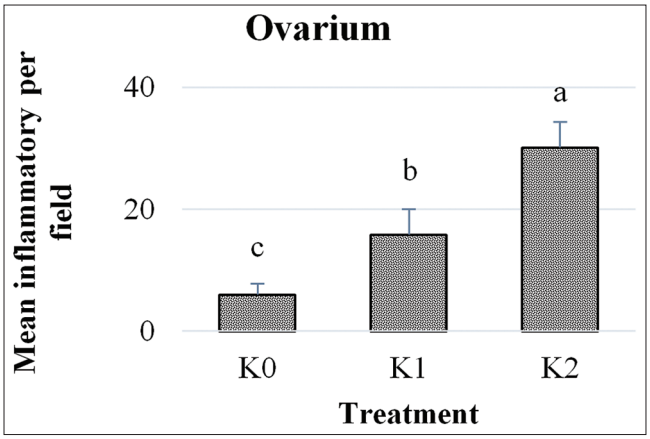

Figure 2: The mean number of inflammatory cells in the control group (K0), vulvar immersion (K1), and vaginal douching (K2). Different notations [a,b] and [c] show significant differences ($p < 0.05$) between treatment groups

Discussion

The rat vaginal, uterus, and ovaries are covered by an endometrium lining composed of mucosa and epithelium. The mucosal epithelium has an excellent ability to absorb fluids, especially during the diestrus stage. The rat vulva is generally composed of a 1 mm wide circular tissue covered by sparse hairs. The vulva consists of the clitoris, vaginal opening, and urethral orifice. The vulva periodically opens and closes following the estrous cycle [25], [26]. The anatomical structure of the rat vulva enables the absorption of arsenic solutions. The vaginal opening is covered by mucosa extending from the vagina. The vaginal opening mucosa on the vulva can also absorb arsenic solution but with a slower mechanism.

The vulvar and vaginal tissues are structurally different from the skin in other body parts. Vulvar tissue is more permeable and very susceptible to friction. Both organs (vulva and vagina) can absorb arsenic, although, anatomically, the vagina absorbs faster. The previous studies demonstrate that arsenic absorbed through the vagina and vulva is distributed more efficiently and effectively than the oral route [12], [27].

Arsenic and its metabolites are toxic and have a substantial impact on the reproductive system [28]. Arsenic exposure to the genital organs triggers the release of free radicals, which are molecules or atoms generated from cellular metabolism. The outer orbit contains one or more unpaired electrons and is, therefore, able to stand independently. Free radicals have an odd number of electrons, are highly reactive, unstable, and short-lived. In principle, all biomolecules are always in pairs. Free radicals seek other free electrons to form a pair, thus achieving stability [29]. The reactive nature of free radicals may damage the lipid, protein, and nucleic acid molecules in tissues and organs [29].

The high levels of H_2O_2 and MDA in the uterus and ovaries caused by arsenic exposure through the genital organs, as presented in Table 1, indicate that arsenic toxic effects have worked thoroughly. Oxidative effects have occurred on the uterus and ovaries. Oxidative stress in these two organs can be triggered by the level of ROS caused by arsenic or a decline in the antioxidant enzyme (SOD) activity. ROS, which is formed due to a homeostatic imbalance between antioxidants and pro-oxidants, is highly toxic. ROS also plays a role in triggering OS by altering deoxyribonucleic acid (DNA) in cell nuclei, cell membranes, lipid peroxides, and proteins [30].

High levels of uterine and ovarian MDA occur after 14 days of vulvar and vaginal exposure to arsenic. It means that the free radicals formed due to arsenic exposure have disrupted the uterus and ovaries' lipid membrane. High levels of MDA indirectly indicate high levels of ROS. Arsenic-triggered free radicals lead to peroxidation of the uterine and ovarian lipid component membrane. Lipid peroxidation occurs when free radicals shift the hydrogen in the methylene group (CH_2). During lipid peroxidation, several products are generated, including MDA [31], [32].

Arsenic exposure affected the H_2O_2 levels of the uterus ($p < 0.01$) and ovaries ($p < 0.01$). Free radicals formed due to arsenic exposure to the genital organs bind to oxygen from the surrounding tissues. Oxygen (O_2) is an electron acceptor, which means that it accepts free electrons even though it has reached stability; thus, forming a superoxide anion radical (O_2). As a result, other substances, in the form of H_2O_2, are generated. This newly formed free radical is a product of ROS. Physiologically, the body produces ROS to kill bacteria; however, if the ratio of free radicals/oxidants (ROS) is greater than anti-free radicals (antioxidants), oxidative stress may occur [33].

Oxygen in the body can mutate into toxic, mutagenic gas, known as ROS, a reactive oxygen compound. After arsenic exposure, tissue indomethacin generation indirectly weakens the vaginal mucosal defense system and leads to infection. The infection can trigger the activation of neutrophils and macrophages. Activation of macrophages and neutrophils into the tissue also triggers ROS [20].

Compared to the control group, the low levels of SOD in the treatment groups indicate that the SOD enzyme in the uterine and ovarian tissue could not maintain antioxidants. As a by-product of ROS, H_2O_2 is usually present in all aerobic organisms; however, the excess amount may cause toxic effects [34]. If tissue H_2O_2 is formed, it will be neutralized by SOD as an antioxidant [35]. SOD, an essential antioxidant to repair the effects of OS [36], is an enzyme that functions as a barrier to fight free radicals. Organisms that rely on oxygen for life have SOD enzymes; therefore, it is also known as the universal enzyme. This enzyme can convert superoxide radicals into oxygen and hydrogen peroxide [35].

Continuous exposure to arsenic for 14 days caused a decrease in the uterine and ovarian SOD. High levels of SOD in the control group led to low levels of MDA and H_2O_2. In contrast, low SOD levels caused an increase in MDA levels in the immersion group.

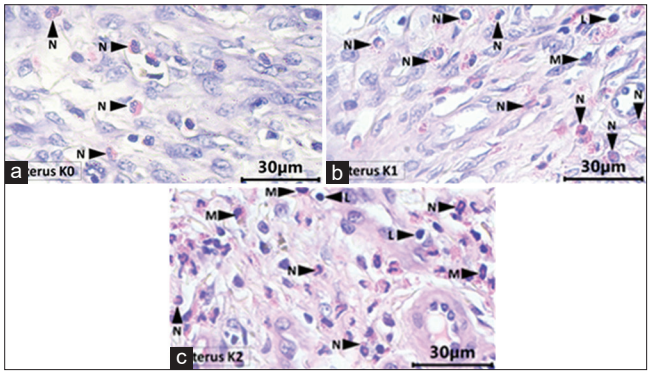

Figure 3: (a) The histological observation of uterine endometrial tissue of R. norvegicus in the untreated control group showed neutrophils infiltration ►N. (b). The appearance of neutrophils ►N, macrophage ►M, and lymphocyte ►L cell infiltration in the uterine myometrial lining of the vulvar immersion group. (c) Infiltration of neutrophils ►N, macrophages ►M, and lymphocyte ►L in the perimetrial lining of the uterus in the vaginal douching group

The difference in mean SOD levels between the immersion and douching groups was due to the level of arsenic exposure. In the douching group, 0.5 mL of arsenic entered the vaginal opening entirely and was absorbed by the mucosal layer, whereas in the immersion group, the arsenic absorption occurred slowly. The anatomy of the external genital organs and the estrous cycle of *R. norvegicus* also influence arsenic absorption into the internal genital organs.

Chronic arsenic exposure harms the female reproductive system's histoarchitecture, with the uterus, ovaries, and uterine tube as the affected organs [37]. A large number of inflammatory cells in both treatment groups (Figure 3 and 4) indicate that arsenic toxic effects have worked thoroughly. The histopathological examination of the uterine and ovarian tissue demonstrates a significant difference between the control and treatment groups. Inflammatory cells are

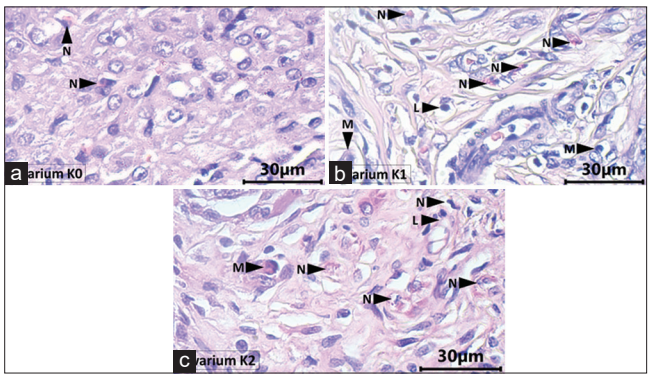

Figure 4: The histological observation of ovarian tissue of R. norvegicus (a) in the untreated control group showed neutrophils infiltration ►N. (b). The appearance of neutrophils ►N, macrophage ►M, and lymphocyte ►L cell infiltration in the vulvar immersion group. (c) Neutrophils ►N, macrophage ►M, and lymphocyte ►L infiltration in the vaginal douching group

generally present in uterine and ovarian tissues that are not exposed to arsenic. Arsenic exposure through genital organs can increase the number of inflammatory cells. The highest number of inflammatory cells, including macrophages, neutrophils, and lymphocytes, occurred in the vaginal douching group, indicating that the oxidative effect happened both in the uterus and ovaries.

As presented in Figures 1 and 2, the large number of inflammatory cells indicates that the oxidative process has caused damage to the uterus and ovaries. Inflammatory cells were also found in the ovaries. However, more inflammation happened in the vaginal douching group compared to the vulvar immersion group. ROS can damage the oocyte and surrounding tissues [38], increase the MDA and H_2O_2 levels, followed by a decrease in the level of SOD. This oxidative mechanism triggers uterine and ovarian tissue inflammation.

ROS, which is a result of a homeostatic imbalance between antioxidants and pro-oxidants, is highly toxic. OS is believed to damage various molecular and cellular structures, as well as alter the body's functions and organ systems [39].

Awareness should be raised regarding the use of various products containing hazardous materials through direct vulvar contact or vaginal douching. The vulva and vagina can absorb toxicants and distribute them to nearby organs, such as the uterus and ovaries. Administration of various harmful products through genital organs, especially during menstruation, can lead to gynecological disorders such as pelvic inflammation, ectopic pregnancy, and cervical cancer [40]. Arsenic and its metabolites are toxic and have a substantial impact on the reproductive system [28]. ROS can be produced at varying times depending on the tissue and the compartments in each tissue. Mitochondria are the main ROS-producing organelles in most tissues [41].

Conclusion

The exposure of 0.8 mg/L arsenic solution to *R. norvegicus* female genitalia through vulvar immersion and vaginal douching reduced the level of SOD, an antioxidant enzyme, and increased the levels of MDA and H_2O_2. The cytotoxic nature of arsenic may cause toxic effects on the cell protoplasm of the genital organs. Oxidative mechanisms occur due to imbalance in the uterus and ovaries. The vaginal mucosa can absorb arsenic thoroughly. Arsenic absorption through vulvar and vaginal mucosa can cause systemic oxidative effects on the uterus and ovaries. Further studies on arsenic exposure with variations in concentrations and exposure duration are required to determine the safe arsenic exposure limit through female genital organs.

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