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TOXIC EFFECTS OF INDUCED SODIUM ARSENITE ON THE OVARY OF SWISS ALBINO MICE

***Shailendra Kumar and Preety Sinha**

Department of Zoology, A. N. College, Patna, Bihar, India.

***Corresponding Author: Shailendra Kumar**

Department of Zoology, A. N. College, Patna, Bihar, India.

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ABSTRACT

Arsenic is an environmental pollutant, which can damage reproductive health and causes disruption of the ovary, quality of ova, decreased ova production and infertility in mice and human. Arsenic creates more and more reactive oxygen species (ROS) in the ovary. The present work provides a detailed analysis of contamination of arsenic in Bihar and further may help to check the main root causes of cancer. Female Swiss albino mice (*Mus musculus*) were selected as the experimental animals. In the treatment group 1.8 mg/kg body weight sodium arsenite and in control group distilled water was given orally by gavage method daily for8 weeks. After the treatment mice were sacrificed and the ovary and blood samples were collected. The effects were assessed by estimating blood arsenic level, serum CA 125 level, LPO level, antioxidant enzymes activity, the apoptotic index in % and histopathological parameters. Sodium arsenite treated group showed no effects on CA 125 in mice blood, LPO level was significantly ($p < 0.0001$) increased, whereas antioxidant enzymes (SOD & CAT) activity were significantly ($p < 0.0001$ & $p < 0.01$) decreased as compared to the control group. Apoptotic index was significantly ($p < 0.0001$) higher in arsenic treated mice compared with control. Sodium arsenite treated group showed vacuolized corpus luteum and ruptured germinal epithelium with Graafian follicle in comparison with the histology of control mice ovary. Our results suggest that sodium arsenite toxicity adversely affected cells of the ovary; caused DNA damage and altered the oxidative stress along with antioxidant enzymes.

KEYWORDS: Sodium arsenite, Ovary, Oxidative stress, Antioxidant enzymes, Apoptosis, Histopathology.

INTRODUCTION

Arsenic, an inessential trace element, tasteless, odorless environmental pollutant, a potent toxin, human carcinogen, modulator of antioxidant defence system, mutagen and xenobiotic metalloid, has recently emerged as a major pollutant of drinking water in a number of districts of Bihar[1], West Bengal[2], India[3], Bangladesh[4], Northern Chile, Thailand, Taiwan, China, Mongolia, Mexico, Argentina, Finland and Hungary.[5] The drinking water of several districts of Bihar contains arsenic more than permissible limit (i.e., 10 ppb in drinking water and 1 ppb in the blood), which is panic for the human population. As these molecules are present and participating in the cellular redox balance, arsenite exposure leads to oxidative stress and followed by cellular responses, which are involved in arsenical toxicity and carcinogenicity.[6] It has been confirmed by different studies that arsenic causes changes in the female reproductive system, including reduced weights of the uterus and ovary and reduced ovarian and uterine peroxidase activities; blockage of steroidogenic enzymes and decreased estradiol levels relative to the controls.[7,8] Exposure to arsenic is a major health problem due to its toxic effect in human and in an animal model. Inorganic arsenic has been linked to developing cancer of skin, prostate, liver, and lung.[9] The non-cancer effects of arsenic include keratosis, diabetes, cardiovascular disease, pigmentation etc.[10]

The arsenic toxicity in the animal model study is very limited. Most of the ingested and inhaled arsenic is well absorbed through the gastrointestinal tract and lung into the bloodstream. Arsenic infiltrates into the human body through these routes, and it is scattered in a number of organs including the lung, liver, spleen, kidney, intestine, skin, and vascular and lymphatic systems, as well as reproductive and nervous systems.[11,12] Reactive oxygen species (ROS) and other free radicals are perpetually produced *in vivo*. Highly reactive radicals such as OH tend to attack different biomolecules (including lipids), initiating free radical chain reaction.[13,14] Lipid peroxidation (LPO) *in vivo* has severe consequences followed by some major clinical problems, cancer being one of them. LPO caused due to oxidative stress ensues eventually in cell damage or death. PUFAs (Polyunsaturated fatty acids) incorporated in lipid bilayer membrane are frequently targeted by free radicals creating lipid peroxides. PUFAs are highly susceptible to oxidation due to the presence of methylene group between double bonds.[15] Free radicals, primarily

produced during electron transport chain in mitochondria; do activate series of chain reaction converting PUFAs into malondialdehyde (MDA). MDA is major aldehyde by-product of LPO[16], which is highly mutagenic and has been denoted to form adducts with DNA bases dG, dA, and dC as m1G, m1A and m1C respectively.[17,18] All cells have intracellular antioxidants (such as superoxide dismutase and glutathione, which are very essential for preventing all cells from oxidative stress at all times. Susceptibility to arsenic curtails the SOD[19] and CAT activity.[20] Apoptosis or programmed cell death is very essential in the homeostasis of the immune system,[21,22] this type of cell death can be also correlated with disease. Arsenic (As) is a very toxic environmental pollutant. It is well known that exposure to this metal causes damage in different tissues, including the reproductive system.[7,8] Arsenic, a human carcinogen, is a worldwide pollutant that is found in soil, water and air. It is an isolate of mitochondrial oxidative phosphorylation that induces production of reactive oxygen species.[23] Several mechanisms have been proposed to explain carcinogenicity of arsenic, including increased formation of reactive oxygen species (ROS) causing oxidative DNA damage, such as single-strand breaks (SSBs) that can be processed to double-strand breaks (DSBs) during replication, inhibition of DNA repair and increasing mutagenicity and carcinogenicity of other factors, like UV light, global changes in DNA methylation and histone modifications and spindle disorder.[24] Therefore, due to global health implications of arsenic toxicity, the present study was undertaken to assess the possible effects of arsenic on the ovary of female mice. Arsenic manifestation has destructive effects on histoarchitecture of ovaries in Swiss albino mice, which indicates that arsenic may have negative results on fertility.

MATERIALS AND METHODS

In the present experiment, 3 months old normal female Swiss albino mice (*Mus musculus*) were selected. These mice were kept in the polypropylene cages containing paddy husk at temperature 26 ± 2°C; the humidity was maintained at 50 ± 10% and in controlled light (12 hrs light and 12 hrs dark). Animals were maintained in ideal conditions as per the ethical guidelines of the CPCSEA, (CPCSEA Regd. No. 1129/bc/07/CPCSEA, dated 13/02/2008) Government of India and Institutional Animal Ethics Committee (IAEC). All the mice were segregated into two groups, each group containing six mice: a control group and arsenic treated group. The inorganic form of arsenic, sodium arsenite (Sigma) was administered to the arsenic treated mice group (except the control group) at the dose of 1.8 mg/kg body weight for 8 weeks by gavage method.

Estimation of arsenic level: The blood samples were digested with an acid mixture containing Triton 'X' solution and nitric acid in the ratio of 1:10, over a hot plate at 200°C. Digested mixture was cooled and the final volume was made up to 10 ml with diluted nitric acid. The estimation was analyzed by atomic absorption spectrophotometer (Perkin Elmer; CT06481-7794, U.S.A.) with graphite furnace.[25]

Estimation of Cancer Antigen 125 (CA125): CA125 Concentration in normal and arsenic treated mice blood serum was estimated using ELISA based CA125 Kit (CALBIOTECH, www.calbiotech.com).Before proceeding with the assay, all reagents, serum, and controls were brought to room temperature (18-26°C). The test was performed according to the protocol of the manufacturer.

Estimation of Lipid Peroxidation (LPO) level: Endogenous lipid peroxidation level from ovarian tissues was estimated. The level of lipid peroxides formed was measured using TBA and expressed as nmol of TBA reactive substances (TBARS) formed per mg of protein using the extinction coefficient of $1.56 \times 10^5 M^{-1} cm^{-1}$.[26]

Estimation of Superoxide dismutase (SOD) activity: Ovarian SOD was estimated spectrophotometrically. SOD activity was determined by quantification of pyrogallol auto-oxidation inhibition and the amount of enzyme necessary for inhibition of the reaction to 50%. Auto-oxidation of pyrogallol in 50 mM Tris- HCl buffer (PH=7.5) was measured by an increase in absorbance at 420 nm.[27,28]

Estimation of Catalase (CAT) activity: Ovarian CAT was estimated spectrophotometrically at 240 nm and expressed as unit/mg of Protein where the unit was the amount of enzyme that liberated half the peroxide oxygen from H_2O_2 in seconds at 25^0C.[29]

Estimation of Protein level: Ovarian Protein Concentration was measured spectrophotometrically with bovine serum albumin as standards.[30]

Apoptosis by TUNEL assay: Apoptotic cells in control and treated ovarian tissues were detected with the TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick - end labeling) technique using an in situ Apoptosis Detection Kit (TaKaRa # MK 500) according to the manufacturer's instructions.

Histological and Histopathological studies: Ovaries were dissected out after 8 weeks and fixed in 10% formalin. After fixing the tissues were washed thoroughly under the running tap water and dehydrated in ascending concentrations of ethanol, cleared in xylene. Tissues were kept in paraffin wax and blocks were prepared. Sections (4-5µm) were cut and fixed on the slide with the help of Mayer's albumin. Double staining was done and the slides were kept on xylene and hydrated in descending alcohol concentration. The slides were stained with hematoxylin and dehydrated up to 70% alcohol. Again, the slides were stained with eosin and then dehydrated in 90% and absolute alcohol and the slide were mounted with DPX.

Histological/histopathological changes for each group of mice ovaries were observed under the light microscope.

Statistical analysis

For two groups comparison, independent samples Unpaired 't'-test was used (GraphPad Software, USA). Level of significance between the group was considered at $p < 0.01$ and $p < 0.0001$.

RESULTS

Results of observations and statistical analysis are recorded in Table – 1 and graphically presented in Figure 1.

Table 1: Showing activities of Arsenic: Sodium arsenite (ppb); CA125: Cancer antigen 125 (U/ml); LPO: Lipid peroxidation (n mol of MDA/mg tissue/60 min); SOD: Superoxide dismutage (unit/mg protein); CAT: Catalase (micro moles of hydrogen peroxide/min/mg protein); DNA damage (apoptotic index) in the blood and ovarian tissue of control and experimental female *Mus musculus*.

Parameter	Control group	Treated group
Arsenic level	1.39 ± 0.16	44.55 ± 8.46 [a]
CA 125 level	2.42 ± 1.35	6.27 ± 2.14 [b]
LPO level	60.03 ± 2.02	82.46 ± 3.02 [a]
SOD activity	0.40 ± 0.02	0.25 ± 0.02 [a]
CAT activity	22.57 ± 1.49	16.49 ± 2.60 [c]
Apoptotic index	3 ± 0.2	16.9 ± 2.1 [a]

Values are expressed as mean ± SD (n = 6); 't' test - for two group comparison; Level of significance, [a] $p < 0.0001$, [b] $p = 0.0001$, [c] $p < 0.01$ control versus treated group.

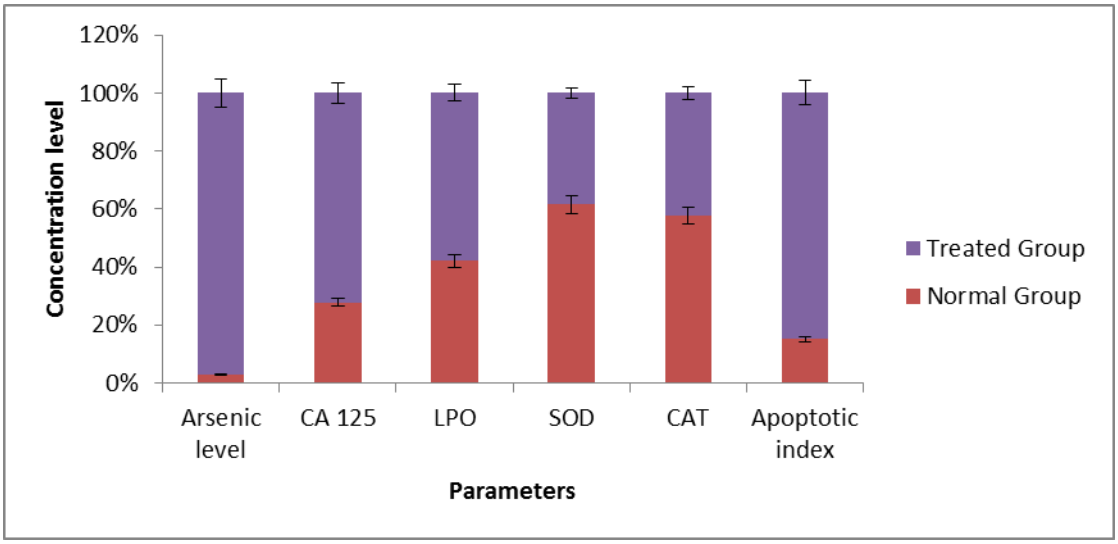


Figure 1: Effect of sodium arsenite on CA125 level, LPO level, SOD activity, CAT activity and apoptotic index of mice ovary. Data represent the mean ± SD (n = 6); [a] $p < 0.0001$, [b] $p = 0.0001$, [c] $p < 0.01$ with respect to control group.

The blood arsenic level of mice treated with sodium arsenite at a dose of 1.8 mg/kg body weight for 8 weeks, increased significantly ($p < 0.0001$) by 44.45 ppb as compared to the control group. The serum CA125 level of mice treated with sodium arsenite showed within normal limit (Normal CA125 < 35 U/ml) i.e., 6.27 U/ml as compared to the control group, hence no significant changes were observed. The level of LPO in the ovarian tissue of mice, treated with sodium arsenite showed, increased significantly ($p < 0.0001$) by 82.46 nmol of MDA/mg tissue/60 min as compared to the control group. Administration of sodium arsenite for 8 weeks significantly ($p < 0.0001$) reduced the SOD activity in ovarian tissue by 0.25 unit/mg protein as compared to the control group. CAT activity in ovarian tissue of sodium arsenite treated mice showed a significant ($p < 0.01$) decrease by 16.49 micromoles of hydrogen peroxide/min/mg protein.

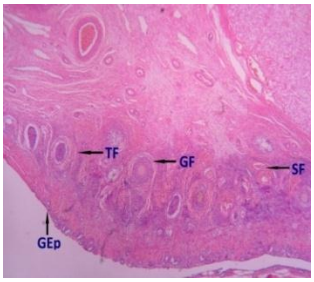


Fig. 2

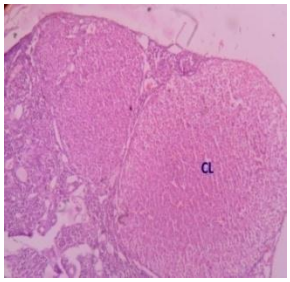


Fig. 3

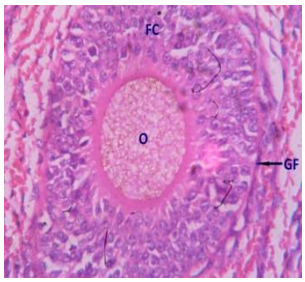
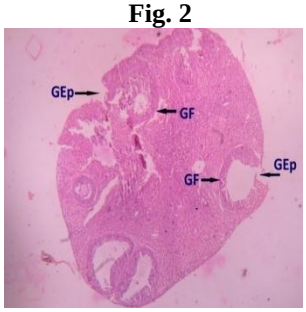


Fig. 4



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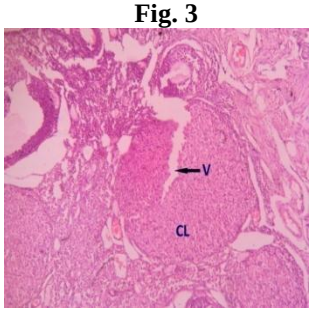

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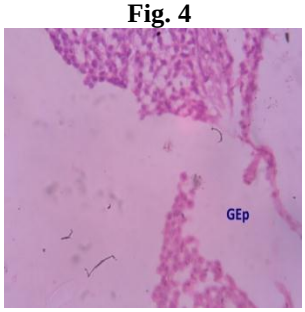
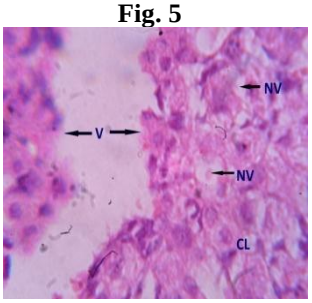


Fig. 7



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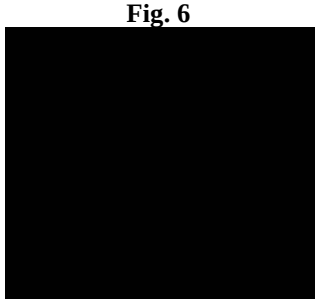

Fig. 9

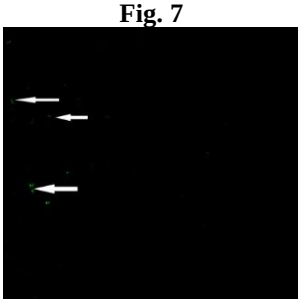

Fig. 10

Figure 2-4: Photomicrograph of histological sections of control ovary of *Mus musculus* with H and E staining.
Figure 5-8: Photomicrograph of histopathological sections of 8 weeks sodium arsenite administered *Mus musculus* with H and E staining.
Figure 9 and 10: Photomicrograph showing apoptosis in the follicles of ovarian cortex from ovarian tissues of control mice (''Fig. 9'') and mice treated with 8 weeks sodium arsenite (''Fig. 10'').

Apoptosis in follicles of ovarian cortex from ovarian tissues of mice treated with sodium arsenite in 8 weeks. Bright fluorescence staining TUNEL is evident as indicated by white arrow ("Fig. 10"). Five slides (5 x 100 cells) from each sample were counted. Apoptotic index was significantly ($p < 0.0001$) higher in arsenic treated mice compared with the control group ("Fig. 9").

Control group: "Fig. 2" Showing normal mature secondary follicle (SF), the tertiary follicle (TF) and Graafian follicle (GF). Germinal epithelium (GE) is continuous. (40x); "Fig. 3" Showing well organized normal corpus luteum (CL). (100x); "Fig. 4" Showing enlarged view of the normal mature Graafian follicle (GF) with ova (O) and follicular cells (FC). (400x).

Treated group: "Fig. 5" Showing ruptured germinal epithelium (GE) with Graafian follicle (GF). (40x); "Fig. 6" Showing corpus luteum (CL) with vacuolization (V). (100x); Fig. 7 - Showing enlarged view of ruptured germinal epithelium (GE). (400x); "Fig. 8" Showing very enlarged view of cytoplasmic and nucleoli vacuolization (V and NV) seen in corpus luteum (CL). (1000x).

DISCUSSION

Arsenic is a potent toxin that alters the ovarian toxicity. It has been confirmed by different studies that arsenic changes in the female reproductive system, including reduced weights of the uterus and ovary and reduced ovarian and uterine peroxidase activities; blockage of steroidogenic enzymes and decreased estradiol levels relative to the controls.[7,8] CA-125 is used to confine the tumor and to determine its stage, subtype, and response to therapy.[31]

In the present study, serum CA125 level of mice treated with sodium arsenite showed within normal limit, hence no significant changes were observed, i.e., there was no relationship between Arsenic and CA125 in mice blood.

LOP is used as a biological marker to show the indicator of oxidative stress and causes plasma membrane damage create a gradual loss of plasma membrane fluidity, reduced membrane potential and increased permeability to ions.[13] Many studies have established that oxidative stress/LPO is directly connected with the developing of ovarian follicles, endometriosis, undetermined female infertility.[32] We noted significant increases of

TBARS/LPO in ovarian tissues after 8 weeks sodium arsenite (1.8 mg/kg body weight) treatment.

SOD and catalase are available in all oxygen – metabolizing cells and its function is to provide a guard against the potentially damaging reactivities of superoxide and hydrogen peroxide. SOD removes the superoxide radicals to H_2O_2, which has been omitted by CAT. Susceptibility to arsenic curtails the SOD[19] and CAT activity.[20] In the present study, both SOD and CAT activities were decreased significantly in the ovarian tissues of sodium arsenite treated mice.

It was assumed that high- level arsenic causes dangerous redox imbalance by decreasing the levels of antioxidant enzymes and increasing the level of ROS through the oxidative stress, which generates apoptosis by activating the cytochrome C-caspase.[33] DNA damage has been observed in ovarian tissue in a dose-dependent appearance.[34] In the present work, Apoptosis was observed in follicles of ovarian cortex from ovarian tissues of mice treated with sodium arsenite in 8 weeks. Bright fluorescence staining TUNEL was evident as indicated by white arrow. Five slides (5 x 100 cells) from each sample were counted. Apoptotic index was significantly ($p < 0.0001$) higher in arsenic treated mice compared with the control group.

The ovaries are composed of an outer cortex and inner medulla or stroma. The cortex is composed of ovarian follicles, interstitial gland cells, and stromal elements. Ovarian follicles are present in the cortex in different stages of development: primordial, primary, secondary (antral, vesicular) and mature (or tertiary or preovulatory or Graafian follicle). In the histopathological study, ovarian follicular degeneration was noted, which is supported by[8]. The present study indicates that ovaries of sodium arsenite treated group showed cytoplasmic and nucleoli vacuolization was seen in corpus luteum with ruptured germinal epithelium.

CONCLUSION

The present research shows that increased arsenic concentration can redesign the oxidative stress and antioxidant enzymes activities bound with free radical metabolism in the ovary of mice. Present work also suggests a possible association between arsenic exposure and DNA damage in Swiss albino mice. The ovary histology has been affected by sodium arsenite, with degeneration of ovarian cells.

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