

## Sodium Arsenite-Induced Histopathological and Oxidative DNA Damage in the Ovary of Mice

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**Abstract:** Arsenic is well-known to promote toxicity in the reproductive system. It alters the ovary of mice at different dose levels. It creates more and more reactive oxygen species (ROS) in the ovary. Female Swiss albino mice were selected as the experimental animal. In the treatment group, 1.8 mg/kg body weight Sodium arsenite and in the control group distilled water was given orally by gavage method daily for 5 months. After the treatment, mice were sacrificed and the ovary and blood samples were collected. Sodium arsenite treated group showed no effects of CA 125 in mice blood, LPO level was significantly ( $p < 0.01$ ) increased, whereas antioxidant enzymes (SOD and CAT) activity was significantly ( $p < 0.05$ ) decreased as compared to the control group. Apoptotic index was significantly ( $p < 0.01$ ) higher in arsenic-treated mice compared with the control. Sodium arsenite treated group showed degenerated follicular structure with vacuolated spaces in stroma filled with RBCs in comparison with the histology of control mice ovary. Our results showed that exposure to Sodium arsenite promoted degenerative changes and caused oxidative stress in the ovary of mice.

**Keywords:** Sodium arsenite, Ovary, LPO, Antioxidant enzymes, Apoptosis, Histopathology.

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### I. Introduction

Arsenic is the twentieth most copious element, natural and omnipresent in earth's crust and mixed with soil and groundwater during weathering of rocks followed by subsequent leaching and runoff. Weathering of rocks converts arsenic sulfides to arsenic trioxide, which enters the arsenic cycle as dust or by dissolution in rain, rivers, or groundwater<sup>1</sup>. Sodium arsenite (As III) is known to be 60 times more toxic than sodium arsenate (As V). The trivalent state of inorganic arsenic (arsenite, As III) has been recorded to be more toxic than the pentavalent form (arsenate, As V), which is efficient in binding with macromolecular thiols<sup>2</sup>. As these macromolecular thiols are present in numerous molecules 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<sup>3</sup>. 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<sup>4,5</sup>. Exposure to arsenic is a major health problem due to its the toxic effect in human and in an animal model. Inorganic arsenic has been linked to developing cancer of skin, prostate, liver, and lung<sup>6</sup>. The non-cancer effects of arsenic include keratosis, diabetes, cardiovascular disease, pigmentation etc<sup>7</sup>.

Study of the arsenic toxicity in an animal model is very limited. Most of the ingested and inhaled arsenic is well absorbed through the gastrointestinal tract and lung into the blood. 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<sup>8,9</sup>. CA-125 is used to confine a tumor and to determine its stage, subtype, and response to therapy<sup>10</sup>. 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<sup>11,12</sup>. 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 the methylene group between double bonds<sup>13</sup>. Free radicals, primarily produced during electron transport chain in mitochondria; do activate a series of chain reactions converting PUFAs into malondialdehyde (MDA). MDA is major aldehyde by-product of LPO<sup>14</sup>, which is highly mutagenic and has been denoted to form adducts with DNA bases dG, dA, and dC as m1G, m1A and m1C respectively<sup>15,16</sup>. 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<sup>17</sup> and CAT activity<sup>18</sup>. Apoptosis or programmed cell death is very essential in the

homeostasis of the immune system<sup>19,20</sup>, this type of cell death can also be 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<sup>4,5</sup>. Arsenic, a human carcinogen, is a worldwide pollutant that is found in soil, water and air. Arsenic is an isolate of mitochondrial oxidative phosphorylation that induces production of reactive oxygen species<sup>21</sup>. 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<sup>22</sup>. In human cell lines exposed to arsenic, an accretion of oxidative DNA damage in the form of 8-hydroxy-29-deoxyguanosine (8-OHdG) has been exhibited, which is transposed by addition of antioxidants<sup>23</sup>. 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. The present work provides the detailed analysis of contamination of arsenic in Bihar and further it may help to check the main root causes of cancer.

## **II. 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  $26 \pm 2^\circ\text{C}$ ; the humidity was maintained at  $50 \pm 10\%$  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) Gov. 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 5 months 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  $200^\circ\text{C}$ . 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<sup>24</sup>.

### **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 for the assay, all reagents, serum, and controls were brought to room temperature ( $18-26^\circ\text{C}$ ). 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  $1.56 \times 10^5 \text{M}^{-1} \text{cm}^{-1}$ <sup>25</sup>.

### **Estimation of Superoxide dismutase (SOD) activity**

Ovarian SOD was estimated spectrophotometrically. SOD activity was determined by quantification of pyrogallol autooxidation inhibition and the amount of enzyme necessary for inhibition of the reaction of 50%. Auto-oxidation of pyrogallol in 50 mM Tris- HCl buffer (PH=7.5) was measured by an increase in absorbance at 420 nm<sup>26,27</sup>.

### **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  $\text{H}_2\text{O}_2$  in seconds at  $25^\circ\text{C}$ <sup>28</sup>.

### **Estimation of Protein level**

Ovarian Protein Concentration was measured spectrophotometrically with bovine serum albumin as standards<sup>29</sup>.

### **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**

After 5 months of treatment, ovaries were dissected out 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  $p \leq 0.05$ .

**III. Results**

The blood arsenic level of mice treated with Sodium arsenite at a dose of 1.8 mg/kg body weight for 5 months, increased significantly ( $p < 0.01$ ) by 191.02 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., 11.13 U/ml as compared to control group, hence no significant changes were observed. The level of LPO in the ovarian tissue of mice, treated with Sodium arsenite increased significantly ( $p < 0.01$ ) by 152.40 nmol of MDA/mg tissue/60 min as compared to the control group. Administration of Sodium arsenite for 5 months significantly ( $p < 0.05$ ) reduced the SOD activity in ovarian tissue by 0.08 unit/mg protein as compared to the control group. CAT activity in ovarian tissue of Sodium arsenite treated mice showed a significant ( $p < 0.05$ ) decrease by 8.49 micromoles of hydrogen peroxide/min/mg protein.

Apoptosis in follicles of the ovarian cortex from ovarian tissues of mice treated with Sodium arsenite in 5 months. 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 ( $p < 0.01$ ) higher in arsenic-treated mice compared with the control group (Fig. 9).

**Control group:** Fig. 2- Showing normal mature Graafian follicle (GF) with ova (O). (100x); Fig. 3 - Showing enlarged view of corpus luteum (CL) with well-organized cells. (400x); Fig. 4 - Showing a very enlarged view of ova (O) and follicular cells (FC) with cytoplasm (C) and nucleus (N). (1000x)

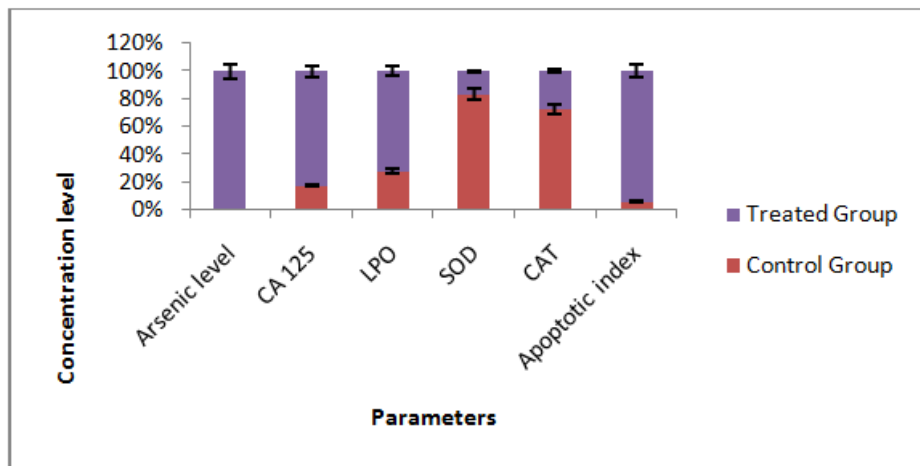
**Treated group:** Fig. 5 - Showing degenerated corpus luteum (dCL) with increased vacuolated spaces in stroma filled with RBCs. (40x); Fig. 6 - Showing degenerated follicular structure (dGF) with vacuolated spaces (V) in stroma filled with RBCs. (100x); Fig. 7- Showing enlarged view of vacuolated spaces (V) in stroma filled with RBCs. (400x); Fig. 8 - Showing a very enlarged view of the degenerated follicular structure with vacuolated spaces (V) in stroma filled with RBCs. Clustered nuclei (Cn) are also observed in the wall of the matured follicle. (1000x)

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

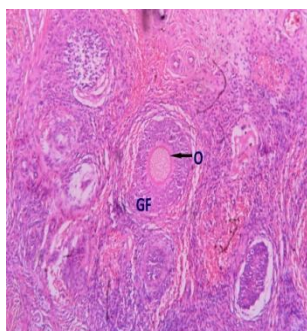
**Table-1:** Showing activities of Arsenic: Sodium arsenite (ppb); CA125: Cancer antigen 125 (U/ml); LPO: Lipid peroxidation (n mol of MDA/mg tissue/60 min); SOD: Superoxide dismutase (unit/mg protein); CAT: Catalase (micro moles of hydrogen peroxide/min/mg protein); DNA damage (apoptotic index) in the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

Parameter	Control group	Treated group
Arsenic level	1.39 ± 0.16	191.02 ± 16.17
CA 125 level	2.42 ± 1.35	11.13 ± 2.41
LPO level	60.03 ± 2.02	152.40 ± 4.18
SOD activity	0.40 ± 0.02	0.08 ± 0.02
CAT activity	22.57 ± 1.49	8.49 ± 1.15
Apoptotic index	3 ± 0.2	48 ± 2.6

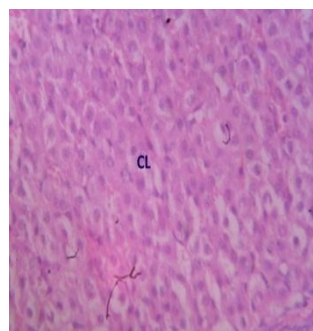
Values are expressed as mean ± SD (n = 6); 't' test - for two group comparison; Level of significance,  $p \leq 0.05$  control versus treated mice groups.



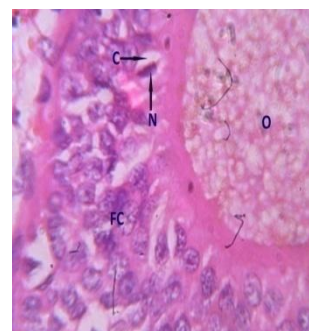
**Figure 1:** Effect of sodium arsenite on the CA125 level, LPO level, SOD activity, CAT activity and apoptotic index of mice ovary. Data represent the mean  $\pm$  SD (n = 6);  $p < 0.0001$  with respect to the control group.



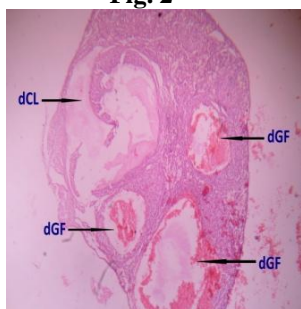
**Fig. 2**



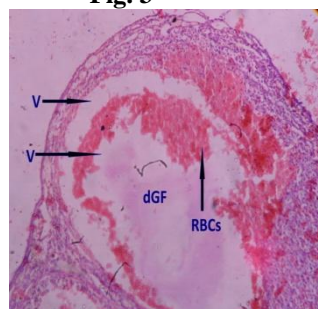
**Fig. 3**



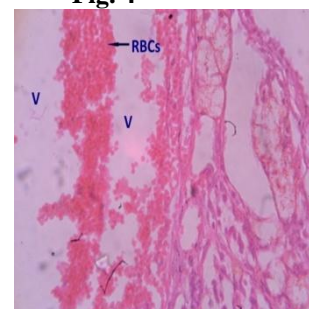
**Fig. 4**



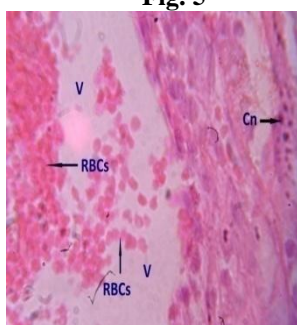
**Fig. 5**



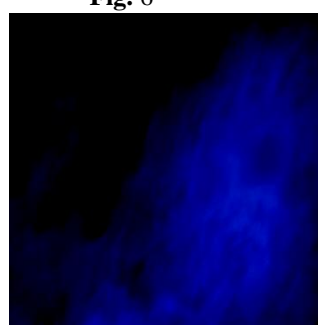
**Fig. 6**



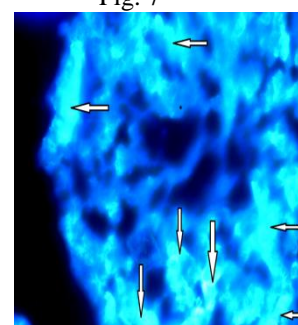
**Fig. 7**



**Fig. 8**



**Fig. 9**



**Fig. 10**

**Figure 2 to 4:** Photomicrograph of histological sections of control ovary of *Mus musculus* with H and E staining.

**Figure 5 to 8:** Photomicrograph of histopathological sections of 5 months 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 5 months sodium arsenite (Fig. 10).

#### **IV. Discussion**

Arsenic is a major environmental pollutant that damages the reproductive toxicity. Thus virulent results are affected by the forms, sources, routes, doses and periods of arsenic exposure. 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<sup>4,5</sup>. CA-125 is used to confine a tumor and to determine its stage, subtype, and response to therapy<sup>10</sup>. 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 the blood of mice.

LOP is used as a biological marker to show the indicator of oxidative stress and causes plasma membrane damage, creates a gradual loss of plasma membrane fluidity, reduced membrane potential and increased permeability to ions<sup>11</sup>. Many studies have established that oxidative stress/LPO is directly connected with the developing of ovarian follicles, endometriosis, undetermined female infertility<sup>30</sup>. We noted significant increases of TBARS/LPO in ovarian tissues after 5 months of Sodium arsenite 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<sub>2</sub>O<sub>2</sub>, which has been omitted by CAT. Arsenic decreases the SOD activity<sup>17</sup> and CAT activity<sup>18</sup>. 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<sup>31</sup>. DNA damage has been observed in ovarian tissue in a dose-dependent appearance<sup>32</sup>. In the present work, Apoptosis occurs in the follicles of the ovarian cortex from ovarian tissues of mice treated with sodium arsenite in 5 months. Bright fluorescence staining TUNEL is evident as indicated by white arrow. Five slides (5 x 100 cells) from each sample were counted. Apoptotic index was significantly (p < 0.01) 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<sup>5</sup>. The present study indicates that ovaries of sodium arsenite treated group showed degenerated follicular structure with vacuolated spaces in stroma filled with RBCs. Clustered nuclei are also observed in the wall of the matured follicle.

#### **V. Conclusion**

The present study suggests that arsenic is an important environmental toxicant that accesses the prospect of oxidative stress in tread mice. High arsenic concentration can significantly change LPO and antioxidant enzymes activities related with free radical metabolism in ovaries of mice. The mechanism of arsenic on antioxidant defense system in the mice appear to assume an increased level of LPO and decreased activities of SOD and CAT, which bound with free radical metabolism. The ovary histology has been induced by Sodium arsenite with full oocyte degeneration. The present study shows a probable relation between arsenic exposure and DNA damage in ovarian tissues. This work provides a complete and clear picture of apoptosis by Sodium arsenite but this may make a future model for the evaluation of DNA damage using TUNEL assay.

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