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ARSENIC INDUCED REACTIVE OXYGEN SPECIES AND THE FATE OF ANTIOXIDANTS IN THE SELECTED TEST TISSUES OF MALE ALBINO RAT

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ABSTRACT

Arsenic (atomic number 33) is the first metalloid that is naturally occurring, highly toxic environmental pollutant that exists in water, soil and food and in some types of rocks and minerals that gets released into the environment from both natural and man-made sources and identified as a human carcinogen. Absorption of arsenic is known to inhibit the activity of many enzymes including SOD, CAT, GST, GPx where the depletion of antioxidants status triggers accumulation of lethal reactive oxygen species (ROS), consequently resulting in oxidative stress. Increased formation of ROS/RNS, by generation of superoxide radicals, singlet oxygen, peroxyl radicals, hydroxyl radical due to Fenton reaction occurs under depleted levels of antioxidants. Acute and chronic arsenic toxicity not only declines the antioxidants status in different tissues it leads to liver diseases, renal diseases, reproductive problems, cardiovascular diseases neurological and gastrointestinal disturbances, including various types of cancers and other health disorders. The fate of antioxidant enzymes due to arsenic toxicity is discussed in the present study.

KEYWORDS: Liver, Kidney, Heart, Lung, Small intestine and Testis.

1. INTRODUCTION

Humans are posed to severe threats due to deterioration in environmental quality and ATSDR priority list showed that high potential for human exposure is indicated by first (arsenic), in ranking and seventh (cadmium) among the 20 hazardous elements.[1] Arsenic is odourless,

tasteless, colourless and nonirritating gas that rapidly destructs red blood cells and promotes kidney failure, which is consistently lethal without any proper therapy. Arsenic displays unpredictable toxicity in mammals, as stated by several factors that depends on form of arsenic (organic or inorganic), valency state, absorption rate and its subsequent elimination, solubility, and particle size. Organs including Liver, Kidney, Testis, Intestine, Spleen, Heart and Muscle promoting multisystem organ damage are more prone to damage under acute toxicity.[2]

Antioxidants are the enzymes that protect body and stabilize free radicals before attacking components of the cell under normal conditions. They diminish free radicals produced in body under toxic stress by reducing their energy or by donating electrons. Antioxidant enzymes are first line of defense against oxidative stress that prevents biological macromolecules degradation from oxidative injury. Deteriorated antioxidant defense system make the cell more prone to toxic compounds invasion. Interruption of prooxidant and antioxidant status causes oxidative stress of biomolecules including lipids, proteins and DNA and also causes tissue injury.[3],[4]

Antioxidant enzymes remove peroxides, superoxides and free radicals produced within the cell and alter their activity in different tissues including Liver, Kidney, Testis, Lung, Heart, Brain and other organs. Positive correlation exists between arsenic toxicity and LPO content in the liver, kidney and heart of rats upon arsenic exposure.[5] Arsenic promotes excessive ROS production including hydroxyl radicals (OH) and superoxide anions (O2-) thus interrupts pro-oxidants and antioxidants status and declines antioxidant activity. Imbalanced pro/antioxidants alter expression of growth factors, inhibition of DNA repair, altered DNA methylation, suppression of cell cycle checkpoint proteins.[6]

SOD (Superoxide dismutase), CAT (Catalase), GST (Glutathione-S-Transferase) and GPx (Glutathione peroxidase) are endogenous antioxidant enzymes that inhibit oxidative cellular damage and scavenge free radicals. Enzymatic (SOD, GST, GPx and catalase) and nonenzymatic (glutathione, thioredoxin, vitamins, etc.) antioxidants tries to detoxifies the free radicals under normal physiological conditions by maintaining the balance between free-radicals and the antioxidants.

SOD and CAT are enzymatic antioxidants that work incoordination to eliminate ROS species produced under arsenic toxicity. Enhanced superoxide radicals inhibit CAT activity, NADH

gets inactivated under toxicity, hence insufficient NADH might decline CAT the activity.[7] GST is involved in chemical disposition of toxic substances and catalyzes the conjugation of glutathione and inactive toxic compounds by non-catalytic binding.[8] GPx synergistically rid the cells of oxidative species that are capable of disrupting cellular homeostasis. Protein carbonylation, alters the protein activity and increases the susceptibility to proteolysis, which is used as a protein oxidation marker and promote ROS-mediated oxidative damage. Rats were examined for oxidative stress related parameters by determination of malondialdehyde (MDA, lipid peroxidation) and carbonyl proteins (protein oxidation) concentration. Hence in the present study we evaluated the antioxidants status in male albino rats in different tissues including Liver, Kidney, Testis, Lung , Heart and Small intestine by estimating MDA, SOD, CAT, GST, GPx and Protein carbonyl content.

2. MATERIALS AND METHODS

2.1 Animals

Adult male albino rats of Wistar strain (120-140g) 90 days old were purchased from a local commercial dealer and housed in polypropylene cages. Animals were acclimatized in a laboratory condition for two weeks prior to the experimentation and maintained at temperature of about 22-25^{0}C in a well-regulated light and dark (12h:12h) conditions in the Animal House Facility of Sri Venkateswara University, Tirupati. Rats were fed with a commercial rat chow daily that is obtained from Sai Durga Feeds and Foods, Bangalore, India and water was given at *ad libitum*. During animal maintenance Rules of the "Institutional Animal Ethics Committee (IAEC) of Sri Venkateswara University" (Resol. No. 58/2012/(i)/a/CPCSEA/IAEC/ SVU/AUR – BC), were strictly followed during the experimentation and steps were taken to protect the experimental animals.

2.2 Chemicals

Arsenic as Sodium arsenite ($NaAsO_2$) is purchased from Himedia Lab Pvt. Ltd. Mumbai. All other chemicals used in the present study were obtained from the standard chemical companies like Sigma Chemical Co. (St Louis, MO, USA) and SD Fine Chemicals, India. The chemical molecules used in the present study are of the maximum purity to obtain the best results. The chemical compounds are given to mice by oral gavage.

2.3 Experimental Design

Male albino rats were divided into 4 groups as given below with 06 rats in each group

Group 1: Not exposed to any treatment and received only deionized water without Arsenic treatment. (Control-Untreated).

Group 2: Rats was treated with Sodium arsenite 1/10 LD50 for 96 h at a dose of 8 mg/kg body weight for a time interval of 15 days.

Group 3: Rats was treated with Sodium arsenite 1/10 LD50 for 96 h at a dose of 8 mg/kg body weight for a time interval of 30 days.

Group 4: Rats was treated with Sodium arsenite 1/10 LD50 for 96 h at a dose of 8 mg/kg body weight for a time interval of 45 days.

After a specific time interval Tissues like Liver, Kidney, Testis, Small intestine, Lung and Heart were isolated to determine Antioxidant enzymes status.

2.4 Antioxidant Defense system

Estimation of Lipid peroxidation

A breakdown product of lipid peroxidation, thiobarbituric acid reactive substance (TBARS) was measured as described by.[9] Briefly, the stock solution contained equal volumes of trichloroacetic acid 15% (w/v) in 0.25 N hydrochloric acid and 2-thiobarbituric acid 0.37% (w/v) in 0.25 N hydrochloric acid. One volume of tissue homogenate and two volumes of stock reagent were mixed in a screw-capped centrifuge tube, vortexed and heated for 15 min in a boiling water bath. After cooling on ice, the flocculent precipitate was removed by centrifugation at 1000x g for 10 min and absorbance was measured at 535 against blank. The values were expressed as µ Moles malondialdehyde produced per min per mg protein. A standard curve was prepared with known amount of malondialdehyde and all the above reagents expect the sample. The optical density was read at 535 nm.

Catalase (CAT) (1.11.1.6)

Catalase activity was measured by a slightly modified version of.[10] The desired concentrations (w/v) of tissue homogenates were prepared in ice cold 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA and then subjected to centrifugation at 10,000 rpm for 10 min at 4◦C. The supernatant obtained was used for enzyme assay. A quantity of 10 µL of 100% ethanol was added to 100 µL of tissue extract and then placed in an ice bath for 30 min. After 30 min the tubes were kept at room temperature followed by the addition of 10 µL of Triton X-100 RS. To a cuvette containing 200 µL of phosphate buffer and 50 µL of tissue extract was added 250 µL of 0.066 M H_2O_2 (in phosphate buffer) and decreases in optical density measured at 240 nm for 60 s in a UV spectrophotometer. A molar extinction

coefficient of 43.6 M cm^{-1} was used to determine CAT activity. The unit of activity is equal to moles of H_2O_2 degraded/mg protein/min.

Superoxide Dismutase (SOD) (1.15.1.1)

Superoxide dismutase activity was determined according to the method of[11] at room temperature. The isolated experimental tissues were homogenized in ice cold phosphate buffer (50 mM; pH 7.0), containing 0.1 mM EDTA to arrive at desired homogenate (5% w/v). The prepared homogenates were subjected to cold centrifugation at 10,000 rpm for 10 min at 4◦C. After the centrifugation, the supernatant was used for further enzyme assays. A quantity of 100 µL of tissue extract was added to 880 µL (0.05 M, pH 10.2, containing 0.1 mM EDTA) carbonate buffer and 20 µL of 30 mM epinephrine (in 0.05% acetic acid) was added to the mixture and the optical density was measured at a value of 480 nm for 4 min using a Hitachi U-2000 spectrophotometer. Activity was expressed as the amount of enzyme that inhibited the oxidation of epinephrine by 50%, which is equal to 1 unit.

Glutathione – S – transferase (GST) (E.C. 2.5.1.18)

Estimate the GST activity with its conventional substrate 1-chloro, 2, 4-dinitro benzene (CDNB) by the method of[12] at 340 nm. Homogenate the tissues in 50mM Tris-HCl buffer pH 7.4 containing 0.25 M sucrose and centrifuged at 4000 g at 40C for 15 min. centrifuge the obtained supernatant at 16,000 g for 1 hour (hr) at 4^0C. Discard the pellet and use supernatant as an enzyme source. Prepare the reaction mixture to a volume of about 3 ml containing 0.1 ml of 30 mM CDNB, 0.1 ml of 30 mM glutathione, 2.4 ml of 0.3 M potassium phosphate buffer at pH 6.9, and the appropriate enzyme source. Initiate the reaction by adding glutathione and read the absorbance against reagent blank at 340 nm and the activity was expressed as µ moles of thioether formed / mg protein / min.

Assay of Glutathione Peroxidase (GPx) (EC.1.11.1.9)

Glutathione Peroxidase activity was assayed as described by.[13] The assay mixture contained 1.59 ml of phosphate buffer (100 mM, pH 7.6), 100 µl of 10 mM EDTA, 100 µl of sodium azide, 50 µl of glutathione reductase, 100 µl of reduced glutathione, 100 µl of 200 mM NADPH, 10 µl of hydrogen peroxide and 10 µl enzyme source. Disappearance of NADPH was measured at 10 sec interval for 3 min at 340 nm against blank containing all components expect enzyme in a Systronics Spectrophotometer. Specific activity of the enzyme was expressed as nmol of NADPH oxidized per min per mg protein.

Protein Carbonyl

Protein carbonyl levels were estimated in control and all treated groups of mice by the method of.[14] Tissue was homogenized in 5ml of normal saline, centrifuged at 2000rpm for 10 min and supernatant was discarded. 2ml of 0.1% digitonin was added to the pellet and incubated with regular shaking for 15 min. Then mixture was centrifuged at 2000 rpm for 10 min and supernatant used for assay. 1ml of supernatant was added to test tube and control run with equal volume of distilled water. 4ml of 10 mM 2,4- dinitrophenylhydrazine (DNPH) prepared in 2.5M HC1 was added to all tubes. The contents were mixed thoroughly and incubated in the dark (room temperature) for 1 hour. The tubes were shaken intermittently every 15 min. Then 5 ml of 20% TCA (w/v) was added to both tubes and the mixture left in ice for 10 minutes. The tubes were then centrifuged at 3500 rpm for 20 min to obtain the protein pellet. The supernatant was carefully aspirated and discarded. This was followed by a second wash with 10% TCA as described above. Finally the precipitates were washed three times with 4ml of ethanol: ethyl acetate (1:1, v/v) to remove unreacted DNPH and lipid remanants. The final protein pellet was dissolved in 2ml of 6M guanidine hydrochloride and incubated at 37°C for 10 min. The insoluble materials were removed by centrifugation. Carbonyl content was determined by taking the spectra at 370 nm. Each sample was read against the control sample (treated with 2.5N HCl). The carbonyl content was calculated from peak absorption (370nm) using an absorption coefficient (e) of 22,000 M-1 cm-1. The protein carbonyl content was expressed as nmole/mg protein.

3. RESULTS

In the present study the Antioxidant enzymes status, Protein carbonyl and Lipid peroxidation was studied in different tissues – Liver, Kidney, Testis, Lung , Heart and Small intestine of Male Albino rat and interpretated the results.

The LPO content increased in all the test tissues under the Arsenic toxicity and more LPO activity is observed in 45 days of treatment when compared 15 and 30 days. In Liver, Kidney and Heart all values are statistically significant at $p<0.001$, but 15 days Testis showed significane at $p<0.01$ in LPO activity (Figure. 1).

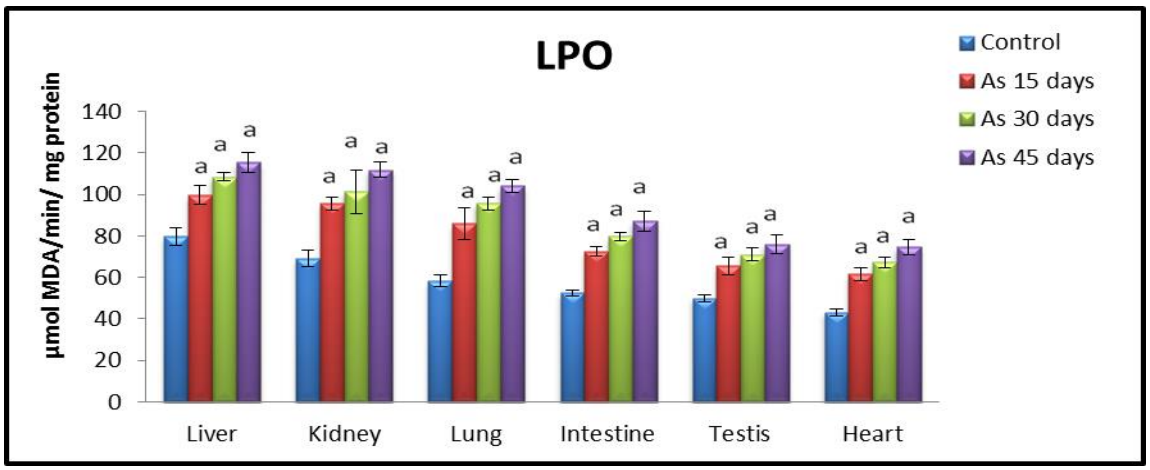

Figure 1: The levels of LPO (µmol MDA/min/ mg protein) in the selected tissues of Arsenic treated male albino rats.

Each bar represents Mean+ SD of six individual observations.

a- Indicates the level of significance $p<0.001$

The activity of antioxidants SOD, CAT, GST and GPx declined in all the test tissues under Arsenic toxicity and more decline in CAT activity is observed in the Liver tissue followed by kidney and less CAT activity is seen in Heart. The maximum decline in antioxidants activity was observed in 45 days of treatment and all values are statistically significant (Figure 2).

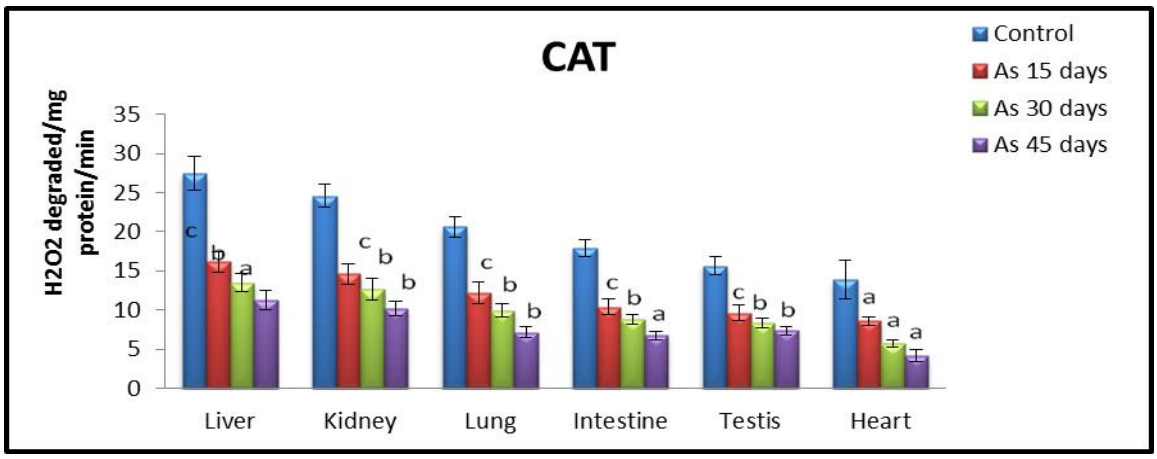

Figure. 2: CAT (H_2O_2 degraded/mg protein/min) activity levels under Arsenic toxicity in the selected tissues of male albino rat.

Each bar represents Mean+ SD of six individual observations.

a- Indicates the level of significance $p<0.001$

b- Indicates the level of significance $p<0.01$

c- Indicates the level of significance $p<0.05$

Similar to CAT activity the SOD also showed decline under Arsenic toxicity and all values are statistically significant. But 15 days Lung did not show significant changes. The changes in Liver and Kidney are more prominent when compared to other organs (Figure 3).

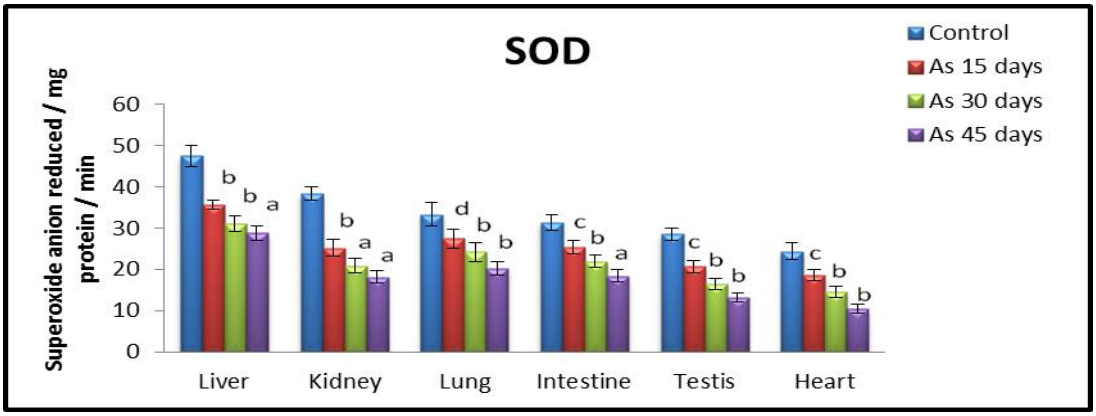

Figure. 3: SOD (Superoxide anion reduced/mg protein/min) activity levels under Arsenic toxicity in the selected tissues of male albino rat.

Each bar represents Mean+ SD of six individual observations.

a- Indicates the level of significance $p<0.001$

b- Indicates the level of significance $p<0.01$

c- Indicates the level of significance $p<0.05$

d- Indicates non significant

The Changes in GST activity under arsenic toxicity is observed and presented in Figure 4. In 15 days Arsenic treated kidney and lung do not show much significant changes when compared to other organs.

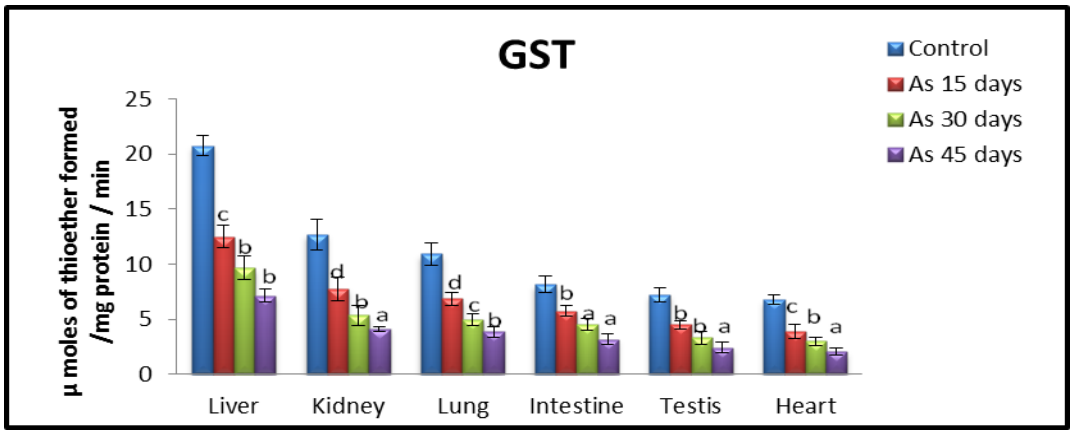

Figure. 4: GST activity levels (µ moles of thioether formed /mg protein / min) in the selected tissues of Arsenic treated male albino rats.

Each bar represents Mean+ SD of six individual observations.

a- Indicates the level of significance p<0.001

b- Indicates the level of significance p<0.01

c- Indicates the level of significance p<0.05

d- Indicates non significant

The activity of GPx under arsenic toxicity also declined in all the test tissues and the changes are more significant in heart tissue (Figure 5).

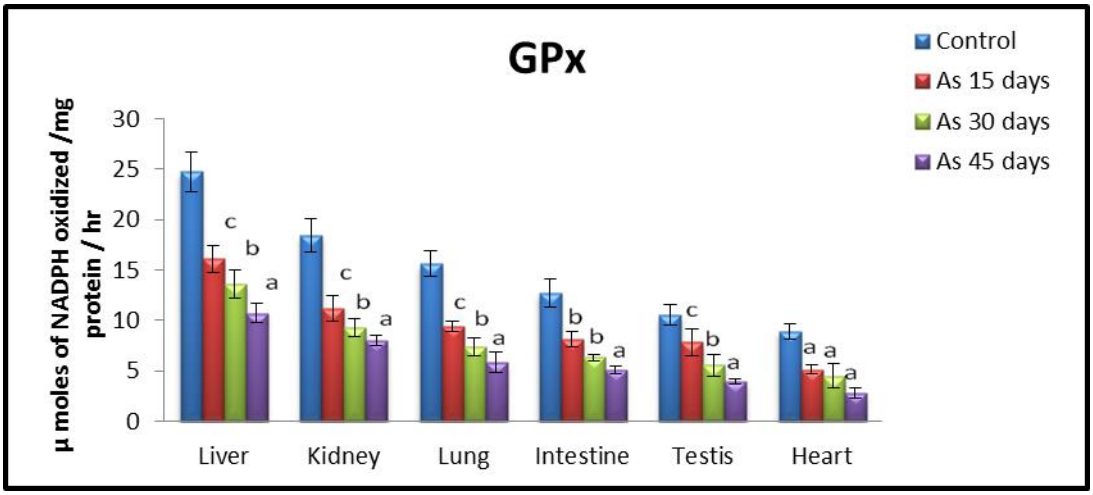

Figure. 5: GPx activity levels (µ moles of NADPH oxidized /mg protein / hr) in the selected tissues of Arsenic Treated male albino rats.

Each bar represents Mean+ SD of six individual observations.

a- Indicates the level of significance p<0.001

b- Indicates the level of significance p<0.01

c- Indicates the level of significance p<0.05

The Protein carbonyl activity increased in all the test tissues under arsenic treatment and all values are statistically significant. The Fig 6 gives vivid details regarding the changes occured in the Test tissues – Liver, Kidney, Testis, Intestine, Lung and Heart.

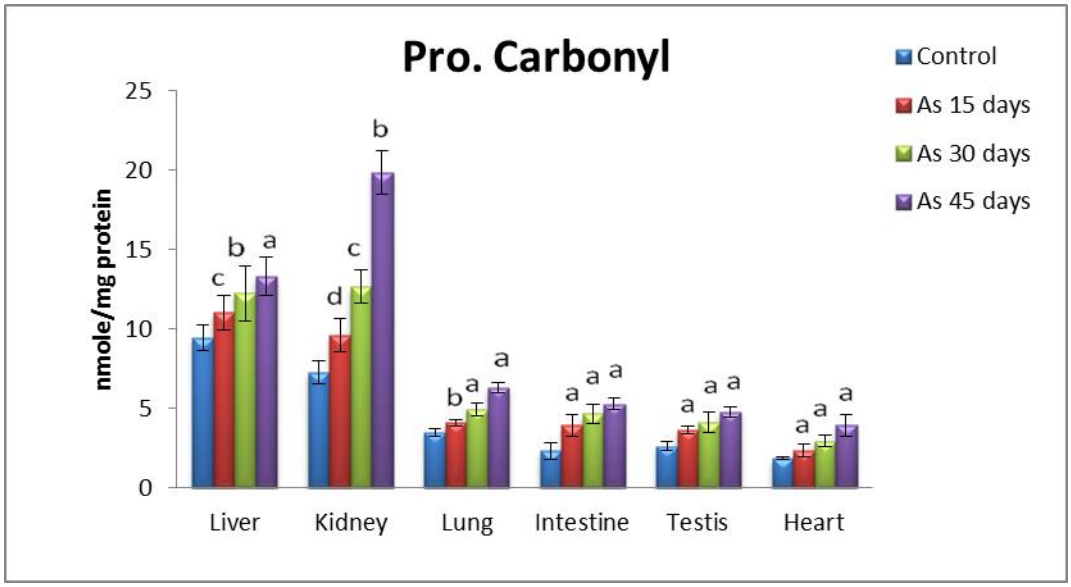

Figure. 6: The levels of Protein Carbonyl (nmole/mg protein) in the selected tissues of Arsenic treated male albino rats.

Each bar represents Mean+ SD of six individual observations.

a- Indicates the level of significance $p<0.001$

b- Indicates the level of significance $p<0.01$

c- Indicates the level of significance $p<0.05$

d- Indicates non significant

4. DISCUSSION

Arsenate or arsenite are the soluble salts of arsenic that gets absorbed upto 80% by gastrointestinal tract and gets distributed and accumulates in different organs. Arsenic gets highly deposited in the liver, kidney, lungs, hair and nails due to chronic exposure generating ROS.[15] The trivalent form of arsenic is more toxic than pentavalent form due to its ability to bind with the sulfhydryl groups of proteins and disrupt the enzyme activity of antioxidants.[16]

Toxicants might alters the enzyme expression, modifies the uptake of antioxidants, structural changes in enzyme and depletion of GSH and vitamin hence reduces the enzyme activity under arsenic stress. Indirect generation of ROS and direct binding with –SH groups arsenic toxicity is enhanced and damage DNA and macromolecules. ROS production is induced in mitochondria that precisely inhibit complexes activity in mitochondria.[17] The levels of Lipid peroxidase significantly increased and the activities of SOD, CAT, GST, and GPx significantly decreased after Arsenic treatment. According to this study, prolonged exposure to arsenic causes the activity of antioxidant enzymes in cells to drastically decrease due to the

possible displacement of Mn, Cu, and Zn ions from the active sites of MnSOD and CuZnSOD, Fe ions from catalase, or Se ions from glutathione peroxidase or Mn, Cu, and Zn ions from the active sites of MnSOD and CuZnSOD.

In Liver reduced SOD, CAT and GPx was also reported by.[18],[19] Reduction of GSH and GST facilitates arsenic accumulation in the liver and causes oxidative stress.[20] GST and GPx activity declined and promoted deformation of renal tubular epithelial cells in kidney. Reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) activities reduced in kidney and promoted necrosis and degenerative changes.[21] Decreased levels of SOD and GSH with increase in MDA with decline in relative weight of kidney and body weight.[22]

Testis showed decline in the activity SOD, CAT, GST and GPx along with the organ and final body weight with increased levels of LPO and protein carbonyl.[23] Arsenic might binds directly to gonads or interferes indirectly with hypothelamopituitary gonadal axis and exerts its toxic effects. SOD, CAT, LPO and Protein carbonyl alterations in testicular tissues is proven by the studies of.[24],[25] Testicular MDA increased with declined SOD, CAT, GST and GPx activity is observed in rat models under arsenite-induced male reprotoxicity are in consonance with the present study results.[26],[27]

Heart showed mild inflammatory infiltration with cardiotoxic effects and promoted decline in the activity of SOD, CAT, GST and GPx with increase in LPO under the toxicity of arsenic trioxide.[28] Reduced SOD, CAT, GST and GPx with increased protein carbonyl content in heart tissues indicated the cardiotoxic effects by altering histological changes in heart. The present studies are in consonance with the previous studies of.[29],[30] where cardiodysfunction promoted the morbidity and mortality in animal and human models.

Protein Carbonylation is a non enzymatic and irreversible post-translational modification that is the indicator of Lipid peroxidation that gets enhanced under arsenic induced stress.[23] The studies have proven that there is a dose-response increase in the protein carbonylation along with arsenic toxicity. Primary Protein Carbonylation occurs due to oxidation of aminoacids, by ROS, RNS and RXS and Secondary Protein Carbonylation that occurs due to aldehydes. Increased protein carbonyl content was proven by the studies of[31],[32] in Liver, Testis, Brain and in other organs. Protein Carbonylation gets induced by hydrogen peroxide, aldehydes and ketones that enhances ROS production.[33]

Arsenic caused the generation of free radicals and imbalanced pro-oxidant and antioxidant homeostasis in biological system and promotes toxic effects due to attraction towards the sulfhydryl groups of protein and thiols of glutathione. Hence Arsenicals produced ROS and RNS that are involved directly in oxidative damage to proteins, lipids, DNA and interact with thiol group of enzymes and proteins which subsequently promote cell death.[34] by enhancing the LPO and protein carbonyl content with decline in SOD, CAT, GST and GPx.

5. CONCLUSION

In the present study Organ dysfunction correlates with the imbalance between ROS and antioxidants status and results in the damage of biological system. Similar changes occurred in the test tissues Liver, Kidney, Testis, Lung, Small intestine and Heart indicating that imbalanced ROS might promote organ damage which is essential to be rectified.

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