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AMELIORATING EFFICACY OF RIBOFLAVIN IN ARSENIC INDUCED CYTOTOXICITY IN THE LIVER OF SWISS ALBINO MICE

Sajal Kumar Dey*
Department of Zoology, Sripat Singh College
Jiaganj, Murshidabad, 742123, West Bengal, India

ABSTRACT■ Arsenic has been found to cause severe cytological alterations in animal hepatic tissues. Oxidative stress induced by arsenic produces reactive oxygen intermediates. Present study aims to explore the extent of damage caused by sub lethal dose of arsenic, and to monitor the biochemical, cytological and histopathological changes caused by the toxic heavy metal. High levels of stress enzymes like phosphatase, peroxidase were noted in the liver. Down regulation of antioxidant enzymes like, peroxidase, dismutase, and catalase were noted in arsenic infected liver. Overall protein metabolism was affected due to arsenic induced cytotoxicity. Present study indicates abnormalities in all such cellular proteins and enzymes. The results also indicate the efficacy of riboflavin in combating arsenic induced cytotoxicity in liver to reduce oxidative stress caused by arsenic in the vitalorgans.

Key Words: *Arsenic, Riboflavin, Cytotoxicity, Liver, Antioxidant*

I. INTRODUCTION

Arsenic is concerned with very serious health problems.The incidence of arsenic contamination of ground water used for irrigation as well as for human consumption or industrial activities has taken the dimension of an epidemiological problem (Mitra et al., 2004). Increasing environmental concentration of this metalloid has drawn attention of many researchers who have studied the intoxication procedure of arsenic, so as to propose remedial mechanism against the cytotoxicity created by arsenic.It has been established that inorganic arsenic is extremely toxic even in sublethal concentrations (Kundu et al., 2000). Initially it enters the human body through ingestion, inhalation, or skin absorption. It is then distributed into a large number of organs including the lungs, liver, kidney and skin. Liver is the main site of As detoxification. Arsenic is also reported to cause oxidative stress in several vital organs.

Several remedial measures have been attempted to combat arsenic poisoning. Hardly anything was found thriving against acute arsenic poisoning, but only similar treatments like cholera and dehydration. Chelating agents like BAL, Penicillamine, DMSA/DMPS helps in clearing melanosis. Riboflavin, pyridoxine, folic acid and vitamin A, C and E significantly modify risk of arsenic

* **Corresponding author** : e-mail: acekaydey@gmail.com

related skin lesions (Flora et al., 1995; Gupta & Flora, 2005a, 2005b). Antibiotics and glucose-methionine has been found useful to some extent, when applied for the treatment of liver damage likeascitis and portal hypertension. Fewer studies have earlier been made on the efficacy of riboflavin to neutralize the loss of body and organ weight and the enzymatic malfunctions caused by arsenic intoxication. Riboflavin is considered a potent antioxidant in reducing oxidative stresses (Massey, 1994). Present study is aimed at the exploring the extent of remedial efficacy of riboflavin against arsenic cytotoxicity in liver.

II. MATERIALS AND METHODS

II:1 – Animals and chemicals used

Inbred strain of Swiss albino mice, *Musmusculus*, weighing 20 ± 5g at the initiation of experiment, reared in comfortable hygienic environment, were used for present study (as per norms of Animal Ethics Committee, ref. no. 892/ac/05/CPCSEA). Arsenic trioxide (E. Merck, India) was used to produce heavy metal toxicity to the Swiss albino mice and Riboflavin tablets, regularly available in medicine market, were used to examine the remedial measure of the heavy metal toxicity.

As_2O_3 was administered through intramuscular injection at 2mg/kg body weight. Earlier works of Kundu et al., (2000) and LC_{50} estimations were used as reference to determine the working dose of toxicant. Several fixation intervals after single dose were used to study the effects of the toxic material, As_2O_3.

Riboflavin, dissolved in water, was administered orally to a group of As_2O_3 injected mice. Equal volume of water was also administered orally to other experimental group as well as control group. Riboflavin was used at 0.2mg/kg body weight (Dey et al., 2015).

II:2 – Study schedule

Three experimental groups were studied – control (cont), arsenic treated (As), and vitamin fed arsenic treated (As+vit) groups. Animals treated with arsenic and riboflavin-fed arsenic intoxicated mice were sacrificed by cervical dislocation after the scheduled periods of 2 weeks, 4 weeks and 6 weeks of injection. Whole liver was dissected out of the animal and used to obtain the organ weights, histological preparations etc. and to estimate total protein, enzymatic activities etc. Native gel electrophoresis was also made of the tissues. Control groups were parallelly examined for similar studies.

A total of 180 animals were used for the whole experiment. 20 for each experimental groups (Cont, As & As + Vit) and each study schedule (2^{nd}, 4^{th} & 6^{th} weeks of treatment).

II:3 – Experimental protocols

II:3:1 – Quantitative study of proteins

Total quantity of protein was estimated following the method of Lowry et al., 1951. Weighing individual tissue of specified mice groups was homogenized in 0.1(N) sodium hydroxide and centrifuged at 3000 g for 15 minutes. The supernatant was collected and quantitatively transferred in a neutral glass test tube. This served as the aliquot.

II:3:2 – Qualitative study of proteins: Native gel electrophoresis

The protocol for native gel electrophoresis is almost similar to that used in SDS-PAGE by Laemmli, 1970. The only difference is proteins are prepared in a non-reducing non-denaturing sample buffer, which maintains the proteins' secondary structure and native charge density. The tissue extract was mixed with a sample buffer that does not contain any detergent. The sample was loaded in gel apparatus and connected with the power pack,

and electrophoresis was performed at 4°C. 30 – 50 mV current was applied. After 5-6 hours, when the tracking dye reached near the end of the gel plates, apparatus was disconnected and gel was removed carefully from the gel plates. The gel was stained by fast blue RR salt solution and analysed.

II:4 – Estimation of enzymes

II:4:1 - Estimation of alkaline phosphatase

Alkaline phosphatase activity was determined by the method of Walter and Schutt (1974). Alkaline buffer was prepared by mixing 1.053g diethanolamine, 8.0 ml of 0.1(N) HCl, 46.4 mg 4-nitrophenyl phosphate in 85 ml doubled distilled water, pH adjusted to 9.8 by addition of 0.1(N) HCl. Volume made upto 100 ml with distilled water. A measured amount of tissues were taken to be homogenised in standard normal saline (0.9% NaCl solution). The supernatant was taken for the protocol.

II:4:2 – Estimation of Lipid peroxidase (LPO)

The isolated tissues of definite amount were homogenised with ice-cold 1.5 % KCl (w/v) followed by centrifugation at 10000 g for 15 minutes at 4°C. The supernatant was quantitatively collected and used for estimation of LPO by the method of Okhawa (1979). The TCA-TBA-HCl buffer required for the protocol was prepared by mixing 15g TCA and 0.375g TBA to 100 ml of 0.25(N) HCl.

II:4:3 – Estimation of Superoxide Dismutase (SOD)

Superoxide dismutase was estimated by the method modified from Gupta and Shukla (1997). SOD activity was measured in tissues after extracting the homogenate with a mixture of chloroform and methanol. SOD activity of the samples was quantified by analyzing the inhibition of pyrogallol auto-oxidation spectrophotometrically at 420 nm, in the presence of catalase enzyme.

II:4:4 – Estimation of Glutathione (GSH)

Estimation of intracellular GSH was performed by homogenising respective tissues with 0.1% EDTA solution. Precipitating reagent (containing 0.21M metaphosphoric acid, 5.4mM EDTA and 5.13M NaCl) was added to it and the final solution was then allowed to stand for 5min before filtration. Finally 0.3M disodium hydrogen phosphate and 0.04% DTNB (0.04%) in sodium citrate solution (10g/l) were consecutively added to the filtrate. Change in colour was analyzed at 412nm (Tietze 1969).

II:5 – Organosomatic indices

Body weight was measured before and after the experimental period. At the end of each experimental period, the animals were scarified and tissues were dissected out and weighed individually to prepare organosomatic indices.

II:6 – Histopathological study

The tissues were fixed in Bouin's fixative, embedded in paraffin and 5μm thick sections were stained with routine hematoxylin-eosin (Kundu et al., 2000). Histopathological changes in the tissues were examined under optical microscope.

II:7 - Statistical analysis

All data are presented as mean ± S.E. of 10 similar experimental results. Two tailed t-test were conducted to test the significance at $p < 0.001$, $p < 0.01$ and $p < 0.05$ level, between data of control and that of both the treated series (Arsenic treated and As+Vitamin fed groups). Comments on significance of difference between any data series were made based on the statistical analysis.

III. RESULTS

III:1 - Behavioral changes

Mice receiving intramuscular injection of

As_2O_3 become restless for 10-15 minutes and then showed a tendency to crowd together in a corner of the cage. Similar behavior was also observed for the mice injected with saline, but the period of restlessness was much less (about 3-5 minutes). As_2O_3 treated mice stopped taking food and water for a while and appeared as dizzy or timid for several hours. Those took almost an overnight rest to become normal. Any kind of disturbance in between lengthens this period of dizziness.

III:2 - Alteration of organ weight

Hepatosomatic index was calculated and compared to observe any effect of treatment on organ weight. The comparison has been presented in graphs. From the results, it is revealed that treatment with As_2O_3 has caused a successive decrease in the organ weights. The hepatosomatic indices show a trend of gain of the weight of liver in 2nd week of treatment, but continued to show loss of weight in 4th and 6th week of exposure, indicating a sign of hepatomegaly during early exposure only (Fig.1).

While comparing the indices, it was recorded that the weight of liver of As_2O_3 + riboflavin mice groups express a satisfactory recovery of loss of organ weights to somewhat similar levels as the control groups do. The recovery, though not optimum, was statistically significant while compared to indices of As_2O_3 injected mice groups.

III:3 – Total protein

A moderate rise in the total protein content in liver tissue was observed in control mice while maturation progresses, though insignificant in statistical analysis. A noteworthy decrease in total protein content in the liver tissue was recorded for As_2O_3 - treated mice, even in the 2nd week of injection. The decline was recorded to be steady and more profound in 6th week of arsenic exposure (Fig.1). This gradual loss of total protein content may be attributed to the chronic toxic effects of arsenic in progressive exposures.

The striking fall in total protein content in liver was found to be recovered successfully in riboflavin-fed As_2O_3 treated mice groups. This group of mice showed a steady and gradual increase in protein content and almost reached to that of the normal saline treated control groups in 6th week of treatment. Thus, the counteractive success of riboflavin may be established, based on the revival of total protein content in liver tissue.

III:4 – Alkaline phosphatase activity

Alkaline phosphatase activity in liver tissues showed a significant linear increase in mice injected with As_2O_3 as compared to that in control mice. The levels of alkaline phosphatase were found to rise steadily along the different exposure intervals (Fig.1).

In the arsenic treated mice simultaneously fed with riboflavin, the activity level of alkaline phosphatase was observed to remain significantly low as compared to As_2O_3 injected group and consistently remained closer to the level of saline treated control at all respective intervals of fixation.

III:5 – Lipid peroxidation (LPO)

The concentration of malondialdehyde (MDA), an indicator of lipid peroxidation, was observed to be high in liver tissues of mice injected with As_2O_3 along all exposure intervals. Results from liver of As_2O_3 treated mice fed with riboflavin showed a reasonable recovery in the level of lipid peroxidation which comes down to almost normal level as found in control group (Fig.1).

III:6 – Activity of Superoxide dismutase (SOD)

The exposure to arsenic decreased the activities of SOD significantly in liver tissue. A gradual decline in activities of SOD was prominent in hepatic tissues of mice injected with arsenic. A decrease in the activity of SOD

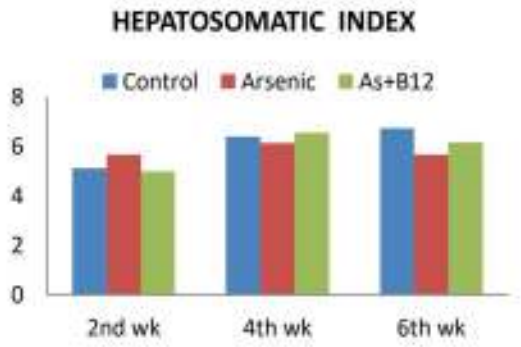
can be owed to an enhanced superoxide production during arsenic metabolism. SOD catalyzes the dismutation of superoxide anions and prevents the subsequent formation of hydroxyl radicals (Fig.1).

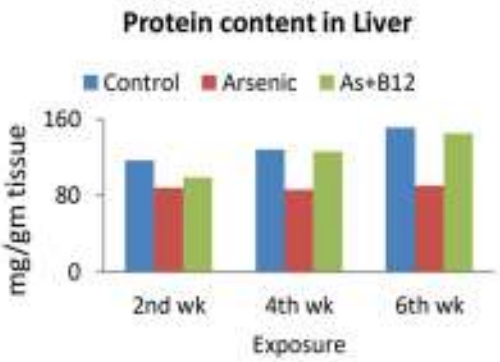
Riboflavin has been observed to be successful in reverting the condition in the organ under study. Liver in riboflavin fed mice groups has shown SOD levels comparable to that of the control groups, and thus suggesting a possible ameliorating success of riboflavin in arsenic induced oxidative stress.

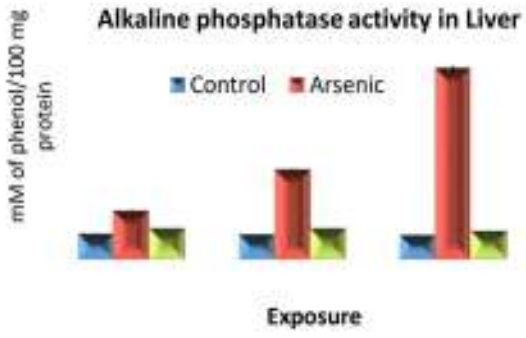
III:7 – Reduced Glutathione content (GSH)

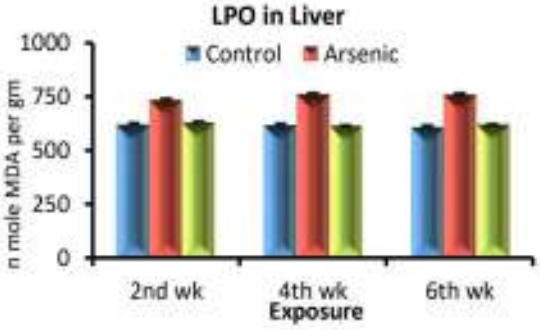
The present study showed that arsenic exposure for longer duration depleted significantly GSH content in liver of growing mice. GSH levels decreased gradually and significantly in the tissue studied of As_2O_3 injected mice while compared to that of control group. Maximum depletion in GSH content was recorded in 6th week of exposure indicating chronic cytotoxicity induced by arsenic (Fig.1).

A potent recovery in GSH content was noticed in riboflavin fed mice groups. Antioxidase activity in liver tissue was observed to be elevated towards the normal level by riboflavin treatment suggesting the efficacy of this vitamin in reducing oxidative stress induced by arsenic.









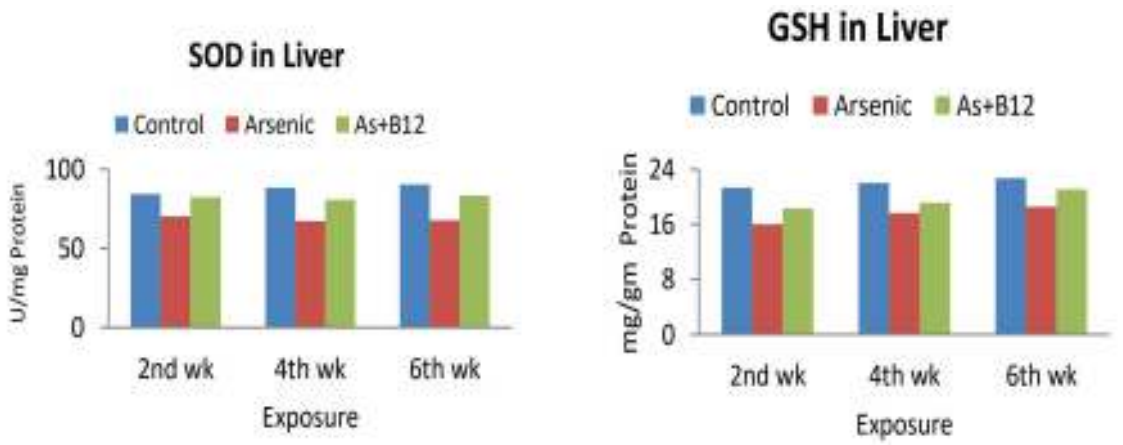


Fig. 1: Graphical presentations of experimental data showing hepatosomatic index, Total protein content, Alk Phos activity, LPO, SOD and GSH activities in Liver tissue of mice

III:8 – Native gel electrophoresis

Study of native gel plates revealed the appearance of new bands (indicated by red arrow in Fig 2) in arsenic treated liver tissue. This might be related to the expression of new proteins due to cytotoxic effects implied by arsenic treatment. These bands are absent both in liver of control and in riboflavin treated mice. Overexpression of some proteins (indicated by blue arrow in Fig 2) is also observed in arsenic treated mice. This can be attributed to genetic malfunction caused due to arsenic intoxication. Such overexpressed bands are found to be absent in riboflavin fed mice group.

III:9 – Histopathological study

The sections of liver in arsenic treated group showed congestion of central vein with moderate degeneration and necrosis in hepatic parenchyma with mild to moderate fatty change. Vacuolation, edema, congestion and condensed nuclei are common phenomena observed in arsenic treated hepatocytes. Nuclear blebbing was observed in some hepatocytes affected by arsenic. The sinusoidal spaces were expanded due to shrinkage and necrosis of hepatic cells. Non-

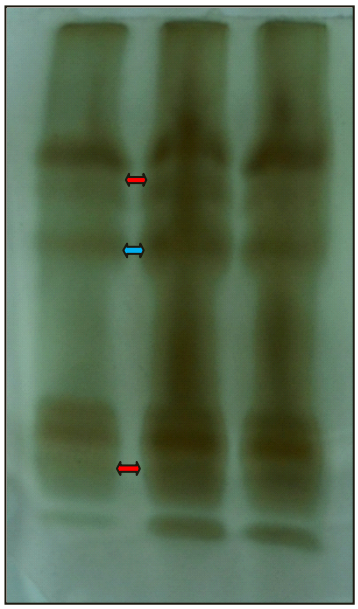


Fig. 2: Native gel plate showing, from the left, Control, Arsenic and As+Vitamin treated Liver (6th week) [Red arrows: New band in Arsenic group ; Blue arrow: Overexpression in Arsenic]

cirrhotic portal fibrosis might have caused due to cytotoxic effect of arsenic. Riboflavin fed mice liver showed a general architecture similar to that of control group. Fatty changes are significantly absent in the tissue. Few

necrotic cells exist, but the remedial action of riboflavin can be suggested from the present analysis of photomicrograph where reappearance of normal hepatocytes has been found to occur in vitamin fed mice liver. Images are shown in Figure 3.

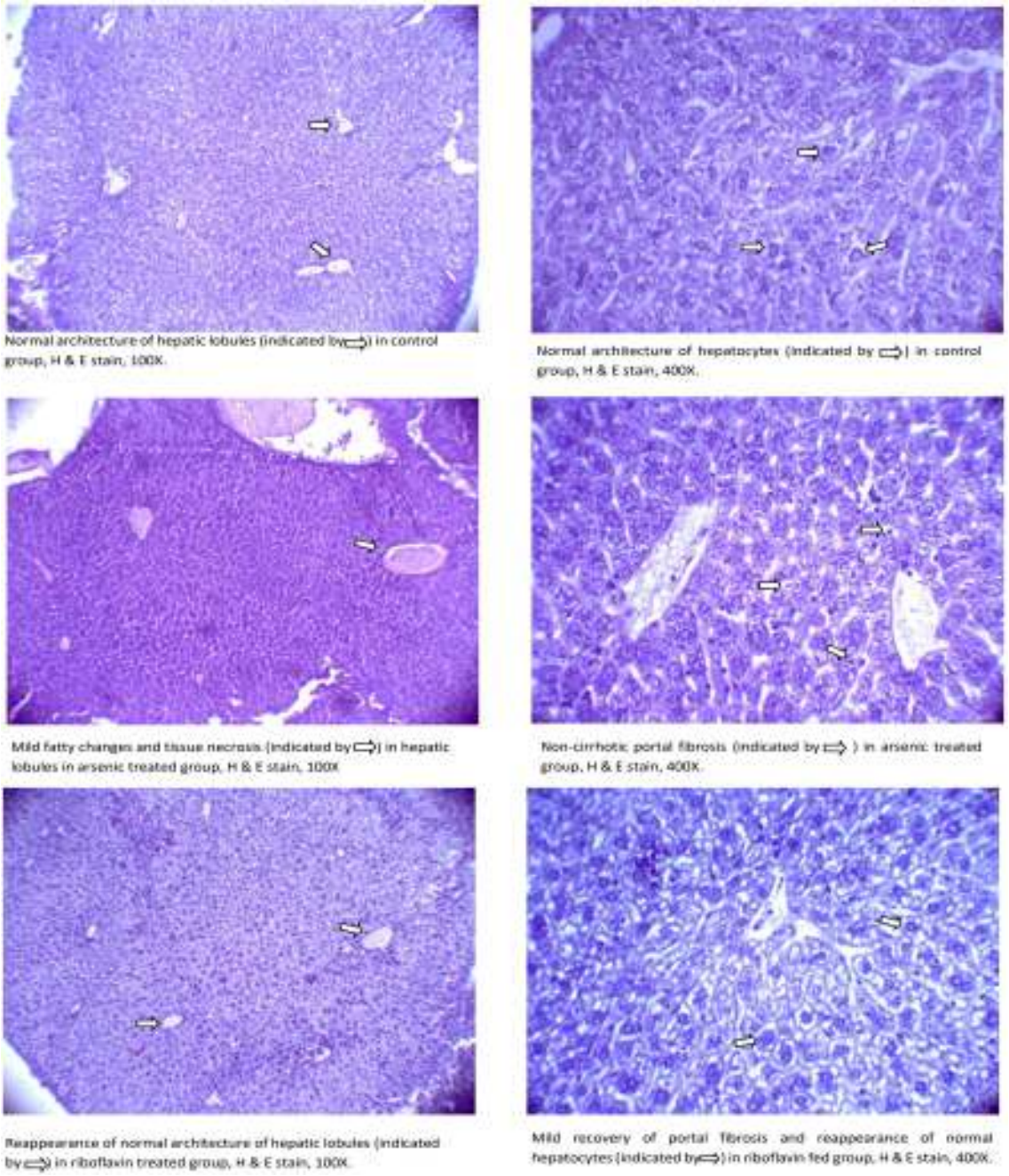


Fig. 3: Photomicrographs on LIVER histology

IV. DISCUSSION

The biochemical and cellular effects of long term exposure to arsenic compounds are well documented (Browning, 1969; Fowler, 1977; Bajra, 2004), and they are believed to inhibit a number of thiol dependent enzyme systems. A single sublethal dose of arsenic trioxide-injection is observed to have long term effects, even gradually increasing in some cases. From the results presented above, it is clearly observed that a single injection of As_2O_3 has brought about significant loss in body weight and organ weights as well. Arsenic is thought to interfere with many essential metabolic enzymes thus affecting the metabolic activity of an organism. The loss of body weight and loss of weight of vital organs indicate that arsenic has caused decreased metabolic activity. General tendency of gain in body weight in control groups was observed to be reverted by the effect of arsenic. This loss of body and organ weight is successive as the experiment progresses. The rapid degeneration of the macromolecules of the vital organs is continued towards the 6^{th} week of exposure. Thus, it can be said that the single sublethal dose of arsenic has created a long-term degenerative effect on the organs concerned. The synthesis of proteins was found to be affected by arsenic intoxication as evident in native gel study in the present work. Some of the vital native proteins are degenerated in liver. Lower expressions of some proteins were also noticed in this organ. All these findings corroborate with lower protein concentration and loss of body and organ weight in arsenic treated mice. Arrival of some protein bands in native gel electrophoresis might suggest cytotoxic alteration induced by arsenic. Arrival of some new proteins may also be concerned with the lowered expression of essential proteins in arsenic affected cells.

Earlier histopathological works of K (Mitra) Dhar, 2000, also describes hepatocellular degeneration in Swiss albino mice caused by arsenic. Present histopathological study shows a general necrotic effect and fatty degeneration caused by arsenic on liver. Hepatic lobular degeneration might be associated with the detoxification of heavy metals in liver tissues. All the adverse effects of arsenic induced cytotoxicity have been found to be reverted towards normal in the riboflavin fed mice group, suggesting a possible ameliorating success of the vitamin in combating toxic effects of arsenic.

The total protein content of liver shows a gradual and significant lowering in the arsenic treated mice, suggesting the failure of protein synthesizing machinery of the respective cells. The heavy degradation of protein content in arsenic treated group is thought to be associated with the cytotoxic effects of arsenic. Gradual loss in the total protein content is caused due to long term effect of single sublethal dose of As_2O_3. The protein synthesizing machinery is under genetic control (Cooper, 1997). Faulty expression of certain genes regulating the metabolic activities of the cells of these vital organs may cause the derangement of protein machinery.

Very significant and gradual increase in the stress enzymes like alkaline phosphatase and lipid peroxidase denotes cytotoxicity caused by oxidative stress. Arsenic induces oxidative stress in the cells of liver. The alkaline phosphatase activity in liver is observed to be increasing gradually, reaching to a high level at the longer exposures, indicating the prolonged stress of single sublethal dose of arsenic. Lipid peroxidation in hepatic cells reaches maximum in 4^{th} week of exposure and hardly increases anymore, suggesting the

cells to face the highest oxidative stress during this phase.

Brown et al., 1976 has shown that arsenic accumulates and affects mitochondria. The dysfunctions in mitochondrial enzymes caused by arsenic, is related with the tissue respiration and hence cytotoxicity. Cellular respiration mediated by NAD linked substrates is particularly sensitive to arsenic. ROS released as a consequence of arsenic intoxication is responsible for the oxidative stress that results in significantly high activity of stress enzymes like phosphatases and peroxidases. Arsenicals disturb the oxidation-reduction equilibria of a cell and thus interfere to the cell signalling pathways (Simeonova & Luster, 2000; Bernstam & Nriagu, 2000). The dramatic rise in the activities of stress enzymes, along the course of exposures, may be attributed to the combination of different signalling malfunctions caused by arsenic and interference of arsenicals to so many proteins that are essential to maintain redox balance in cellular systems.

Tissues are susceptible to free radical damage due to suppressed activities of antioxidant enzymes (antioxidases) like superoxide dismutase (SOD), glutathione (GSH) etc. Overproduction of superoxide anions may be the causative factor of arsenic-induced inhibited SOD and GSH activities. Decreased GSH content in all vital tissue due to arsenic indicates GSH-linked oxidative damages. Glutathione is well known for its pivotal role on the intrinsic antioxidant system of mammalian cells. The present study showed that relative high single sublethal dose of arsenic and duration depleted significantly GSH content in liver of arsenic treated mice. The effect of GSH depletion on the mutagenicity of arsenic has also been examined by many scholars (Xu et al., 1999; Liu et al., 2003).

Based on the above view of arsenic toxicity, an approach was made to investigate the ameliorating efficiency of riboflavin on the cytotoxicity caused by arsenic. Vitamin B_2 was suggested to play an important role in the detoxification of ingested arsenic (Gamble et al., 2005b; Mitra et al., 2004). Consumption of B_3 also increases methylation of arsenic. All the forms of vitamin-B can reduce the risk of arsenic related skin lesions (Lydia et al., 2008). Riboflavin was selected in present study because of being a potent antioxidant and having been the active part of FMN & FAD, enzymes related to reduce oxidative stress (Marmol et al., 2007).

The remarkable changes in the activity of stress enzymes in the riboflavin fed group also support that this vitamin has significant ability to regulate the important enzyme systems associated with combating arsenic poisoning. The isoalloxazine ring of riboflavin molecule provides it with significantly high affinity to react with molecular oxygens, liberated due to the oxidative stress caused by arsenic intoxication (Massey, 1994). Along with the scavenging function, the flavoenzymes are known to take part in various cellular signalling pathways and maintenance of redox potential in cell.

Riboflavin is observed to have the efficiency of lowering the stress caused by arsenite even in the 2nd week of exposure. The levels of phosphatases and peroxidases in riboflavin fed As intoxicated mice tissues reach almost near the control values in early intervals of present experiment, suggesting that the daily uptake of this vitamin can effectively ameliorate oxidative stress even in 2 weeks of treatment. The declined SOD and GSH activities due to enhanced superoxide production during arsenic metabolism were found to be reverted successfully by riboflavin, suggesting the efficacy of riboflavin in

combating arsenic induced cytotoxicity. Present study clearly indicates that riboflavin can efficiently mitigate the hepatotoxicity and reduce oxidative stress in liver.

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