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The Protective Role Of High Dietary Protein On Arsenic Induced Hepatotoxicity In Albino Rat Model;A Possible Role Of Antioxidants.

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Abstract: *The objective of the present investigation was to study the protective role of High dietary protein on arsenic induced hepatotoxicity model in adult male albino rats. Hepatotoxicity in rats was caused by arsenic tri oxide at a dose of 3mg- /ml/kg body weight. Hepamerz, a drug used as standard hepatoprotective agent, was administered orally as standard hepatoprotective agent for 14 consecutive days prior to arsenic treatment at a dose of 10mg- /ml/kg body weight. This drug has many side effects. These side effects have prompted the scientific world for the search of alternative natural remedies of liver damage. The High dietary protein was administered orally to rats along with arsenic. The biochemical parameters were investigated. The results indicated that biochemical changes produced by arsenic were restored to almost normal by High protein diet. The High protein diet produced hepatoprotective effect through the modulation of antioxidant - mediated mechanism by altering serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), superoxide dismutase (SOD) and catalase (CAT) activities and reduced glutathione (GSH) and lipid peroxidation (LPO) levels - against arsenic induced hepatotoxicity model in rats.*

Key words:- *Arsenic trioxide, Hepamerz, hepatotoxicity, High protein diet.*

I. Introduction

Arsenical compounds are environmentally toxic with multiple effects in animal and human populations. We are exposed to arsenic mainly through water, food and drugs. The main source of environmental arsenic exposure is drinking water. The frequent uses of arsenic are as herbicides, insecticides, rodenticides and food preservatives. It has become evident that increasing human activities have modified the global cycle of heavy metals and metalloids, including the toxic non-essential elements like arsenic (As) [1]. Arsenic is one of the most abundant and potential carcinogen [2] which is present in the nature in the stable from as trivalent arsenate (As^{+3}) or Pentavalent arsenate (As^{+5}). The permissible limit for arsenic in drinking water is 0.05 mg/lit as per WHO [3]. A survey of 25000 tube wells in West Bengal in India reveals that the average As Concentration reaches to 0.3 mg/lit and even it reaches up to 3 mg/lit of water. During the process of arsenic metabolism, Inorganic arsenic is first methylated to monomethyl arsenic (MMA) and then to dimethyl arsenic acid (DMA) followed by its excretion from the body. In this process of biomethylation, constant depletion of methyl causes DNA hypomethylation that leads to generation of mutation followed by carcinogenesis [2]. Arsenic affects the mitochondrial enzymes, impairs the cellular respiration and causes cellular toxicity. It can also substitute phosphate intermediates, which could theoretically slow down the metabolism and interrupt the production of energy.On the other hand Hepamerz has been reported as a liver protective drug which partly corrects these toxicities [4]. But it has many side effects like vomiting,nausea, headache etc. It is reported earlier that aqueous leaf extract of moringa olifera has an antioxidant effect [5]. Dietary Proteins have also been found to have antioxidant activities [6]. Wheat and pea are both good sources of dietary plant protein, while casein is an animal protein. The antioxidant activities of pea, wheat and casein has been studied using different liposomal models and the results show a minimization in lipid per oxidation, thus preventing the damage produced by the free radicals [6].The main aim of our study is to find out the relationship between arsenic generated oxidative stress and cellular damage using rat as a model and also the dietary protein how far counteract this arsenic induced oxidative stress.

1.1. Animals Used and Maintenance

Thirty-six male Wistar strain adult albino rats of age approximately 120 days and weighing 150- 200 g were used in the following studies. The animals were individually housed and maintained under standard laboratory conditions with natural dark and light cycle (approximately 12--h light/10--h dark cycle) and room temperature (27 ± 1^{o}C) and constant humidity (60%) in accordance with the 'Institutional Ethical Committee' rules and regulations. A control diet composed of 71% carbohydrate,18% protein,7% fat and a mixtureof salt and vitamins were given. Drinking water was supplied ad libitum. Animals were randomly divided equally into

six groups of six each which are as follows: control group, arsenic treated experimental group, high protein supplemented control group, Hepamerz treated control group, high protein along with arsenic treated experimental group and Hepamerz pretreated arsenic treated experimental group. The high protein diet means pea (37g/100g of diet) which contributed 8.5% of protein and casein (9g/100g of diet) which contributed additional 9% protein in formulation of a high protein (27%) diet [6]. In addition to the normal diet, high protein diet was given to the group of high protein supplemented control group and high protein along with arsenic treated experimental group.The control group was kept in the laboratory condition for 42 (28+14=42) days. A dose of 3 mg/ml/kg body weight /day of arsenic was given orally through orogastric cannula daily for 28 days to each of the 2^{nd}, 5^{th} and 6^{th} group of the animals. The dose was standardised in our laboratory. In the 4^{th} and 6^{th} groups, i.e., Hepamerz treated control group and Hepamerz pretreated arsenic treated experimental group, a dose of 5 ml of liver protective drug Hepamerz was administered for 14 days. After that, arsenic at a dose of 3 mg /ml/kg body weight was administered orally for about 28 days. Body weights of the rats were recorded everyday and maintained in the laboratory throughout the experimental period**.**

II. Biochemical Estimations

The biochemical results are obtained by estimation of parameters (e.g. SGOT, SGPT, ALP, SOD, CAT, GSH and LPO) attached to the study on rats.

1.2. Collection of serum

Rats were sacrificed by cervical dislocation after 28 days of arsenic treatment. Blood sample was collected by heart puncture and serum was separated by centrifugation (3000 rpm at 4^oC for 10 min). The liver was immediately removed.

1.3. Estimation of SGPT, SGOT and ALP levels

Tissue and serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) activities were measured according to the method of Kind and King [7], alkaline phosphatase (ALP) activity was measured according to the procedure of Reitman and Frankel[8].

1.4.Measurement of SOD

Superoxide dismutase (SOD) was estimated by the method of Mishra and Fridovich **[9]** and Roy *et al*[10].Brain tissue samples were homogenised with 5 ml of ice--cold 0.1 M phosphate buffer (pH--7.4). The homogenates are then centrifuged at 3000 rpm for 10 min. Afterthat, 0.1 ml of sample was mixed with 0.8 ml of TDB. Reaction was started by the addition of 4 µl of nicotinamide adenine dinucleotide phosphate (NADPH). Then, 25 µl of ethylenediaminetetraacetic acid- manganese chloride (EDTA-$MnCl_2$) mixture was added to it. Thereafter, spectrophotometric readings were recorded at 340 nm. After recording of the spectrophotometric readings, 0.1 ml of mercaptoethanol was added to this mixture and again spectrophotometric readings were recorded at 340 nm.

1.5.Measurement of LPO

Lipid peroxidation was measured according to the method of Bhattacharya *et al.* **[11]** .and Roy *et al.[10]*Brain tissue samples were homogenized with 5 ml of ice-cold 0.1 M phosphate buffer (pH-7.4). The homogenates were then centrifuged at 3000 rpm for 10 min. After that, 0.5 ml of sample was mixed with 1 ml of TDB and then the mixture was incubated at 37^oC for 1 hour. To this, 0.5 ml of trichloroacetic acid (TCA) was added, vortexed and the absorbance was read at 350 nm. After recording of the spectrophotometric reading, 1 ml sample was mixed with 500 µl mercaptoethanol and again the absorbance was read at 350 nm.

1.6.Measurement of CAT

Catalase activity was estimated by the method of Cohen *et al.* [12] and Roy *et al.*, [10].Brain tissue samples were homogenised with 5 ml of ice-cold 0.1 M phosphate buffer (pH-7.4). The homogenates were then centrifuged at 3000 rpm for 10 min. The precipitate was then stirred with the addition of 15 ml of ice-cold 0.1 M phosphate buffer and allowed to stand in cold condition with occasional shaking. The shaking procedure was repeated for thrice. 1 ml of sample was added to 9 ml of H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically from the changes in absorbance at 350 nm. The activity of CAT was expressed as percentage of inhibition unit.

1.7.Measurement of GSH

Reduced glutathione was measured according to the method of Ellman [13].Equal quantity of homogenate was mixed with 10% TCA and centrifuged to separate the proteins. To 0.01 ml of this supernatant, 2 ml of phosphate buffer (pH-8.4), 0.5 ml of 5, 5-dithiobis (2-nitrobenzoic acid) and 0.4 ml of double distilled

water were added. The mixture was vortexed and the absorbance was read at 412 nm within 15 min. The concentration of GSH was expressed as μg/g of tissue.

III. Statistical Analysis

The data were expressed as MEAN ± SEM (SEM means standard error of mean) and were analysed statistically using one-way analysis of variance (one-way ANOVA), followed by multiple comparison't'- test, which was used for statistical evaluation of the data. In addition to this, two-tailed Student's't'- test was performed to determine the level of significance between the means. Difference below the probability level 0.05 was considered statistically significant.

IV. Results

Twenty-eight days after the introduction of arsenic treatment, the SGOT, SGPT, ALP, SOD and CAT activities, GSH and LPO levels were estimated.

There was a sharp rise ($P<0.001$) in SGOT, SGPT and ALP activities in the arsenic treated experimental group as compared to the control group. The SGOT, SGPT and ALP activities were significantly ($P<0.001$) decreased in High protein supplemented control group compared to the control group. High protein supplement significantly ($P<0.001$) made reduction of SGOT, SGPT and ALP activities in High protein pretreated arsenic treated experimental group compared to the arsenic treated experimental group. There were a sharp declines ($P<0.001$) in SGOT, SGPT and ALP activities in the Hepamerz treated control group compared to the control group. The SGOT, SGPT and ALP activities were significantly ($P<0.001$) decreased in Hepamerz treated control group in comparison to High protein treated control group. The SGOT, SGPT and ALP activities were significantly ($P<0.001$) decreased in Hepamerz pretreated arsenic treated experimental group when compared to arsenic treated experimental group. The results are shown in Table 1.

There was a sharp decline ($P<0.001$) in SOD activity both in serum and liver in the arsenic treated experimental group as compared to the control group. The SOD activity was significantly ($P<0.001$) increased in High protein treated control group compared to the control group, both in serum and liver. High protein led to significantly ($P<0.001$) increase SOD activity in High protein pretreated arsenic treated experimental group in comparison to arsenic treated experimental group, both in serum and liver. There was a sharp increase ($P<0.001$) in SOD activity both in serum and liver in the Hepamerz treated control group compared to the control group. The SOD activity was significantly ($P<0.001$) increased in Hepamerz treated control group in comparison to High protein treated control group both in serum and liver. The SOD activity was significantly ($P<0.001$) increased in Hepamerz pretreated arsenic treated experimental group compared to the arsenic treated experimental group both in serum and liver. The results are shown in Tables 2 and 3.

There was a sharp rise ($P<0.001$) in LPO level both in serum and liver in the arsenic treated experimental group as compared to the control group. The LPO level was significantly ($P<0.001$) decreased in High protein treated control group compared to the control group, both in serum and liver. High protein significantly ($P<0.001$) decreased LPO level in High protein pretreated arsenic treated experimental group in comparison to arsenic treated experimental group, both in serum and liver. There was a sharp decline ($P<0.001$) in LPO level both in serum and liver in the Hepamerz treated control group when compared to the control group. The LPO level was significantly ($P<0.001$) decreased in Hepamerz treated control group compared to the High protein treated control group, both in serum and liver. The LPO level was significantly ($P<0.001$) decreased in Hepamerz pretreated arsenic treated experimental group when compared to the arsenic treated experimental group, both in serum and liver. The results are shown in Tables-2 and 3.

There was a sharp decline ($P<0.001$) in CAT activity both in serum and liver in the arsenic treated experimental group as compared to the control group. The CAT activity was significantly ($P<0.001$) increased in High protein treated control group when compared to the control group, both in serum and liver. High protein significantly ($P<0.001$) increased CAT activity in High protein pretreated arsenic treated experimental group in comparison to arsenic treated experimental group, both in serum and liver. There was a sharp increase ($P<0.001$) in CAT activity, both in serum and liver in the Hepamerz treated control group compared to the control group. The CAT activity was significantly ($P<0.001$) increased in Hepamerz treated control group in comparison to High protein treated control group, both in serum and liver. The CAT activity was significantly ($P<0.001$) increased in Hepamerz pretreated arsenic treated experimental group when compared to the arsenic treated experimental group, both in serum and liver. The results are shown in Tables-2 and 3.

There was a sharp decline ($P<0.001$) in GSH level both in serum and liver in the arsenic treated experimental group as compared to the control group. The GSH level was significantly ($P<0.001$) increased in High protein treated control group compared to the control group, both in serum and liver. High protein significantly ($P<0.001$) increased GSH level in High protein pretreated arsenic treated experimental group in comparison to arsenic treated experimental group, both in serum and liver. There was a sharp increase ($P<0.001$) in GSH level, both in serum and liver in the Hepamerz treated control group compared to the control group. The

GSH level was significantly (P<0.001) increased in Hepamerz treated control group when compared to the High protein treated control group, both in serum and liver. The GSH level was significantly (P<0.001) increased in Hepamerz pretreated arsenic treated experimental group compared to arsenic treated experimental group both in serum and liver. The results are shown in Tables-2 and 3.\

V. Discussion

The present study evaluates the protective role of High dietary protein on arsenic induced hepatotoxicity in albino rat model with the possible involvement of the antioxidants. SGPT, SGOT and ALP are the most sensitive tests employed in the diagnosis of hepatic disease. Extensive liver damage by arsenic itself decreases its rate of metabolism and other substrates for hepatic microsomal enzymes. It is evident from the results of the present investigation that treatment with High dietary protein significantly decreased the SGPT, SGOT and ALP activities .These findings can be explained by alterations of the LPO level and antioxidant activities such as that of SOD, CAT and GSH, both in serum and liver tissue.

Free radicals play a crucial role in the pathogenesis of liver damage. LPO can be used as an index for measuring the damage that occurs in membranes of tissue as a result of free radical generation [14,15]. In our present study, oral administration of arsenic, significantly increased the LPO level. Significant elevation of LPO level observed in arsenic treated experimental- group is possibly due to the generation of free radicals via auto-oxidation or through metal ion or superoxide catalysed oxidation process. In the present study, High dietary protein significantly decreased LPO level at a dose of 400 mg/kg body weight compared to other groups. High dietary protein was found to have excellent scavenging effect on LPO, which was well comparable with the standard drug Hepamerz. Endogenous antioxidant status in arsenic-induced experimental rat group was evaluated here by noting the activities of CAT, SOD and GSH as these are the important biomarkers for scavenging free radicals[16].

The primary role of CAT is to scavenge H_2O_2 that has been generated by free radicals or by SOD in its removal of superoxide anions, and convert it in to water **[17].** The destruction of superoxide radicals is catalysed by SOD, which is an important defense system against oxidative damage. From our experimental results of the aforesaid antioxidant enzyme activities in serum and liver tissues, it is clear that arsenic significantly led to reduction of SOD, CAT, GSH activities in arsenic treated experimental group compared to control group, High dietary protein treated control group, Hepamerz treated control group, Hepamerz pretreated arsenic treated experimental group and High dietary protein pretreated arsenic treated experimental group..

Glutathione is an endogenous antioxidant, which is present majorly in the reduced form within the cells. It prevents the hydroxyl radical generation by interacting with free radicals. During this defensive process, GSH is converted to oxidised form under the influence of the enzyme glutathione peroxidase (GPX). The decreased level of GSH in arsenic treated experimental group seen in our study indicates that there was an increased generation of free radicals and the GSH was depleted during the process of combating oxidative stress **[18,19].** This has probably been possible either from the low level of reactive oxygen species (ROS) production or through a rapid dissolution of ROS that has further been strengthened by the elevated activities of important antioxidant defense enzymes CAT and SOD, studied in this experiment. Extract showed the presence of flavonoids. Arsenic causes lipid peroxidation by generation of Reactive Oxygen Species (ROS) [20,21]. Our results also support this view. In recent studies [6-8] dietary proteins have been found to have antioxidant activities. Wheat and Pea are both good sources of dietary plant protein while casein is an animal protein. The antioxidant properties of milk casein have been established [22]. It is also reported that casein phosphopeptides (Cpp) and casein hydrolyses bind with per oxidant and thus lipid per oxidation is suppressed [22-24]. Our findings also supported that pea and casein has an antioxidant properties. In our present experiment arsenic trioxide induced oxidative stress. On the other hand supplementation of specific proteins with normal diet causes significant recovery from all of these toxic effects.

The salient findings of our present study suggest that High dietary protein provide a good source of antioxidants that could offer potential protective effects against LPO and which could be exploited to make a hepato protective formulation.

Table 1: Effect of High dietary protein (HDP) on SGPT, SGOT and ALT activity in arsenic- induced hepatotoxic rat model

Group (n)	SGPT (IU/l)	SGOT (IU/l)	ALT (IU/l)
Control	51.89 ± 0.09	57.85 ± 0.02	58.83 ± 0.05
Arsenic treated	132.34 ± 1.04***	131.66 ± 0.42***	134.02 ± 0.25***
HDP+ Control	44.31 ± 0.02*	45.72 ± 0.03*	43.76 ± 0.04*
HDP+ Arsenic	100.23 ± 0.45**	103.46 ± 0.09**	105.38 ± 0.06**
Hepamerz + Control	41.66 ± 0.02*	41.43 ± 0.03*	38.02 ± 0.04*
Hepamerz + Arsenic	83.61 ± 0.12**	85.84 ± 0.09**	85.37 ± 0.14**

Values are mean ± SEM, n = 6. Data were analyzed statistically using one-way ANOVA test followed by multiple comparison *t*–tests. $^{*}P < 0.001$ when compared with control group; $^{**}P < 0.001$ when compared with arsenic treated group; $^{***}P < 0.001$ when compared with other mentioned groups.

Table 2: Effect of HDP on serum antioxidant enzymatic changes in arsenic- induced hepatotoxic rat model

Group (n)	SOD (% inhibition unit)	LPO (nmol of [TBARS /-g] mol of tissue)	CAT (% inhibition unit)	GSH (μg/g of tissue)
Control	13.61 ± 0.09	5.01 ± 0.01	14.61 ± 0.02	32.35 ± 0.09
Arsenic treated	$25.23 \pm 0.05^{***}$	$12.62 \pm 0.02^{***}$	$26.45 \pm 0.03^{***}$	$3.59 \pm 0.04^{***}$
HDP + Control	$10.24 \pm 0.02^{*}$	$2.37 \pm 0.02^{*}$	$10.17 \pm 0.03^{*}$	$35.54 \pm 0.09^{*}$
HDP + Arsenic	$17.27 \pm 0.04^{**}$	$9.57 \pm 0.03^{**}$	$20.13 \pm 0.05^{**}$	$11.34 \pm 0.06^{**}$
Hepamerz + Control	$16.92 \pm 0.02^{*}$	$1.69 \pm 0.03^{*}$	$8.47 \pm 0.04^{*}$	$38.78 \pm 0.04^{*}$
Hepamerz + Arsenic	$16.15 \pm 0.02^{**}$	$7.12 \pm 0.03^{**}$	$18.36 \pm 0.03^{**}$	$14.43 \pm 0.10^{**}$

Note: TBARS- Thio-barbituric acid reactive substances.

Values are mean ± SEM, n = 6. Data were analyzed statistically using one-way ANOVA Test followed by multiple comparison *t*–test. $^{*}P < 0.001$ when compared with control group; $^{**}P < 0.001$ when compared with arsenic treated group; $^{***}P < 0.001$ when compared with other mentioned groups.

Table 3: Effect of HDP on tissue antioxidant enzymatic changes in arsenic induced hepatotoxic rat model

Group (n)	SOD (% inhibition unit)	LPO (nmol of [TBARS /-g] mol of tissue)	CAT (% inhibition unit)	GSH (μg/g of tissue)
Control	12.24 ± 0.03	6.13 ± 0.01	15.02 ± 0.02	32.25 ± 0.03
Arsenic treated	$24.23 \pm 0.03^{***}$	$13.33 \pm 0.02^{***}$	$27.66 \pm 0.04^{***}$	$3.75 \pm 0.06^{***}$
HDP + Control	$10.22 \pm 0.02^{*}$	$2.76 \pm 0.03^{*}$	$10.11 \pm 0.03^{*}$	$37.58 \pm 0.03^{*}$
HDP + Arsenic	$17.21 \pm 0.01^{**}$	$8.53 \pm 0.04^{**}$	$18.94 \pm 0.04^{**}$	$13.56 \pm 0.04^{**}$
Hepamerz + Control	$14.87 \pm 0.02^{*}$	$1.68 \pm 0.01^{*}$	$8.83 \pm 0.04^{*}$	$39.47 \pm 0.02^{*}$
Hepamerz + Arsenic	$16.32 \pm 0.01^{**}$	$6.78 \pm 0.04^{**}$	$16.32 \pm 0.06^{**}$	$14.54 \pm 0.02^{**}$

Note: TBARS- Thio-barbituric acid reactive substances.

Values are mean ± SEM, n = 6. Data were analyzed statistically using one-way ANOVA Test followed by multiple comparison *t*–tests. $^{*}p < 0.001$ when compared with control group; $^{**}p < 0.001$ when compared with arsenic treated group; $^{***}p < 0.001$ when compared with other mentioned groups.

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