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RESEARCH ARTICLE

ARSENIC INDUCED TOXIC EFFECTS ON OXIDATIVE SYSTEM AND mRNA EXPRESSION LEVELS OF GPX IN ALBINO RAT BRAIN: PROTECTIVE EFFECT OF VITAMIN-E.

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Abstract

Induction of reactive oxygen species by arsenic and subsequent depletion of antioxidant cell defense can result in disruption of the pro oxidant / antioxidant balance in mammalian tissues. Therefore the brain is very susceptible to oxidative stress due to its high oxygen consumption. In the present study the young albino rats (3 months) were exposed to low dose of As (2.5 mg/kg body weight) and high dose of As (5 mg/kg body weight) through intraperitoneal injection daily for a period of 3 weeks. After the period of dosage, the As exposed animals were divided into two groups of which one group of both the doses were given Vit E at a dose of 5 mg/kg bw for a period of one week. Then the oxidative stress enzymes i.e. isoforms of Superoxide dismutase (Mn SOD & Cu/Zn-SOD), Glutathione peroxidase (GPx) and Lipid peroxidase (Lpx) were assayed. In this study, it was observed that the Mn-SOD, Cu/Zn-SOD, Glutathione peroxidase (GPx) were decreased significantly in high and low dose of As exposure in different brain regions (cerebral cortex, cerebellum and hippocampus), suggesting that As leads to alteration in the activity of antioxidant enzymes. Decrease in antioxidant enzymes were more evident in high dose compared to low dose. In this study, we have also observed that the Lpx was increased in As supplemented rats as compared to control rats. As-induced neurotoxicity is dose dependent. From our observation, it was clear that the exposure of Vitamin-E provides a protective role against toxic influence of As on high and low doses in all examined parameters in rat brain regions. In addition to this, we have also examined the mRNA expression levels of GPx in cerebral cortex of rats. The GPx expression levels were significantly decreased in As-exposed rats (both in low-high dose). The effect was more pronounced in high dose of As exposed animals. However, supplementation with Vit-E significantly reversed the As-induced decrease in gene expression levels of Gpx. The recovery in expression was more evident in low dose when compared to high dose of As exposed animals. Our results showed that the nutritional antioxidant Vit-E ameliorated oxidative stress and loss of cellular antioxidants and suggested that Vit-E efficiently protects cerebral cortex and cerebellum from arsenic induced oxidative damage. This protection may include

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the capability of Vit-E to alter the distribution of arsenic in tissues and to induce binding of the complexes to proteins, which are similar to metallothionein.

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Introduction:-

The World Health Organization (WHO) estimates that more 200 million people worldwide are chronically exposed to arsenic at levels above proposed safety standards (WHO). Arsenic and its inorganic compounds have long been known to be neurotoxic. Common symptoms of acute inorganic arsenic poisoning are nausea, anorexia, vomiting, epigastric and abdominal pain and diarrhea. Dermatitis, muscle cramps, cardiac abnormalities and hepatotoxicity, bone marrow suppression, haematologic abnormalities and vascular lesions have also been reported (U.S. Air Force, 1990; ATSDR, 1989; Franzblau and Lilis, 1989; U.S. EPA, 1984; Armstrong et al., 1984; Hayes, 1982). Acute arsenic exposure is also known to cause neurologic symptoms such as hyperpyrexia, convulsions, tremor, coma, etc. (Ellenhorn, 1997). Jha et al., 2002, have reported hyperkeratosis together with peripheral neuropathy, weakness and sensory motor flaccid quadriparesis in acute and chronic arsenic toxicity. Central nervous system deficits (hearing loss, eye damage, abnormal EEGs, mental retardation, epilepsy) occurred in infants who had been fed arsenic contaminated milk for 1-2 months (Hamamoto E, 1955). Chronic exposure to arsenic dust in smelter workers at a concentration of 0.5 mg/m^3 caused a decrease in peripheral nerve conduction velocities, encephalopathy, polyneuropathy, tremor and axonal degeneration (Ellenhorn, 1997; Feldman et al., 1979; Blom et al., 1985; Landau et al., 1977; Lagerkvist and Zetterlund, 1994).

Arsenic easily crosses the blood brain barrier (Tripathi et al., 1997) and accumulates in the brain leading to neurobehavioral abnormalities (Itoh et al., 1990). Although not much information about the precise target of arsenic in brain is known, the basal ganglia has been shown to be quite vulnerable (Ghafgazi et al., 1980; Rodriguez et al., 2001). Studies have been carried out in whole brain (Flora et al., 2005; Gupta and Flora, 2006) and brain regions to understand the mechanisms of arsenic induced neurotoxicity (Shila et al., 2005a, b, c). It was observed that arsenic has marked effect on corpus striatum, cortex and hippocampus (Shila et al., 2005c). Delayed maturation of Purkinje cells and their defective migration have been reported in rats exposed to sodium arsenite during the rapid brain growth period from postnatal days 4 to 10 (Dhar et al., 2007). Impaired learning and memory in arsenic exposed individuals and children have been reported (Danan et al., 1984). Alteration in motor behavior has also been reported in arsenic exposed rats (Rodriguez et al., 2001, 2003).

The present study was focused to evaluate the antioxidant potential of vitmin E against arsenic induced organ toxicity in male wistar albino rats. The results of the present study revealed that treatment with Vit-E at the dose of 5mg/kg exerted marked amelioration from arsenic intoxication as evidenced by significantly restoring the altered antioxidant enzymes such as superoxide dismutase (Mn-SOD &Cuzn SOD), glutathione peroxidase and levels of lipid peroxidation.

Material and Methods:-

Procurement and maintenance of experimental animals

Young albino rats (Wistar) were purchased from IISc, Bangalore and maintained in the animal house of Watson Life Sciences, Tirupati. The animals were housed in clear plastic cages with hardwood bedding in a room maintained at $28^0 \pm 2^0$ C and relative humidity 60 ± 10% with a 12 hour light/day cycle. The animals were fed in the laboratory with standard pellet diet supplied by Sri Venkateswara Traders, Bangalore and water *ad libitum.*

Chemicals

Arsenic (As) and Vit – E were selected as test chemicals. The chemicals used in this study namely Thiobarbutric acid, Glutathione oxidized, NADPH, DTNB, Reduced glutathione were obtained from Sigma, USA. The remaining chemicals were obtained from Qualigens, India.

Animal exposure to As and Vit - E

The young albino rats (3 months) were exposed to low dose of As (2.5 mg/kg body weight) and high dose of As (5 mg/kg body weight) through intraperitoneal injection daily for a period of 3 weeks. After the period of dosage, both the low and high As exposed animals were divided into two groups of which one group of both doses were given Vit

E at a dose of 5mg/kg body weight for a period of one week. After the period of dosage the animals were sacrificed through cervical dislocation and the tissues were stored at -80^{0}C for further biochemical analysis.

Biochemical Studies:-

Preparation of Brain Mitochondrial Fraction

Brain mitochondrial fractions were prepared following Lai and Clark, 1979. Briefly, the tissue was homogenized in 5 volumes (w/v) of SET buffer (0.25 M sucrose, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.4). The homogenate was first centrifuged at 800 *g* for 10 min at 4°C, and then the supernatant was centrifuged at 10,000 *g* for 20 min at 4°C. Then the pellet of mitochondrial fraction was suspended in SET buffer.

Superoxide dismutase (SOD) (E.C. 1.15.1.1) activity

SOD activity was determined by using the epinephrine assay of Misra and Fridovich (1972). At alkaline pH, superoxide anion O^-_2 causes the autooxidation of epinephrine to adenochrome; while completing this reaction, SOD decreases the adenochrome formation. One unit of SOD is defined as the amount of extract that inhibits the rate of adenochrome formation by 50%.

The reaction mixture in a final volume of 2.0 ml contained 1.760 ml of 0.05 M carbonate buffer (pH 10.2), 0.04 ml of 30 mM epinephrine (freshly prepared) and 0.2 ml of s the enzyme extract. 1-3 mM potassium cyanide will inhibit both Cu/Zn SOD and extracellular SOD resulting in Mn SOD activity only. Changes in absorbance were recorded at 480 nm, measured at 10 sec intervals for 1 min in a spectrophotometer. The enzyme activity was expressed as Units/mg protein.

Lipid peroxidation

The level of lipid peroxidation in the tissues was measured in terms of malondialdehyde (MDA; a product of lipid peroxidation) content and determined by using the thiobarbituric acid (TBA) reagent. The reactivity of TBA was determined with minor modifications of the method adopted by Hiroshi *et al.* (1979).

To 2.5 ml of homogenate, 0.5 ml of saline (0.9% sodium chloride), 1.0 ml of (20% w/v) trichloroacetic acid (TCA) were added. The contents were centrifuged for 20 minutes on a refrigerated centrifuge at 4000 x g. To 1.0 ml of supernatant, 0.25 ml of TBA reagent was added and the contents were incubated at 95°C for 1hr. 1ml of n-butanol was added to it. After thorough mixing, the contents were centrifuged for 15 minutes at 4000 g in a refrigerated centrifuge. The organic layer was transferred into a clear tube and its absorbance was measured at 532 nm. The rate of lipid peroxidation was expressed as µ moles of malondialdehyde formed/g wet wt. of tissue.

Glutathione peroxidase (GPx) (EC 1.11.1.9) activity

GPX activity in the mitochondrial fraction of rat brain was assayed as described by Rotruck et al., (1973). The reaction mixture contained 0.2 ml of EDTA, 0.2ml of 4mM sodium azide, 0.2ml of glutathione reduced, 0.2ml of H_2O_2, 0.4ml of 0.32M Sodium pyrophosphate buffer (pH-7.0), and 0.1ml of enzyme source. Then the reaction mixture was incubated at 37º C for 10 min. Then the reaction was arrested by adding of 0.5 ml 10% TCA and centrifuged at 2000 rpm for 10 min. To 0.5 ml of supernatant, 3.0 ml of 0.3M disodium hydrogen phosphate and 1.0 ml of DTNB were added and the reaction was read at 412 nm in spectrophotometer. The enzyme activity was expressed as µmole/min/mg protein.

Estimation of protein content

Protein content of the brain was estimated by the method of Lowry et al. (1951). 1% (W/V) homogenation was prepared in 0.25 M ice cold sucrose solution. To 0.5ml of crude homogenate, 1ml of 10% TCA was added and the samples were centrifuged at 1000 g for 15min. The residue was resuspended in 0.5ml of 1N NaOH and 4ml of alkaline copper reagent was added followed by 0.4ml of folin-phenol reagent (1:1 folin: H_2O). The color was measured at 600 nm in a UV- Vis spectrophotometer (Hitachi model U-2000) against blank. The protein standard graph was prepared using Bovine serum albumin. The protein content of the tissues was calculated using the standard graph.

RT-PCR (Reverse transcriptase PCR) Analysis

The expression of gene encoding glutathione peroxidase (GPx) was evaluated by RT-PCR. Total RNA was isolated from cerebral cortex using RNA-X press Reagent (HIMEDIA, India). The purity and concentrations of the RNA samples were assessed by OD 260/OD 280 spectrophotometric measurements and by agarose gel electrophoresis.

The ratio of OD 260/OD 280 of all extracted RNA samples was between 1.8 and 2.0. RNA was transcribed into first-strand cDNA using revertAid first strand cDNA synthesis kit (Fermentas, India). The synthesized cDNAs were amplified by one cycle of 95^0C for 5 min and amplified by 30 cycles of PCR (denaturation at 94^0C for 1min, annealing at 60.7^0C for 1 min, extension at 72^0C for 2 min. Final extension was at 72^0C for 5 min. The sequences of oligonucleotide primers used for PCR amplification of GPx was as follows: Forward primer 5'- TGC AAT CAG TTC GGA CAT CA -3' and Reverse primer 5'- ACC ATT CAC CTC GCA CTT C -3'. Primers were the same as those reported before (Lestaevel et al. 2009). The PCR products were separated by electrophoresis using 1% agarose gels stained with ethidium bromide to visualize cDNA products. Bands of each target transcript were visualized by ultraviolet transillumination and captured using a digital camera. ODs for each band were quantified by image J analysis software.

Statistical treatment of the data

The mean and standard deviation (SD), analysis of variance (ANOVA) and test of significance or Student's 't' test was calculated using standard statistical software package.

Results:-

Mn-SOD and Cu/Zn SOD activities:

A decrease in the activity of Mn-SOD in cerebral cortex, cerebellum and hippocampus was observed in arsenic treated rats as compared to controls (Fig. 1). The arsenic treated rats also showed a decrease in the activity of Cu/Zn SOD in cerebral cortex, cerebellum and hippocampus as compared to controls (Fig. 2). Simultaneous treatment with arsenic and Vit-E caused an increase in the activities of Mn-SOD and Cu/Zn-SOD in the cerebral cortex, cerebellum and hippocampus as compared to rats treated with arsenic alone (Figs. 1 and 2). However, the effect of decrease in enzyme activity was highly pronounced in high dose exposed animals.

GPx Activity:

A significant decrease in glutathione peroxidase (GPx) activity in cerebral cortex, cerebellum and hippocampus was observed in arsenic treated rats compared to those in the control group (Fig. 3). Interestingly, co-treatment with Vit-E and arsenic in rats caused an increase in the activity of glutathione peroxidase in cerebral cortex, cerebellum and hippocampus as compared to rats treated with arsenic alone. Among the brain regions studied, hoppocampus was found to be more susceptible compared to the other brain regions.

Protein content:

Rats exposed to arsenic exhibited decreased level of protein content in cerebral cortex, cerebellum and hippocampus in comparison to control (Fig. 4). Simultaneous treatment with Vit-E and arsenic in rats increased the levels of protein content in cerebral cortex, cerebellum and hippocampus simultaneously as compared to those treated with arsenic alone (Fig. 4).

Lipid Peroxidation:

A significant increase in lipid peroxidation levels in cerebral cortex , cerebellum and hippocampus was observed in rats following exposure to both low and high dose of arsenic as compared to control (Fig. 5). Lipid Peroxidation was normalized in the animals supplemented with Vit-E along with low and high dose As-exposed animals.

RNA Expression levels of GPx:

The graph in Fig.6 depicts the mRNA Expression levels of GPx in cerebral cortex of rat brain. The GPx expression levels were significantly decreased in As-exposed rats (both in low and high dose). The effect was more pronounced in high dose As exposed animals. However, supplementation with Vit-E significantly reversed the As induced decrease in gene expression levels of GPx. The recovery in expression was more in low dose when compared to high dose As exposed animals.

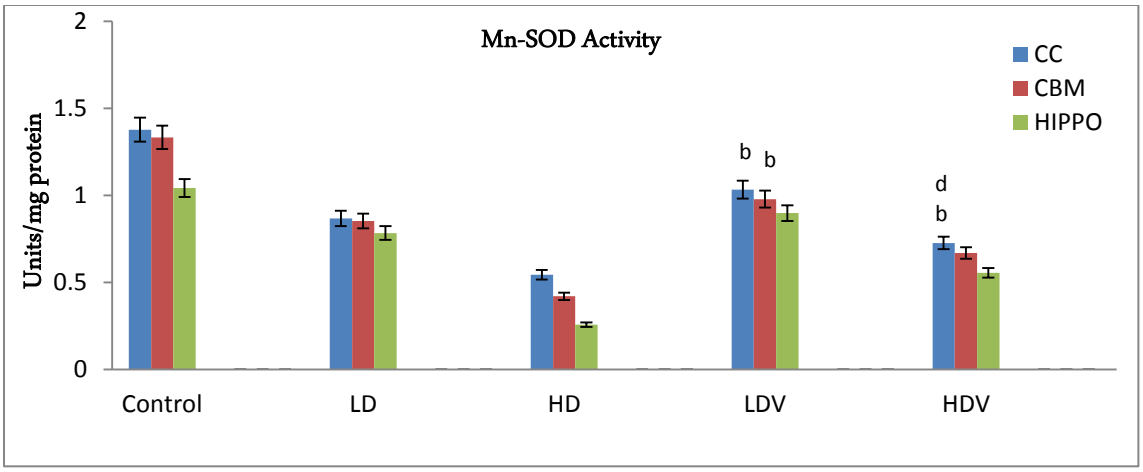

Fig 1:-Effect of arsenic on Mn -SOD activity in brain mitochondria of control and As-exposed rats to low dose (2.5mg/bw) and high dose (5mg/bw) through intraperitoneal injections for 3 weeks. The recovery of As toxicity is shown in two separate groups of albino rats exposed to As intraperitoneally low and high dose treated with Vit-E (5mg/bw) for a week. Each bar represents mean±SD (n=6) and statistical significance at $p<0.05$, except the values (bars) marked with a: compared with control, b: compared with LD, c: compared with HD, d: compared with LDV. CC- Cerebral cortex, CBM- Cerebellum, HIPPO- Hippocampus, LD- Low Dose, HD-High Dose, LDV- Low Dose+Vit-E, HDV-High Dose+Vit-E.

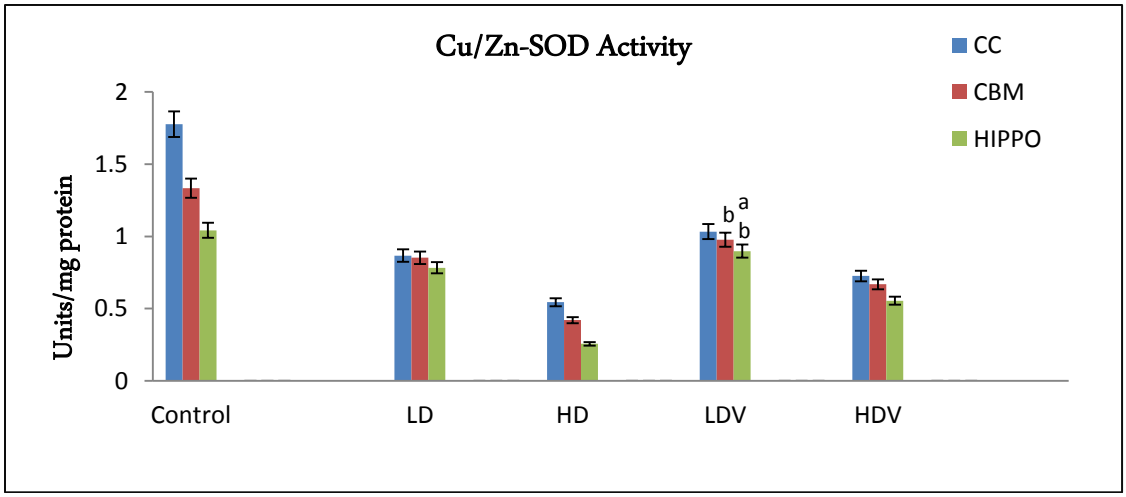

Fig 2:-Effect of arsenic on Cu/Zn -SOD activity in brain mitochondria of control and As-exposed rats to low dose (2.5mg/bw) and high dose (5mg/bw) through intraperitoneal injections for 3 weeks. The recovery of As toxicity is shown in two separate groups of albino rats exposed to As intraperitoneally low and high dose treated with Vit-E (5mg/bw) for a week. Each bar represents mean±SD (n=6) and statistical significance at $p<0.05$, except the values (bars) marked with a: compared with control, b: compared with LD, c: compared with HD, d: compared with LDV. CC- Cerebral cortex, CBM- Cerebellum, HIPPO- Hippocampus, LD- Low Dose, HD-High Dose, LDV- Low Dose+Vit-E, HDV-High Dose+Vit-E

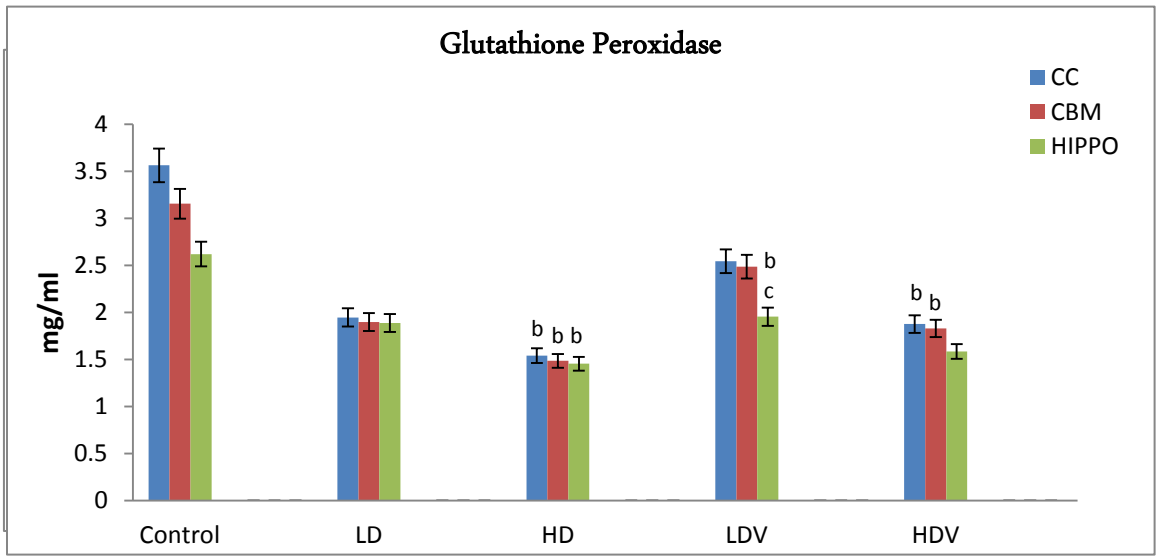

Fig 3:-Effect of arsenic on GPx activity in brain mitochondria of control and As-exposed rats to low dose (2.5mg/bw) and high dose (5mg/bw) through intraperitoneal injections for 3 weeks. The recovery of As toxicity is shown in two separate groups of albino rats exposed to As intraperitoneally low and high dose treated with Vit-E (5mg/bw) for a week. Each bar represents mean±SD (n=6) and statistical significance at $p<0.05$, except the values (bars) marked with a: compared with control, b: compared with LD, c: compared with HD, d: compared with LDV. CC- Cerebral cortex, CBM- Cerebellum, HIPPO- Hippocampus, LD- Low Dose, HD-High Dose, LDV- Low Dose+Vit-E, HDV-High Dose+Vit-E.

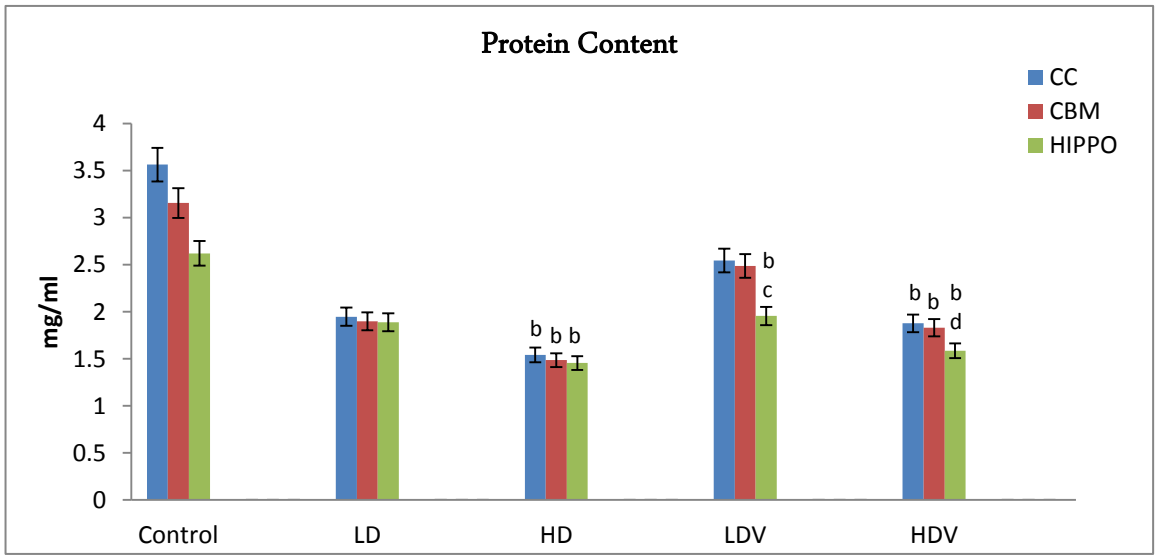

Fig.4: Effect of arsenic on protein content in brain mitochondria of control and As-exposed rats to low dose (2.5mg/bw) and high dose (5mg/bw) through intraperitoneal injections for 3 weeks. The recovery of As toxicity is shown in two separate groups of albino rats exposed to As intraperitoneally low and high dose treated with Vit-E (5mg/bw) for a week. Each bar represents mean±SD (n=6) and statistical significance at $p<0.05$, except the values (bars) marked with a: compared with control, b: compared with LD, c: compared with HD, d: compared with LDV. CC- Cerebral cortex, CBM- Cerebellum, HIPPO- Hippocampus, LD- Low Dose, HD-High Dose, LDV- Low Dose+Vit-E, HDV-High Dose+Vit-E.

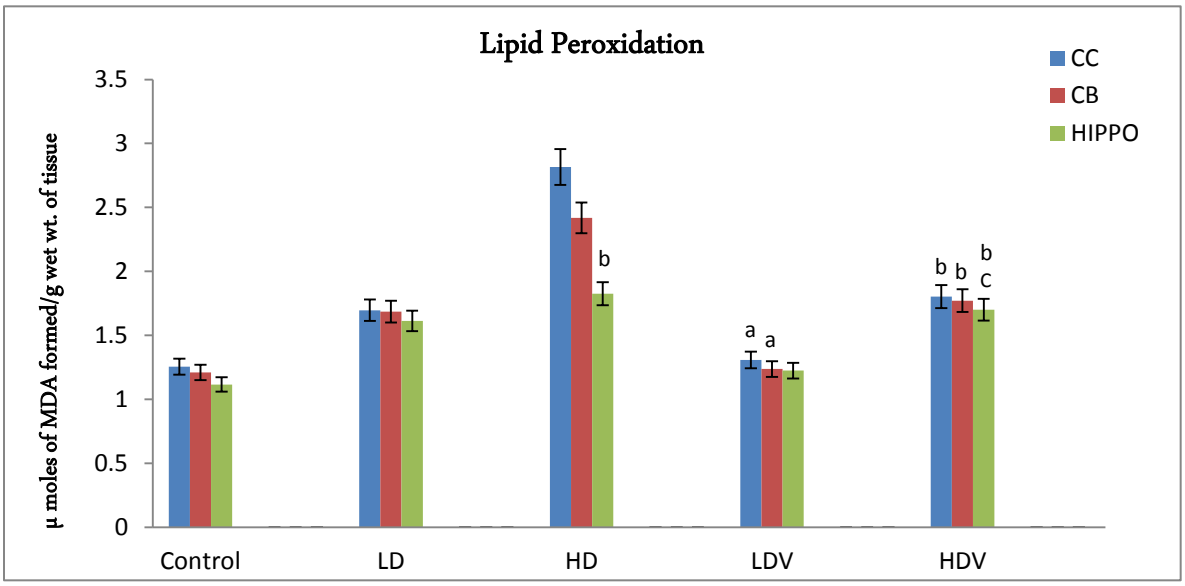

Fig. 5:-Effect of arsenic on lipid peroxidation in brain mitochondria of control and As-exposed rats to low dose (2.5mg/bw) and high dose (5mg/bw) through intraperitoneal injections for 3 weeks. The recovery of As toxicity is shown in two separate groups of albino rats exposed to As intraperitoneally low and high dose treated with Vit-E (5mg/bw) for a week. Each bar represents mean±SD (n=6) and statistical significance at $p<0.05$, except the values (bars) marked with a: compared with control, b: compared with LD, c: compared with HD, d: compared with LDV. CC- Cerebral cortex, CBM- Cerebellum, HIPPO- Hippocampus, LD- Low Dose, HD-High Dose, LDV- Low Dose+Vit-E, HDV-High Dose+Vit-E.

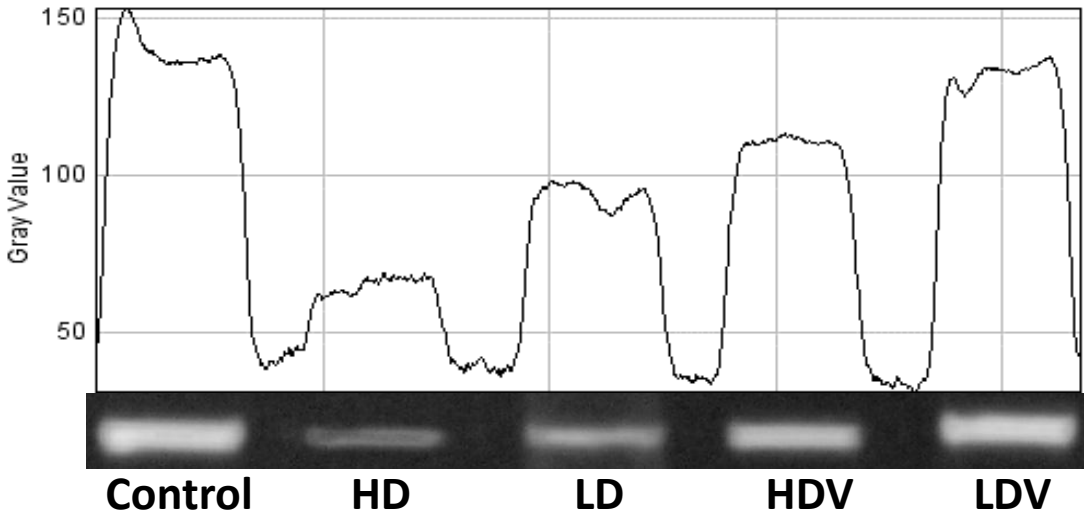

Fig.6:- Effect of arsenic on GPx gene expression levels in Cerebral Cortex in control and As-exposed rats to low dose (2.5mg/bw) and high dose (5mg/bw) through intraperitoneal injections for 3 weeks. Graph shows the recovery of As toxicity in two separate groups of albino rats exposed to As intraperitoneally low and high dose treated with Vit-E (5mg/bw) for a week. The graph depicts the density of amplicon bands through the grey value indicator.

Discussion:-

Enhanced oxidative stress has been suggested to be an important mechanism in arsenic induced neurotoxicity. Arsenic exposure has been found to cause oxidative damage to the biological system by enhancing generation of free radical species (Yamanaka et al., 1991, Flora et al., 2005) which, in turn, may be responsible for increased lipid peroxidation, protein carbonyl and decreased GSH levels (Flora et al., 2005, Shila et al., 2005a, b, c). Glutathione is an important biomolecule involved in the defense against toxicants (Nordmann, 1994). A decrease in the activity of superoxide dismutase, Catalase and GPx in the brain has been observed in arsenic exposed rats (Gupta et al., 2005; Gupta and Flora, 2006; Flora and Gupta, 2007; Shila et al., 2005a). In the study of R. S. Yadav et al., (2009), an increase in malanoldehyde and proteincarbonyl levels was observed in frontal cortex, corpus striatum and hippocampus regions of the brain in arsenic treated rats. Also, a decrease in glutathione levels and in the activity of superoxide dismutase, catalase and glutathione peroxidase observed in these brain regions is consistent with earlier reports and suggests enhanced oxidative stress following arsenic exposure in these rats. In the present study, our data confirm that chronic intoxication with arsenic causes significant increase of LPx concentrations in Cerebral cortex, Cerebellum and hippocampus of rats. Shila et al., (2005c) found that arsenic exposure in rats has pronounced effect on striatum, cortex and hippocampus.

Arsenic, along with several other heavy metals, generates reactive oxygen species (ROS) in developing rat brains (Rai, 2010). In another study, (Xi, 2010), sodium arsenite in drinking water was found to lead to the generation of ROS and subsequent lipid peroxidation in the brains of developing rat pups. In addition to demonstrating ROS generation, the pups' levels of the antioxidant glutathione (GSH) as well as the activity of the antioxidant enzyme glutathione peroxidase (GPx) were reduced following arsenic exposure. (Xi, 2010), GSH and GPx play essential roles in protecting an organism from oxidative damage; a reduction in these reduces the capacity of an organism to defend itself from the damage caused by ROS. In another study focusing on oxidative stress, rats administered arsenic in drinking water, even at the "permissible limit"(50mg/L, the national standard in Bangladesh), displayed increased lipid peroxidation, decreased GSH levels, and reduced superoxide dismutase and glutathione reductase activities in the brain, indicating free radical-mediated cellular degeneration (Chaudhuri et al., 1999).

In the present study, our results also corroborate with those studies, in this the SOD, Gpx activities were decreased in cerebral cortex and cerebellum. This is probably a consequence of the intracellular accumulations of ROS with subsequent development of cerebral cortex, cerebellum and hippocampus injury. Accumulation of As-SOD inhibition was highest in cerebral cortex followed by cerebellum, indicating a direct effect of As on SOD activity. This suggests a role of free radicals in causing cellular damage during long term exposure to arsenic. The decreased activity of GPx can be explained by competition of As-metallothioneins and GPx for sulphur containing amino acids. Studies of other authors have shown that As inhibits the activity of majority of enzymes involved in anti oxidative system inducing an increased production of free radicals, lipid peroxidation, and destruction of cell membranes. As also inhibits the activities of many enzymes by binding to their sulfhydryl groups or by inhibiting the protein synthesis.

In the present study, lipid peroxidation and protein content in different adult brain regions following arsenic exposure were decreased in rats co-administered with arsenic and Vit-E. Activity of superoxide dismutase, catalase and glutathione peroxidase, involved in the antioxidant defense were found increased with reversal effects in rats supplemented with arsenic and Vit-E as compared to those treated with arsenic alone. Vit-E has also been shown to protect developing neurons *in vitro* (Chattopadhyay et al., 2002). Our results suggest that Vit-E protects against the oxidative nervous damage caused by arsenite exposure. Treatment with Vit-E reduced oxidative damage, probably through its capacity to quickly and efficiently scavenge lipid peroxyl radicals before they attack membrane lipids. This ability might be related to the fact that lipid peroxyl radicals react more rapidly (by four orders of magnitude) with Vit-E than with membrane lipids, as suggested by Halliwell and Gutteridge (2002).

Conclusion:-

It can be concluded from the present study that arsenic accumulation in cerebral cortex and cerebellum of rats, due to intra peritonial injections of arsenic, is associated with marked alternations of enzymatic (SOD, GPx) and non-enzymatic compounds. Data suggest that lipid peroxidation was associated with arsenic toxicity in brain tissues, cerebral cortex, cerebellum and hippocampus. Our results showed that the nutritional antioxidant Vit-E ameliorated oxidative stress and loss of cellular antioxidants and suggested that Vit-E efficiently protect cerebral cortex, cerebellum and hippocampus from arsenic induced oxidative damage. This protection may be attributed to the

capability of Vit-E to alter the distribution of arsenic in tissues and to induce binding of the complexes to proteins, which are similar to metellothion.

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