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RESEARCH ARTICLE

Assessment of Alterations in Antioxidant Enzymes and Histology of Liver and Cerebral Cortex of Developing Chick Embryo in Acrylamide Toxicity

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Abstract

The acrylamide effect studied on antioxidants and histoarchitecture of 11th day old developing chick embryo liver and cerebral cortex that was exposed to 0.2 to 0.6mg concentrations at 72h incubation showed maximum superoxide dismutase and glutathione-S-transferase activities up to 0.4mg acrylamide treatment than control activity. These tissues catalase and glutathione peroxidase also showed a decrease in activity to minimum when embryo was administered with 0.6mg acrylamide than control. The decreased activities might be related to oxidative damage that occurs variably in these two tissues. The histological sections of tissues on microscopic analysis have showed mild degeneration, vacuole formation, necrosis and total damage in liver, and mild degeneration in cortex was found at 0.6mg acrylamide treatment. So our experimental results conclude that acryalmide was capable of producing major alterations in liver than in cerebral cortex of chick embryo at 72h incubation doses of 0.6mg acrylamide.

Introduction

Acrylamide (AC) is an alpha, beta unsaturated vinyl monomer of poly-acrylamide (Bohi et al., 2011), used extensively in the production of polyacrylamide for several uses (Gold and Schaumburg, 2000). It is used in water purification, making of cosmetics and paper, as a soil stabilizer and for the production of polyacrylamide gel to electrophoresis (Tareke et al., 2002). Growing evidence exists that some ingredients of preserved foods can induce epigenetic phenomena in genotoxic and nongenotoxic ways, with long-term effects on human health. Conversely, the short-term effects of harmful food components can cause structural damage to various systems and organs from the direct toxic effects that cause functional failure of tissues (Koletzko et al., 2010). Genotoxic and non-genotoxic pathways have been suggested for the carcinogenic effect of acrylamide. The possible nongenotoxic mechanisms of acrylamide may influence the redox status of cells and thus gene transcription be interfered with DNA repair or hormonal balance (Besaratinia and Pfeifer, 2007).

Acrylamide is produced in starchy foods that are baked, roasted or fried at high temperature (Rommens et al., 2008). Acrylamide concentration in fried potato chips are ranged from 376 to 2348 mg/ kg (Nojska et al., 2008). Potentially toxic acrylamide is largely derived from heat-induced reactions between the amino group of the free amino acid aspargine and carbonyl groups of glucose and fructose in cereals, potatoes, and other plant-derived foods (Friedman and Levin, 2008). The genotoxic and non-genotoxic action of acrylamide with special emphasis on DNA-adduct targeted mutagenesis, and in mechanistic data of various experimental systems including *in vitro* experiments and *in vivo* rodent and human studies with special focus on mouse models were known. Updated human exposure data, estimates of daily intake of dietary acrylamide in different populations, and the corresponding cancer risk assessments were studied well. The significant gaps in knowledge, which currently preclude a more definitive evaluation of human cancer risk due to exposure to dietary acrylamide future directions for

research on acrylamide and cancer are outlined, and potential challenges are underscored (Besaratinia and Pfeifer, 2007). Marsh et al., (2007) have observed both deficit and excess overall mortality risks among the U.S. cohort for cancer sites implicated in experimental animal studies: brain central nervous system, thyroid gland, testis, male genital organs respiratory system, esophagus, rectum, pancreas, and kidney.

Acrylamide is an important industrial chemical that is neurotoxic in rodents and humans, and carcinogenic in rodents. The observation of cancer in endocrine responsive tissues in Fischer 344 rats has prompted hypotheses of hormonal dysregulation, as opposed to DNA damage, as the mechanism for tumor induction by acrylamide (Marsh et al., 2007). Three developmental toxicity studies have reported in rats, on developmental neurotoxicity based on endocrine dysfunction. Other early studies on embryo development in rodents have demonstrated that acrylamide induces cumulative neurotoxicity linked to nerve terminal damage in the CNS where distal axon swelling and subsequent to be the hallmark degeneration was considered, as morphological features of this toxic axonopathy (Lehning et al., 1998). At the molecular level, it has been postulated by Carlson and Weaver (1985) that covalent binding of acrylamide to CNS proteins may play an important role in acrylamide toxicity, resulting in inhibition of a number of enzymes and essential compounds. Considering the endocrine and neurological influences of acrylamide in rodents. The aim of present study was to evaluating the alteration levels of antioxidants, histological observation of hepatic and brain damages by the Acrylamide in chick embryo during its development. The experiments conducted on this study and results achieved were discussed as below.

Material and Methods

The chemicals purchased from indigenous companies were of pure and used for the analysis of various samples of our research.

Source of fertilized eggs and incubation conditions

Freshly laid *Bobcock strain* zero day old fertilized eggs were purchased from Sri Venkateswara Veterinary University, Tirupati and Sri Balaji hatcheries, Chittoor, Andhra Pradesh. The eggs were incubated horizontally at 37.5±0.5˚C with a relative humidity of 65% in an egg incubator, we consider day1 (d1) as an incubation period of 24h. the egg incubation and collection of tissues were made based on the experimental condition of Ruxana Begum and Thyaga Raju, (2010). The embryos were treated with 0.2mg, 0.4mg and 0.6mg of acrylamide as single dose and incubated for 72h. The tissues were collected after 72hof incubation and stored at -20º C until further use and a piece of tissue was collected for histological studies.

Tissue processing for assay of antioxidant enzymes

Normal and treated chick embryo liver, and brain were thawed slowly, minced with scissors and homogenized in 50 mM Tris-HCl buffer, pH 8.0, containing 0.25M sucrose and 1mM PMSF using a glass homogenizer. The homogenate was passed through two layers of cheese cloth to remove fat and the filtrate was centrifuged at 12000rpm on high speed refrigerated centrifuge (Remi) for 30minutes. The resulted supernatant was used as an enzyme source. All the purification procedures were conducted at 4^{o}C unless otherwise stated.

Protein determination

In all the enzyme preparations protein was determined by the method of Lowry et al., (1951) using bovine serum albumin (BSA, Sigma) as standard.

Superoxide dismutase (SOD) assay

The activity of SOD was assayed according to Misra and Fridovich (1972). The assay mixture in a total volume of 3 ml contained 50 mM sodium carbonate/bicarbonate buffer (pH 9.8), 0.1 mM EDTA, 0.6 mM epinephrine and enzyme. Epinephrine was the last component to be added. The adrenochrome formation in the next 4 min was recorded at 475 nm using UV-Vis spectrophotometer. One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions.

Catalase (CAT) assay

In partial modification to above the tissues were homogenized in 50 mmol/L potassium phosphate buffer (pH 7.4) with a weight-to-volume ratio of 1:10. The homogenate was centrifuged at 40, 000x*g* for 30 minutes. Supernatant (50µl) was added to a cuvette containing 2.95µl of 19 mmol/L H_2O_2 solution prepared in potassium phosphate buffer (Aebi et al., 1984). The disappearance of H_2O_2 was monitored at 240 nm wavelength at 1-minute intervals for 5 minutes. Specific activity of the enzyme was expressed as mmol of H_2O_2 consumed /mg protein.

Glutathione peroxidase (GPx) assay

To 2.4ml of 75mM phosphate buffer, p^H 7.0, in a cuvette added 50µl of 60mM GSH, 10µl of glutathione reductase (1Unit), 50µl of 120mM sodium azide, 100µl of 15mM sodium EDTA, 100µl of NADPH, 100µl enzyme and made up to 2.9µl using distilled water. The reaction was initiated by the addition of 100µl of substrate, H_2O_2/CHP. GPx activity was expressed as nanomoles of reduced NADPH oxidized to NADP per minute per milligram protein (Paglia and Valentine, 1967).

Glutathione-S- Transferases (GSTs) assay

GST activity was assayed spectrophotometrically as described by Habig et al., (1974) to measure the rate of conjugation of GSH to CDNB, a standard substrate. The cuvettes (final volume of 3.0 ml) contained 1 mM (CDNB) and 5 mM (GSH) in 0.1 M sodium phosphate buffer, pH 6.5. Reagents and 1 mM of either substrate or suitable aliquots (usually 20 µl) of appropriately diluted enzyme from the different sources were prepared in fresh and used for assay. The reaction rates were measured at 340nm for 5minutes. The GST activity per individual was calculated in mmol CDNB conjugated/min/mg using the published extinction coefficient of 9.6 and 8.5 for CDNB. Specific activity was calculated by correcting for protein content.

Preparation of slides for histopathology

The developing brain and liver tissues of d11 chick embryo control and treated were dissected and they were gently rinsed with physiological saline to remove blood and debris adhering to them. They were cut into pieces and fixed in Bouin's solution until processing. The tissues were washed with running tap water, overnight to remove Bouin's solution. After dehydrating through a graded series of alcohols, the tissues were cleared in methyl benzoate and embedded in paraffin wax. Sections were cut at 6µ thickness and stained with haematoxylin (Harris et al., 1900) and counter stained with Eosin dissolved in 70% alcohol. After dehydration and cleaning, sections were mounted in Canada balsam. Histological examinations of the tissues were followed according to Humason, (1972) and the specimens were observed under the light microscope. Photomicrographs were taken by Ricoh 35 mm SLR camera.

Statistical Analysis

All results obtained were expressed as the mean±SD. For a statistical analysis of the data, group means were compared by one-way ANOVA, and Duncan's test was used to identify differences between groups. The statistical differences were assessed between the control and acrylamide-treated developing chick embryo by an Independent-sample t-test (Graphpad PRISM version 5; Graphpad Software Inc., San Diego, CA, USA).

Result

SOD activity

The SOD activities of liver and cerebral cortex of brain are represented in **Figure-1**. Our results SOD activity showed on a reduced activity with an increasing doses of acrylamide ($p<0.0001$), in liver and cerebral cortex of brain when chick embryo was administered with acrylamide ($p<0.0001$). The liver and brain cerebral cortex SOD activity is in 0.2 and 0.4mg acrylamide treated chick embryos were increased and extremely significant when compared to control tissue samples. However the decreased activities of SOD were found in both tissues.these results have no significant difference in 0.6mg acrylamide treated embryonic liver and cerebral cortex compared to control.

Catalase activity

The activities of catalase liver and cerebral cortex of brain are represented in **Figure-2**. Liver and brain CAT showed lowest activity than the control when chick embryo was administered with 0.6mg acrylamide ($p<0.0001$). A significant decrease of catalase activity was found upon increasing doses of acrylamide treatment to chick embryos.

GPx activity

Figure-3 represents the activities of GPx in liver and cerebral cortex of brain. Our results showed that GPx activity was reduced with increasing doses of acrylamide, in liver and brain when chick embryo was administered with 0.6mg acrylamide ($p<0.0001$) in comparison with control. Liver and cerebral cortex GPx activity in 0.2, 0.4 and 0.6mg acrylamide treated chick embryo were 1.46, 2.05, 2.25, 1.08, 1.24, and 1.70 fold decreased when compare to controls. While there is no significant difference ($p<0.32$) in 0.2mg acrylamide treated embryonic cerebral cortex compared to control and significant difference ($p<0.04$, 0.002) in 0.4 and 0.6mg treated cerebral cortex brain.

GST activity

The activities of liver and cerebral cortex brain GSTs are represented in **Figure-4**. From this–it is known that the liver and brain GSTs showed maximum activity when chick embryo was administered with 0.2 to 0.4mg acrylamide ($p<0.0022$) in comparison with control. Liver GST activity in 0.4 and 0.6mg acrylamide treated chick embryo were very

significant when compared to enzyme activity in controls.

Histological Results

Examination of liver sections of the different groups illustrated that: Liver tissue of the normal group showed hepatic lobules with normal architecture **(Figure-5a**). In case of liver sections of chick embryo administrated with 0.2, 0.4 and 0.6mg acryamide with 72h incubation, showed Mild degenerative changes, such as formation of vacuole, and damage of liver central veins (**Figures-5b-d**).

Brain

Figure-6a showing the cerebral cortex of control indicates the normal architecture of developing chick embryo as well as 0.2 and 0.4mg acrylamide-treated embryos showed the same histological structure consisting of molecular layer **(Figure-6b-c)** Purkinje cell layer and granular layer. Where in 0.6mg acrylamide treated has mild distortion of brain tissue indicates degeneration in Purkinje cell layer of cerebral cortex **(Figure-6d)**.

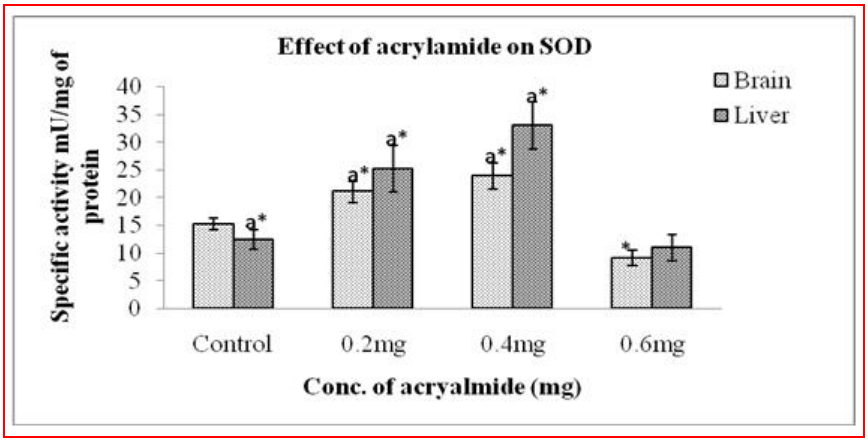

Figure 1. Effect of acrylamide on SOD activity in liver and cerebral cortex of brain of chick embryo. The enzyme activity is expressed as micromoles of epinephrine unit/mg protein. The statistical differences were assessed between the control and acrylamide-treated developing chick embryo by an Independent-sample t-test, P> 0.01 not Significant, $^{*}P \leq 0.01$ Significant, $^{**}P \leq 0.001$ More Significant, and $^{a*}P \leq 0.0001$ Extremely Significant.

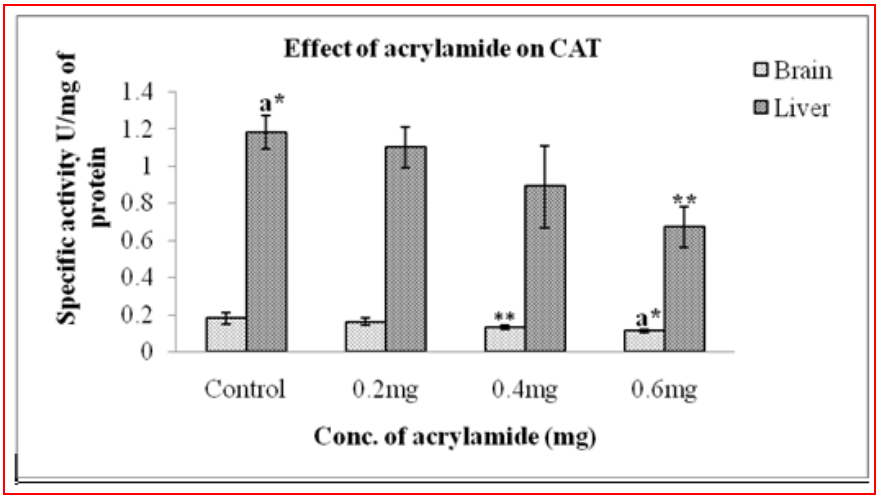

Figure 2. Effect of acrylamide on Catalase activity in liver and cerebral cortex of brain chick embryo. The enzyme activity is expressed as micromoles of H_2O_2 consumed per minute and milligram of protein. The statistical differences were assessed between the control and acrylamide-treated developing chick embryo by an Independent-sample t-test, P> 0.01 not Significant, $^{*}P \leq 0.01$ Significant, $^{**}P \leq 0.001$ More Significant, and $^{a*}P \leq 0.0001$ Extremely Significant.

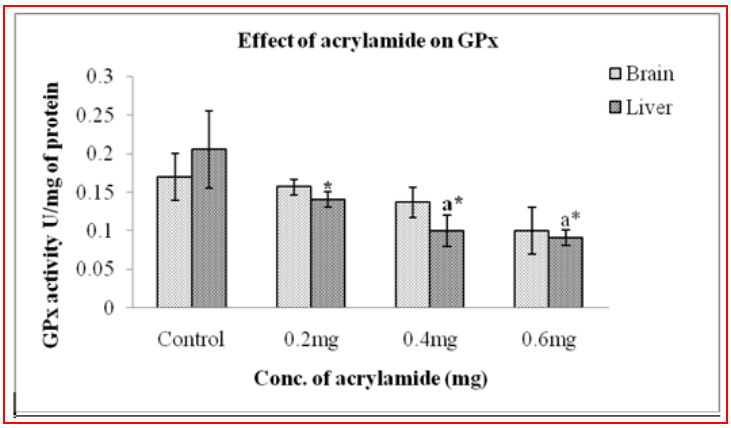

Figure 3. Effect of acrylamide on GPx activity in liver and cerebral cortex brain chick embryo. The enzyme activity is expressed as nanomoles of NADPH oxidized /minute/ milligram protein. The statistical differences were assessed between the control and acrylamide-treated developing chick embryo by an Independent-sample t-test, P> 0.01 not Significant, $^{*}P \leq 0.01$ Significant, $^{**}P \leq 0.001$ More Significant, and $^{a*}P \leq 0.0001$ Extremely Significant.

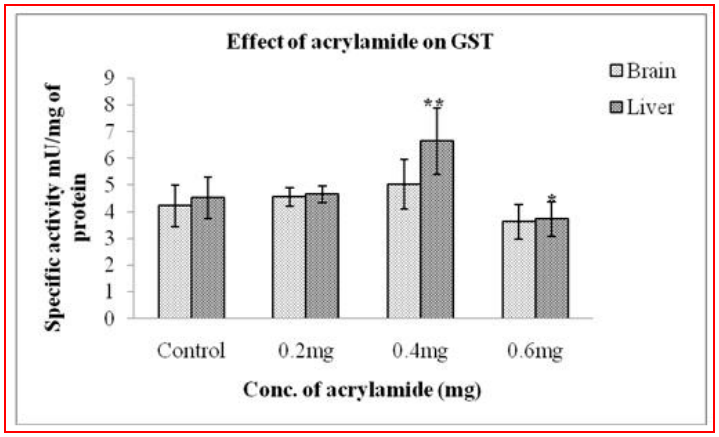

Figure 4. Effect of acrylamide on GST activity in liver and cerebral cortex brain of chick embryo. The enzyme activity is expressed as µmoles of GS-DNB conjugate formed per minute per mg protein. The statistical differences were assessed between the control and acrylamide-treated developing chick embryo by an Independent-sample t-test, P> 0.01 not Significant, $^{*}P \leq 0.01$ Significant, $^{**}P \leq 0.001$ More Significant and $^{a*}P \leq 0.0001$ Extremely Significant.

Figure 5a

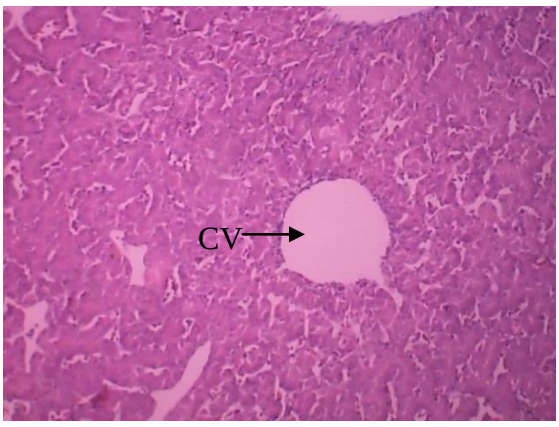

Figure 5b

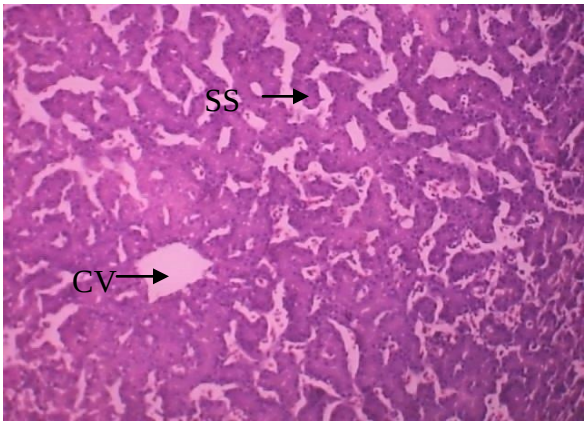

Figure 5c

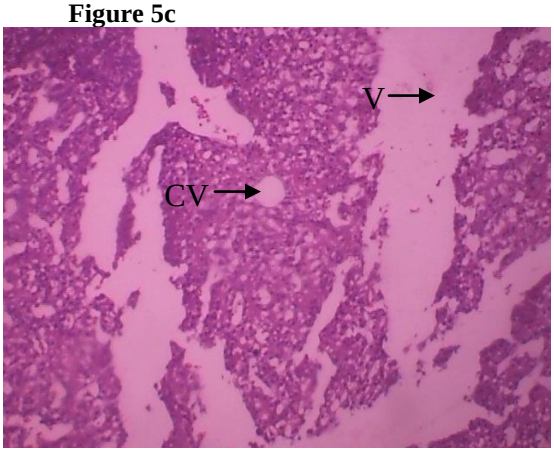

Figure 5d

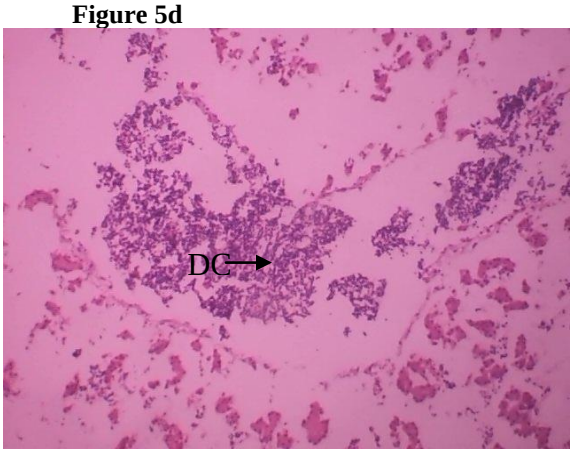

Figure 5a. Liver of 11-day old chick embryo showing normal cytoarchitecture (Control). **5b).** Section in liver of chick embryo with acrylamide dose of 0.2mg showing Mild degenerative changes at bottom arrow Central Vein (CV), and top arrow Sinusoids Space (SS). **5c).** liver of chick embryo with acrylamide dose of 0.4mg showing degeneration of tissue shows formation of vacuole at Central Vein (CV) and Vacuolization (V). **5d).** liver of chick embryo with acrylamide dose of 0.4mg showing degeneration of tissue indicates complete damage of liver with 10X.

Figure 6a

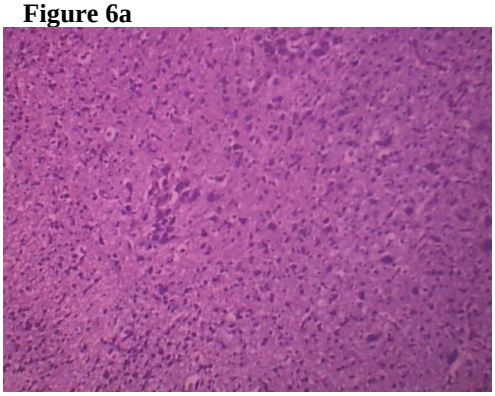

Figure 6b

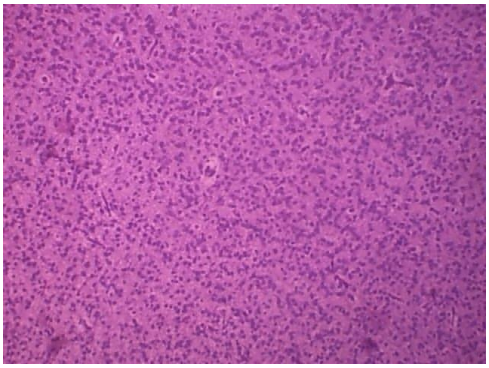

Figure 6c

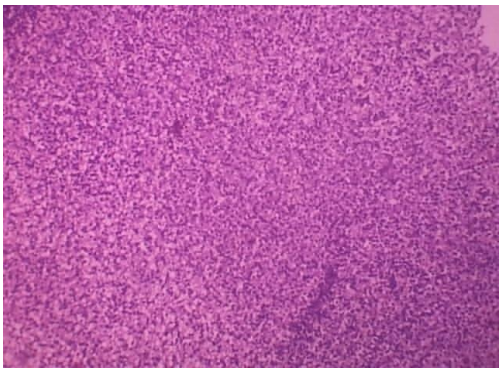

Figure 6d

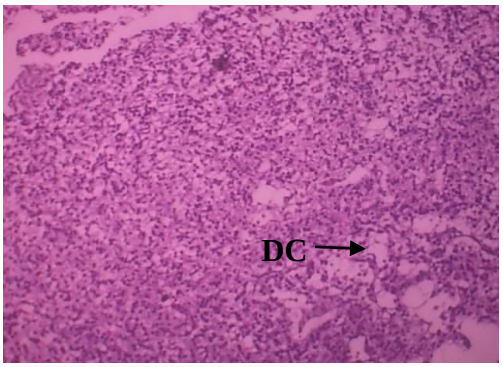

Figure 6a. Brain of Cerebral cortex of 11-day old chick embryo showing normal cytoarchitecture (Control). **6b and c).** Cerebral cortex shows similar to normal architecture as control of chick embryo with acrylamide doses of 0.2mg and 0.4mg. **6d).** Cerebral cortex of chick embryo with acrylamide dose of 0.6mg showing mild distortion of cerebral cortex region indicates degeneration with 10X.

Discussion

The oxygen radical formation is damage to an array of biomolecules found in tissues, including nucleic acids, membrane lipids, enzymes, and receptors (Iseri et al., 2008). Cells are able to defend themselves from damaging effects of oxygen radicals by way of their own antioxidant mechanisms, including enzymatic and non-enzymatic systems (Sehirli et al., 2008). The SOD, an antioxidant enzyme of cells rapidly converts superoxide anion (O_2^-) to less dangerous hydrogen peroxide (H_2O_2) and then GPx and CAT can decompose H_2O_2 to water (Fatema et al., 2005). Although, H_2O_2 is not a particularly reactive product, it may be reduced to the highly reactive metabolites hydroxyl radicals and/or singlet oxygen. The present study showed that the treatment of acrylamide to developing chick embryo has resulted in several hepatic and cerebral neurological manifestations as loss of metabolic equilibrium. Thus it is assumed that exposure acrylamide increases the risk in the formation of mutations and cancers. On the other hand signs of acrylamide toxicity in animals exposed for longer periods of time (months to year) were gastrointestinal bleeding, respiratory distress syndrome, hepatotoxicity and peripheral neuropathy (Achim et al., 2008). The steady weight gain due to inflamation in groups treated with acrlamide possibly may also due to organs failure and shrinkage in subsequent toxicity by compounds investigated (Hamaoka and Kusunok, 1986). Examination of control liver sections of male rats pronounced large extent of corresponds with liver structure previously mentioned in rodents and in other mammals (Junqueira and Carneiro, 2005; Hummdi and Habashi, 2010)**.** The most marked signs of liver tissue impairment and vacuolations in the present study were observed. These results agree with (Bhattacharya et al., 2011) work, were it was noted the vacuolations of hepatocytes were more pronounced around the central vein in the developing chick embryo injected with acrylamide for 72h. In addition, acrylamide treatment induced vacuolations and necrosis in liver hepatocytes of chick embryos at dose of 0.1mg/kg (Kedam et al., 2012). Cheville, (2009) reported that centrolobular hepatocytes are typically the primary site of toxic injury; they have more surface receptors for toxins and less oxygen. Abdel Hameed, (2004) described the vacuolation of hepatocytes as ballooning degeneration and interpreted it as a kind of cellular defensive mechanism against injurious substances. Cheville, (2009) added that these vacuoles are responsible for collecting the injurious elements and preventing them from interfering with the biological activities of these cells.

The increased activity of SOD could lead to overproduction of hydroperoxides; in consequence, GPx might be stimulated in response to the accumulated peroxides. The results of the present study revealed significant decreased in hepatic and cerebral cortex of brain anti oxidant enzymes activity in chick embryos treated with acrylamide **Figure-1-4**, the damage effects of acrylamide are mainly related to its physiological antioxidant properties, and hence, increased generation of ROS and RNS. Thus, acylamide administration could decrease the activity of SOD, CAT, GPx and GST enzymes responsible for the loss of detoxification of such free radicals. Acrylamide induced the depletion of glutathione and ascorbic acid levels in hippocampus region of chick brain, (Thyaga Raju et al., 2013) induce hepatocyte, oxidative damage and thus decreses of liver enzymes as in the cases of cadmium hepatotoxicity (Newairy et al., 2007). This biochemical result is supported by the histopathological examination of liver sections of the different groups which illustrated that, in the liver and brain sections of chick embryo administrated acrylamide.

Conclusion

Based on the obtained results, acryalmide is capable of producing alterations in antioxidant enzymes, extremely in the liver and cerebral cortex tissues when consumed in high doses. In conclusion, the decreasing antioxidant levels might be related to oxidative damage that occurs variably in both liver and cerebral cortex. Acrylamide administration produced partial, but significant, however, such genotoxic compound induced hepatic damage, and mild damages in cerebral cortex as evidenced by the biochemical measurements and histopathological examinations of the hepatic and cerebral cortex of brain tissues. It is recommended that further research has to be carried out in order to investigate the effects of acrylamide on specific organ tissues.

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