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Toxic Effect of Acrylamide on Body Weight, the Study of Antioxidants and Histoarchitecture of Heart in the Developing Chick Embryo

KEYWORDS: Acrylamide, body weight, developing chick embryo, antioxidants, histology, heart

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ABSTRACT *Acrylamide, used in many fields, from industrial manufacturing to laboratory work, is also formed during the heating process through the interactions of amino acids. Therefore, acrylamide poses a significant risk for both human and animal health. The present study carried out to investigate the toxic effects of acrylamide in alterations of cardiac, total body weight, antioxidant enzyme activities and histoarchitecture of heart, at the end of experiment (after 72h) the tissues were collected from the 11th day (d11) developing chick embryos exposed to different doses of acrylamide toxicity. The present results showed that catalase, superoxide dismutase, glutathione peroxidase, and glutathione-S-transferase enzymatic activities were reduced with increasing doses of acrylamide, the decreased activities antioxidant enzymes levels in discrete regions of heart indicating oxidative stress and inhibited antioxidant defense. However low doses of acrylamide significantly increases the heart and total body weights and increased the doses of the acrylamide induces the loss of heart and body weights. Light microscopic examination revealed severe structural changes in acrylamide-treated embryos. The dose of 0.2mg tissue showing mild degeneration at myocardial fibers, 0.4mg showing tissue degeneration with necrotic and degenerative changes and dose of 0.6mg showing collapsed myocardial fibers indicates degeneration of tissue. These findings concluded that high doses of acryalmide toxicity induced the oxidative stress, loss of heart and total body weight and collapsed myocardial fibers.*

Introduction

Acrylamide (H2C=H–CO–NH2) is a highly reactive water soluble, vinyl monomer, and has multiple chemical and industrial applications used extensively in electrophoretic techniques (Friedman 2003) and in molecular laboratories for gel chromatography used as a chemical intermediate in the production of N-methylol acrylamide and N-butoxy acrylamide, and as a superabsorbent in disposable diapers, medical products, and agricultural products. Small amounts of acrylamide are also used in sugar beet juice clarification, adhesives, binders for seed coatings and foundry sand, printing ink emulsion stabilizers.

The worldwide concern about acrylamide toxicity began in 2002 when the Swedish National Food Administration announced that high concentrations of acrylamide are present in some food products. Acrylamide is produced in starchy foods that are baked, roasted or fried at high temperature (Rommens et al., 2008). After degradation, highly reactive compounds like furfural, reductones, acetol or pyruvaldehydes condense with free amino groups forming aldehydes and a-aminoketones. Mottram et al., (2002) have established that –NH2 groups of asparagine and methionine in a presence of dicarbonyls from the Millard reaction are the main substrates for acrylamide synthesis. However, Zyzak et al., (2003) have revealed that rather carbonyls are required for acrylamide formation from aspargine instead of dicarbonyls. Polyacrylamide is not a toxic agent whereas its monomer has been reported to be neurotoxic, genotoxic and carcinogenic in rodents (Park et al., 2002). Manna et al., (2006) and Mogda et al., (2008) studied the oxidative stress caused by acrylamide like affection of lipid peroxidation as well as reduction of glutathione (GSH) and catalase enzyme.

Recently, oxidative stress has been demonstrated to be one of the key mechanisms in many chemical-induced cell injuries. Oxidative stress in the cells or tissues refers to enhanced generation of ROS and/or depletion of antioxidant defense system, causing an imbalance between prooxidants and antioxidants, potentially leading to damage and the role of reactive oxygen species (ROS) in disease and toxicity have been two major issues in biomedical sciences (Omurtag et al., 2005 and Omurtag et al., 2008). Free radicals are continuously produced in vivo and there are number of protective antioxidant enzymes (superoxide dismutase, catalase, glutathione S-transferase, glutathione peroxidase, and antioxidant glutathione) for dealing with these toxic substances. The delicate balance between the production and catabolism of oxidants is critical for maintenance of the biological function (Sridevi et al., 1998). Changes in the embryonic development occurring under a moderate excess of oxygen in medium indicate that metabolic processes under these conditions are not disturbed. Energy metabolism in embryos is disturbed only under a large excess of oxygen. Developmental delay in this case is believed to be determined by inactivation of some respiratory enzymes, inhibition of some cofactors of these enzymes, and suppression of oxidative phosphorylation. During embryonic development, simultaneously with growth and differentiation of the embryo, its energy metabolism develops, which is expressed in the complication of oxygen consumption pathways. The aim of the present study was to examine effects of acrylamide on the antioxidants defense system, cardiac, total body weight, and histopathological changes in developing chick embryo.

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Materials and Materials
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 humidity of the incubator was maintained by keeping the trough full of water inside. The water was replaced for every alternate day and the water level was maintained to keep the same percentage of humidity throughout the incubation. Eggs were rotated manually four times a day and were examined through the Candler every day for the proper growth and viability. The dead eggs were removed immediately from the incubator. During all experiments, the embryos were maintained at 37±0.5˚C except for brief intervals (60-120 seconds) required during the different treatment conditions (Thyaga Raju et al., 2013). During this interval embryos experienced ambient room temperature (29-30˚C).

Tissue processing for assay of antioxidant enzymes
Normal and treated chick embryos heart 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 resulting supernatant was centrifuged at 35000 x g on high speed refrigerated centrifuge (Remi) for 30 minutes. The resulted supernatant was used as the enzyme source. All the purification procedures were conducted at 4oC unless otherwise stated.

Protein determination
In the entire 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 in a 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
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 000g for 30 minutes. Supernatant (50 mL) was added to a cuvette containing 2.95 mL of 19 mmol/L H2O2 solution prepared in potassium phosphate buffer (Aebi 1984). The disappearance of H2O2 was monitored at 240 nm wavelength at 1-minute intervals for 5 minutes. Specific activity of the enzyme was expressed as mmol/mg protein.

Glutathione Peroxidase (GPx) assay
GPx activity was expressed as nanomoles of reduced NADPH oxidized to NADH per minute per milligram protein (Rotruck et al., 1973). Cytosolic GPx was assayed in a 3-ml cuvette containing 2.4 ml of 75 mM phosphate buffer (pH 7.0). The following solutions were then added: 50 ml of 60 mM reduced glutathione, 100 ml of glutathione reductase (30 U), 50 mL of 120 mM NaN3, 100 ml of 15 mM Na2EDTA, 100 ml of 3.0 mM NADPH, and 100 ml of cytosolic fraction obtained after centrifugation of the heart homogenate at 20000g for 25minutes. The reaction was initiated by the addition of 100 ml of 7.5 mM H2O2, and the conversion of NADPH to NADP was assayed by measuring the absorbance at 340 nm at 1-minute intervals for 5 minutes.

Glutathione-S- Transferases (GSTs)
GST activity was assayed spectrophotometrically essentially as described by Habig et al., (1974) to measure the rate of conjugation of GSH to CDNB. 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 were prepared fresh prior to use and 1 mM of either substrate or suitable aliquots (usually 20 µl) of appropriately diluted enzyme from the different sources. The reaction rates were measured at 340 nm for 5 min.

Preparation of slides for histopathology
The developing heart tissue of d11 chick control and treated was 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 (Harries 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 are presented 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 assed 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).

Results
Antioxidant enzyme activities
The activities of heart SOD represented in Figure-1-. Our results showed that SOD activity was reduced with increasing doses of acrylamide ($p<0.0008$ and 0.0001). The SOD activity 31.83, 90.34, and 142.85% were decreased in 0.2, 0.4 and 0.6mg acrylamide treated chick embryo compared with control. While there is extremely significant difference in acrylamide treated embryos ($p<0.0001$) compared to control. The CAT activities were represented in Figure-2. The results showed that heart CAT activity was reduced with increasing doses of acrylamide. The CAT activities 8.33, 149.31, and 243.39% decreases were observed in treated embryos compared with control. The 0.4 and 0.6mg treated are extremely significant decreased with control, while there is no significant 0.2mg with control.

The activities of heart GPx was represented in Figure-3 and the results showed that GPx activity was reduced with increasing doses of acrylamide, the GPx showed lowest maximum activity when chick embryo was administered with 0.6mg acrylamide in comparison with control. The GPx activity in 0.2, 0.4 and 0.6mg acrylamide treated chick embryo were 0.190, 0.125, and 0.095. While there is very significant difference ($p<0.01$ and 0.002) in 0.2 and 0.4mg acrylamide treated embryo compared to control and significant difference ($p<0.001$) 0.6mg treated heart with control. The Figure-4 represents the GST activities; the GST activity was reduced with increasing doses of acrylamide. There is very and extremely significant decreases were observed ($p<0.001$ and 0.0009) in 0.4 and 0.6mg acrylamide treated compared with control. While there no significant in 0.2mg with control.

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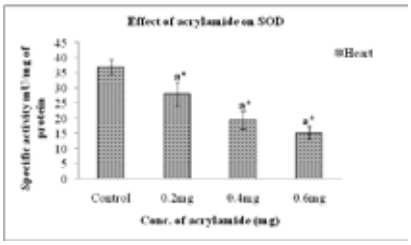

Figure-1: Effect of acrylamide on heart of chick embryonic SOD activity. The enzyme activity is expressed as micro unit/ mg protein. The statistical differences were assed between the control and acrylamide-treated developing chick embryo by an Independent-sample t-test, P> 0.01 not Significant, *P ≤ 0.01 Significant **P ≤ 0.001 More Significant, and a*P ≤ 0.0001 Extremely Significant.

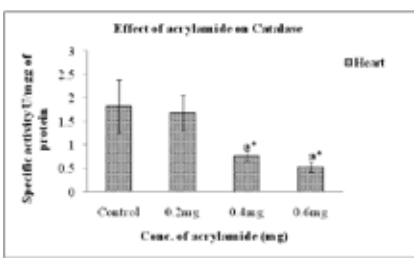

Figure-2: Effect of acrylamide on heart of chick embryonic CAT activity. The enzyme activity is expressed as micromoles H2O2 consumed per minute and milligram of protein. The statistical differences were assed between the control and acrylamide-treated developing chick embryo by an Independent-sample t-test, P> 0.01 not Significant, *P ≤ 0.01 Significant, **P ≤ 0.001 More Significant, and a*P ≤ 0.0001 Extremely Significant.

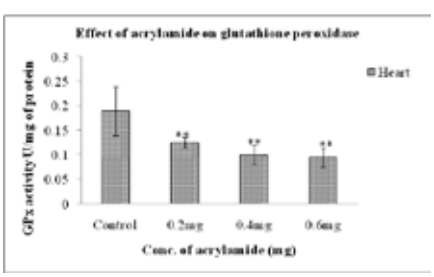

Figure-3: Effect of acrylamide on heart GPx activity in chick embryo. The enzyme activity is expressed as nanomoles NADPH oxidized /minute/ milligram protein. The statistical differences were assed between the control and acrylamide-treated developing chick embryo by an Independent-sample t-test, P> 0.01 not Significant, *P ≤ 0.01 Significant, **P ≤ 0.001 More Significant, and a*P ≤ 0.0001 Extremely Significant.

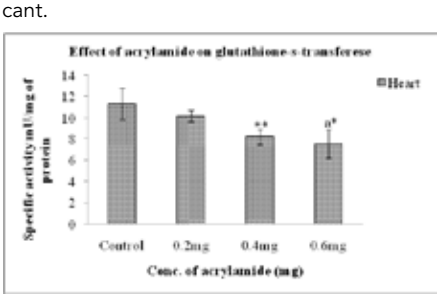

Figure-4: Effect of acrylamide on heart GST activity in chick embryo. The enzyme activity is expressed as µmoles of CDNB-GSH conjugate formed per minute per mg protein. The statistical differences were assayed between the control and acrylamide-treated developing chick embryo by an Independent-sample t-test, P> 0.01 not Significant, *P ≤ 0.01 Significant, **P ≤ 0.001 More Significant and a*P ≤ 0.0001 Extremely Significant.

Total body and heart weight

Table-1 represents the body and heart weight of the developing chick embryo. The results showed that the total body and heart weights were increased with initial doses of the acrylamide. The maximum weight of heart and total body when the chick embryo was administered with 0.4mg acrylamide compared with control. There is extremely significant increases were observed (p<0.001) in 0.4mg acrylamide treated in both heart and total weight of embryo compared with control. While there no significant in 0.2 and 0.6mg with control.

Table-1: Total body and heart weight of 11th day old developing chick embryo

Treatment (in mg)	Control	0.2mg	0.4mg	0.6mg
Total body weight (g)	31.41±1.08	32.75±1.09	37.64±1.91a*	31.25±2.35
Heart weight (g)	0.201±0.01	0.218±0.00*	0.273±0.01a*	0.197±0.02

The statistical differences were assed between the control and acrylamide-treated developing chick embryo by an Independent-sample t-test, P> 0.01 not Significant, *P ≤ 0.01 Significant, **P ≤ 0.001 More Significant and a*P ≤ 0.0001 Extremely Significant.

Histological Results:

Cardiac histopathological results showed that; in the control sections (Figure-5a), the cardiac muscle fibers were grouped in bundles with connective tissue in between. Figure-5b and 5c are the cardiac sections obtained 11-day old developing chick embryo administrated with 0.2, 0.4mg of acrylamide, tissue showing mild degeneration at myocardial fibers, tissue degeneration with necrotic and degenerative changes and heart of chick embryo with acrylamide dose of 0.6mg showing collapsed myocardial fibers indicates degeneration of tissue with 10X (Figure-5d).

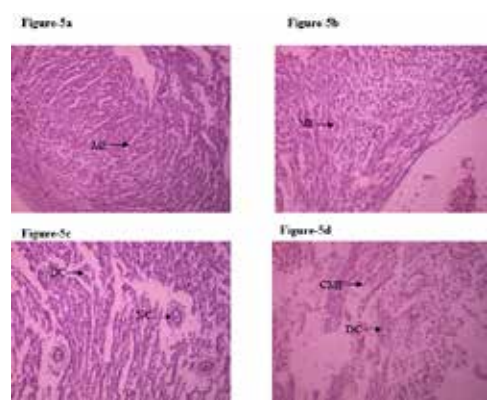

Figure-5a: Heart of 11-day old chick embryo showing normal cytoarchitecture (Control). 5b): Sections with acrylamide dose of 0.2mg tissue showing mild degeneration at myocardial fibers. 5c): Heart with acrylamide dose of 0.4mg showing tissue degeneration with necrotic and degenerative changes. 5d): Heart with acrylamide dose of 0.6mg showing collapsed myocardial fibers indicates degeneration of tissue with 10X.

Discussion

Most dry pet foods contain cereal grains or potatoes, and are processed at high temperatures and high pressure, making acryalmide formation possible. On the other hand, the content and potential effects of acrylamide formation in pet foods are unknown. The spontaneous formation of AA during the cooking of food has led to its description as a cooking carcinogen (Tareke et al., 2002). In addition to the evidence of AC mutagenicity [Besaratinia and Pfeifer, 2003), genotox-

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icity (Dearfield et al., 1988) and carcinogenicity (Hogervorst et al., 2007) have also been reported. AC therefore poses a potentially significant risk both for human and animal health. In light of the concern expressed by various European Union (EU) member states and of the public alarm, a consultation was convened by FAO/WHO in 2002. The consultation recognized the presence of acrylamide in food "as a major concern in humans based on the ability to induce cancer and heritable mutations in laboratory animals". Based on the data available at that time, food was estimated to make a significant contribution to total exposure of the general population to acrylamide. However, the mechanism by which AC exposure causes cellular dysfunction in experimental animals and humans is not clear. AC may cause alterations in thirst and hunger regulation centers in hypothalamus.

To the best of our knowledge, this study is the first to investigate the effects of acrylamide toxicity on the heart tissues of the developing chick embryo by evaluating biochemical, antioxidant markers and histoarchitecture. Oxidative stress is usually caused by the increase of intracellular pro-oxidant species such as hydrogen peroxide, hydroxyl radicals and superoxide anion radicals. Highly intracellular levels of ROS can lead to the damage of mitochondria, DNA modification, lipid peroxidation, and even cell death (Gonzalez 2005). ROS generated in tissues is efficiently scavenged by enzymatic antioxidant system (such as SOD, GPx, CAT, GR and GSTs).

The role of antioxidant enzymes in embryogenesis and tissues of adult animals, it should be taken into account that superoxide radical may fulfill several functions: oxidize SH groups, undergo dismutation to form H2O2 and singlet oxygen, and react with oxidized iron ions to restore them [Andreev et al., 2005]. Each of these pathways may cause changes in embryo metabolism and change the properties of cell membranes. Moreover, the depletion of cellular antioxidant enzymes and non-enzymes stores and the change in the redox status of the cell, in turn, may modulate gene expression directly or via the transcription factors that are redox-regulated, and may lead to apoptosis, cell proliferation, or transformation (Awad et al., 1998).

In the current study, AC exposure was found to markedly decrease the anti oxidant enzyme contents (in heart) and the anti-ROS activity in a dose-dependent manner (Figure-1-4), indicating that the antioxidant activity of heart tissues was gradually impaired following AC treatment. Significant and extremely significant increase of heart weights were observed in the low acrylamide dose treatment, suggesting the enhancement of the total body weight (Table-1), These data strongly indicated that the impairment of antioxidative capacity were involved in the development of AC-induced toxicity. SOD and GPx are, which can effectively scavenge free radicals directly and indirectly, are the major enzymatic antioxidant in cells. GST also plays an important role in antioxidant defense, and regulation of cellular events. GSH deficiency contributes to oxidative stress, which takes effect in the pathogenesis of many diseases, e.g., Alzheimer's disease (Wu et al., 2004). Previous studies showed that conjugation with glutathione (GSH) is a mechanism for the detoxification of AC (Tong et al., 2004). AC-GSH was the major metabolic route in rats (i.p., 47 mg/kg), accounting for 69% (Sumner et al., 2003). Glycidamide, an active neurotoxic metabolite of acrylamide, can also conjugate with GSH [Sumner et al., 1997].

Current work revealed degenerative myocardial changes, necrosis and collapsed myocardial fibers of cell heart may indicate the loss of functional efficiency of the cells (Figure-5b-d). Similar results have been demonstrated by (Ortiz et al., 2006) on liver of male rats. Also, this result is consistent with the findings by Kedam et al, (2012) that indicating hypertrophy of nuclei and pycnotic nuclei in acrylamide treated chick liver.

Conclusion

In conclusion, high doses of acryalmide toxicity induced the oxidative stress with the loss of free radical scavenging activity. The antioxidant enzymatic activities were reduced with increasing doses of acrylamide, the decreased activities of catalase, superoxide dismutase, glutathione peroxidase, and glutathione-S-transferase levels in discrete regions of heart indicating oxidative stress and inhibited antioxidant defense. However low doses of acrylamide significantly increases the heart and total body weights and increased the doses of the acrylamide induces the loss of heart and body weights. The mild degenerations at low doses, necrotic and collapsed myocardial fibers at high doses of acrylamide treatment of developing chick embryo.

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