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GLUTATHIONE ADMITS ENHANCED RATE OF CHICK EMBRYO LIFESPAN FROM LIPID DEGENERATIVE STRESS DURING INCUBATION

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ABSTRACT

Acrylamide is carcinogenic to experimental animals, causing tumors at multiple organ and chromosomal sites in mice, rat and chick embryo when given in drinking water or by other means. The level of genetic integrity of human populations is increasingly under threat due to industrial activities that result in exposure to chemical and physical xenotoxins. The excess concentration of chemicals can cause damage to defence system and modifies tissue to lead to cancer. To encounter the above changes, the organisms are well equipped with certain defence enzymes like superoxide dismutase, catalase, peroxidases, glutathione S- transferases, mixed function oxygenase etc. These enzymes can participate either to catabolise or excrete the molecules from the body. Some of these enzymes are induced for secondary defence by using glutathione as primary substrate and the other chemicals as secondary substrates. The present study was focused on effect of glutathione (GSH; 0.1mg), the variable concentrations of acrylamide and combination exposure of acrylamide and GSH (Glutathione) on chick embryo liver for different time intervals i.e. 96hrs, 120hrs and 144hrs has indicated that acrylamide administration significantly increases damage in the antioxidant system with increased levels of time intervals ($P< 0.05$), However it has been observed that glutathione addition might protect the system from damage. The MDA content has been decreased in chick embryo liver due to the glutathione effect. With 0.025mg GSH 14.8, 6.51, 5.73 fold decrease, with 0.05mg GSH 7.45, 5.87, 4.43 and with 0.075mg 2.12, 1.92, 1.79 fold decrease has been observed at 96hrs, 120hrs and 144hrs. Glutathione by itself has enhanced the viability rate of chick embryo 75% in the presence of 0.1mg in vivo supply in the form of injection, because of decrease of MDA

content in 2.125, 1.92 and 1.79 folds in the time intervals of 96hrs, 120hrs and 144 hrs respectively. In addition to these the antioxidant enzyme activities were found to be reduced less in liver by GSH compared to acrylamide treatment to chick embryos.

KEY WORDS: Acrylamide, GSH, Lipid peroxidation, Chick embryo liver, Antioxidants.

INTRODUCTION

Acrylamide a neurotoxicant, is extensively studied and has a large database on its complex toxicity, pharmacokinetic and mode of action. The organisms have an antioxidant defence in order to minimize oxidative damage that occur due to internal or external molecules on to cellular components such as lipids, proteins and DNA. Reactive oxygen species (ROS) produced by an external molecule acrylamide, are the most studied biomarkers to evaluate the biochemical alterations of organic contaminants on terrestrial organisms. The ROS that include are superoxide anion radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) ion. The most important antioxidant enzymes which involved in the elimination of ROS include are superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), xanthine oxidase and glutathione peroxidase (GPx). The other group of enzymes which involved are glutathione S-transferases which participate in the conjugation of glutathione (GSH) to various electrophilic molecules, and play a role in prevention of oxidative stress induced damage. Hence forth organisms can adapt new ways for the elimination of oxygen species by up-regulating antioxidant enzymes. [1] Failure of defence to detoxify the regulation of ROS production can lead to significant oxidative damage including enzyme inactivation, protein, DNA and lipid degradation due to oxygenation. Therefore considering the significant function of acrylamide in induction of toxicity in developing chick embryo liver, the aim that was selected in the present work was to assess the effects of acrylamide on oxidative stress of chick embryo liver.

Glutathione (GSH, γ-glutamylcysteinylglycine), the primary non-protein sulfhydryl molecule in aerobic organisms, is synthesized in most cells. [2] Glutathione conjugation is of particular importance in being of the major defense mechanisms in the body against electrophilic xenobiotics. Thiol-containing GSH scavenges ROS and RNS, as well as nucleophilic compounds, using its reduced thiol group [i.e., *S*-glutathionylation]. [3] By reacting with ROS and RNS (i.e., *S*-nitrosylation), GSH is oxidized to produce GSSG by GSH peroxidase (GPx), then is recycled back to two GSH molecules by GSH reductase and NADPH, effectively completing this detoxification cycle. [3, 4] From our laboratory studies it is known that the

acrylamide enhances decrease in vitamin c, glutathione and other antioxidant enzymes. Also our studies has revealed that it enhances the death rate of embryo level itself. [5] Therefore considering these consequences the influence of GSH was studied under the stress of acrylamide in chick embryos.

MATERIALS AND METHODS

Source of fertilized eggs and incubation conditions

Freshly laid *Bobcock* strain zero day old fertilized chick eggs were purchased from Sri Venkateswara Veterinary University, Tirupati, and Sri Balaji hatcheries, Chittoor, Andhra Pradesh. They 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 tray full of water inside.

Treatment

Acrylamide treatment

A group of six eggs were incubated for each dose and each time point. Every fertilized egg of a group (n=6) separately has received a dose of acrylamide (0.1, 0.2 and 0.3mg) for each single dose for the time interval periods of 96hrs, 120hrs and 144hrs.

Group of six eggs (n=6) were also maintained for each time point and dose 0.025mg, 0.05mg, 0.075mg and 0.1mg concentration of GSH in saline of 10µl was administered to fertilized chick embryos on each set of eggs of same group and have received equivalent quantity of GSH for 96h, 120h and 144h as single dose.

Another group of six eggs (n=6) were maintained for each time point and dose. 0.1, 0.2, 0.3mg concentration of acrylamide in saline and GSH (0.1mg concentration) of 10µl was administered to fertilized chick embryos on each set of eggs of same group and have receive equivalent quantity of mixture of Acrylamide and GSH for 96h, 120h and 144h as single dose. In our experimental analysis control eggs have received the same volume of saline. The egg shell was opened at the blunt end at the top to obtain access to the air sac, where the respective test substance (10µl) was injected directly on to the inner shell membrane. Covering the hole by wax could ensure the embryos vitality for the remaining time until blood sampling and dissection. Chick embryonic liver of six groups was collected on d14 after 96h (d10), 120h (d9) and 144h (d8) administration of the test substance. The tissue was

washed with normal saline to remove blood and fat debris and stored at -20°C until further use.

Preparation of test samples

The Acrylamide of 0.1, 0.2 and 0.3mg was prepared by dissolving 10mg, 20mg and 30mg in 1ml volumes of saline separately, to get a concentration of 0.1, 0.2 and 0.3mgs, respectively, in 10µl for each injection. Similarly 10mg of GSH was dissolved in one ml of saline to get 0.1mg concentration of GSH for each injection of 10µl.

Measurement of tissue lipid peroxides

The level of lipid peroxidation was measured in terms of MDA determination by the method of Okhawa *et al*. [6]

Each group of liver tissue was homogenated in 100 ml of 1.15% of KCl for determination of lipid peroxides. To 0.1ml of the tissue homogenate, added 0.2ml of 8.1% SDS and 1.5ml of 0.8% TBA. The total volume was made up to 4ml with distilled water and the tubes were kept at 95°C for 60min, and then cooled. To this added 1ml of distilled water along with 5ml of n-butanol-pyridine mixture (15:1 v/v) and the contents were mixed vigorously. Then the tubes were centrifuged at 4000rpm for 10min and the colour of the organic layer was measured at 532nm using spectrophotometer.

The results obtained in the experiments were calculated for statistical significance using mean ± standard deviation and in other places the single arithmetic calculations were made to determine the significance based on area and circumference studies, such as radar and doughnut representations.

RESULTS

Determination of lipid peroxidation in liver tissue

The effect of GSH on lipid peroxidation in liver of d14 chick embryo is represented in Table 1 and Figure 1. The MDA levels were significantly ($p<0.05$) decreased in GSH treated liver in a dose dependent manner. The maximum percentage of reduction was found in 0.1mg GSH (55.7%) treatment when compared to controls and other lower GSH treatments to chick embryos.

Table 1: Effect GSH on Lipid peroxidation in Liver of 14th day old Chick Embryo.

Effect of GSH on MDA levels			
Concentration of GSH	Time intervals of incubation		
	96hrs	120hrs	144hrs
Control	2.386±0.181	2.386±0.181	2.386±0.181
0.025mg	2.225±0.017	2.02±0.016	1.972±0.029
0.05mg	2.066±0.029	1.980±0.017	1.848±0.03
0.075mg	1.809±0.025	1.590±0.025	1.380±0.030
0.1mg	1.263±0.017	1.146±0.029	1.056±0.027

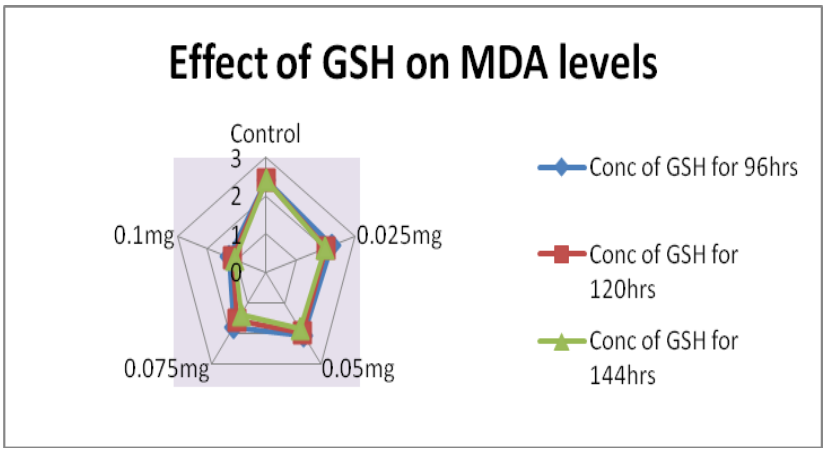

Figure-1: Radar distribution data comparison on MDA levels of GSH treated Chick embryo liver at different time intervals.

To make the conclusive decisions the arithmetic area studies of triangle and quadrangle were calculated and used.

The formation of MDA in low levels of GSH was 80% and in high concentration it was reduced to 20% even when compared to control tissues. Therefore 0.1mg of GSH was selected for further analysis of Acrylamide treatment due to increased survival rate of embryo when it was treated with 25, 50, 75 and 100µgs concentration of Glutathione (Table 2, Figure 2).

Table 2: Survival rate of Embryo due to the administration of GSH.

Survival rate of Embryo in the presence of GSH			
Concentration of GSH	Time intervals of incubation		
	96hrs	120hrs	144hrs
Control	95%	95%	95%
0.025mg	50%	50%	50%
0.05mg	60%	60%	60%
0.075mg	70%	70%	70%
0.1mg	75%	75%	75%

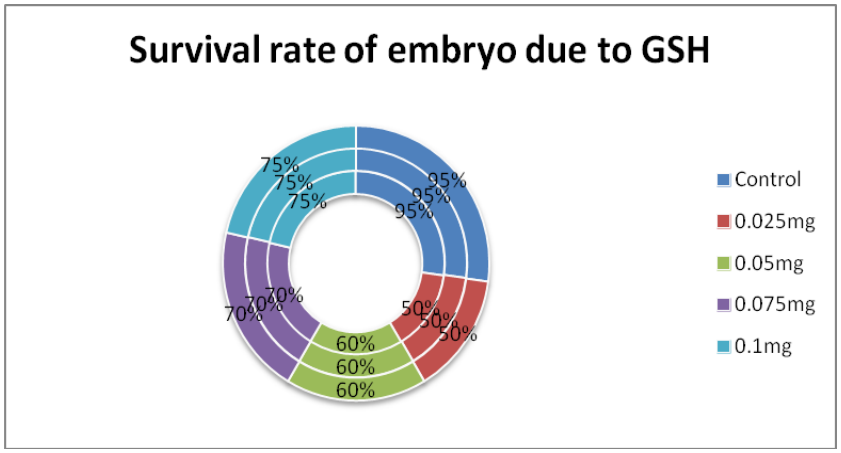

Fig 2: Doughnut representation in data variation of survival percentage of embryo treated with glutathione with differrent concentrations for different time intervals Lower circle represents 96hrs, Middle circle 120hrs and outer circle 144hrs of Glutathione treatment.

To make the conclusive decisions the arithmetic area studies of triangle and quadrangle were calculated and used.

From the Table 2 and Figure 2 survival rate of embryo was observed to be increased to about 75%. The loss when compared to control was just 20% with the 100µgs of administration of GSH. Further on conversion of data into present decrease (Table 3 and Figure 3)

Table 3: Effect of GSH on MDA levels.

Conc of GSH	96hrs	120hrs	144hrs
0.025mg	6.70%	15.10%	17.20%
0.05mg	13.40%	16.80%	22.50%
0.075mg	23.90%	33.30%	42%
0.1mg	46.90%	51.90%	55.70%

To make the conclusive decisions the arithmetic area studies of triangle and quadrangle were calculated and used.

Shows that reduction in MDA levels were about 50% on average with 0.1mg GSH administration to chick embryo liver. More amount of decrease i.e. about 55.7% was observed in 0.1mg GSH treated chick embryo liver at 144hrs incubation. After knowing about the role of GSH in the reduction of MDA levels and survival of embryo the effect of acrylamide and Acrylamide mixed with GSH was tested on lipid peroxidation in liver of d14 chick embryo. The results of acrylamide treatment were represented in Table 4 & Figure 4. The MDA levels after acrylamide treatment were significantly ($p<0.05$) increased in liver in a

dose dependent manner. The maximum percentage of induction was seen in 0.3mg AC (43.8%) treatment when compared to controls. In 0.3mg AC treated embryos; 1.55, 1.79 and 1.86 fold increase in the induction of MDA levels in 96, 120 and 144hrs treatment were observed.

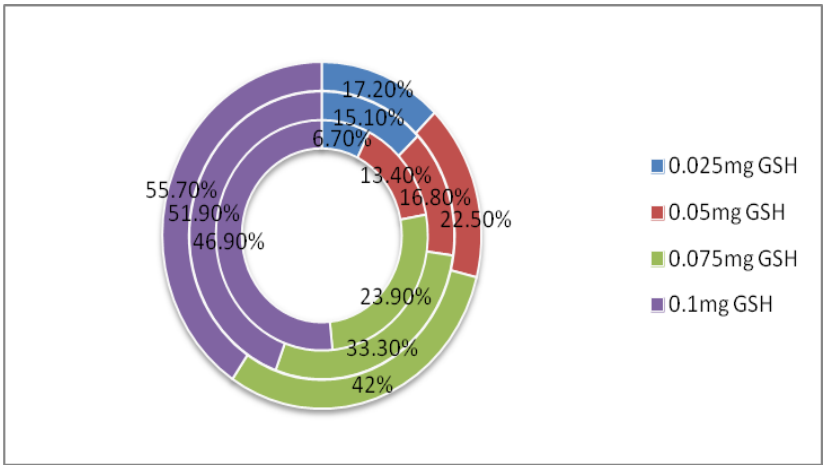

Fig 3: Doughnut representation of MDA levels reduction in embryo liver treated with glutathione with differrent concentrations for different time intervals Lower circle represents 96hrs, Middle circle 120hrs and outer circle 144hrs of Glutathione treatment.

Table 4: Effect of Acrylamide on Lipid peroxidation in Liver of 14th day old Chick Embryo.

Treatment	96hrs	120hrs	144hrs
Control	2.38±0.18	2.38±0.18	2.38±0.18
0.1mg	3.19±0.16	3.33±0.17	3.46±0.09
0.2mg	3.52±0.10	3.70±0.22	3.81±0.14
0.3mg	3.93±0.12	4.17±0.08	4.24±0.08

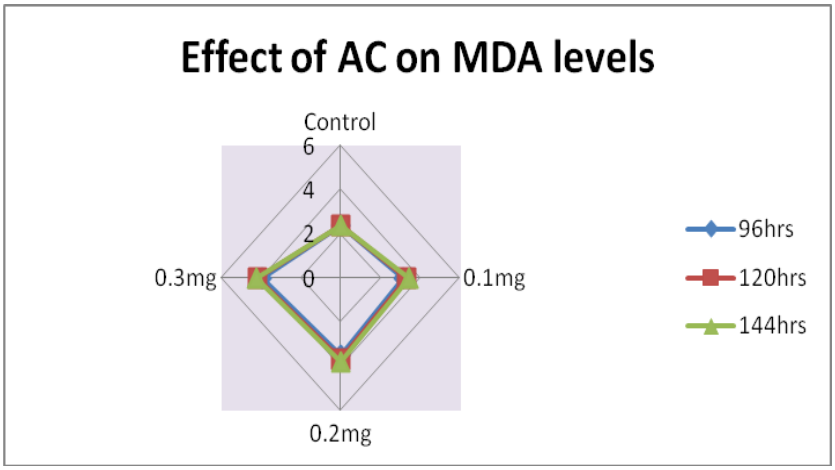

Fig 4: Radar distribution data comparison on MDA levels of Acrylamide treated Chick embryo liver at different time intervals.

To make the conclusive decisions the arithmetic area studies of triangle and quadrangle were calculated and used.

The above Table (4) on conversion to radar distribution (Fig 4) and calculation to percentage increase in MDA formation (Table 5 and Figure 5) it was found that the acrylamide in low concentrations has generated 38% MDA levels and in high concentrations increased to 68% of MDA levels in chick embryo liver.

Table 5: Effect of Acrylamide on MDA levels.

Conc of AC	96hrs	120hrs	144hrs
0.1mg AC	25.30%	28.50%	31.20%
0.2mg AC	32.30%	35.60%	37.50%
0.3mg AC	39.40%	42.90%	43.80%

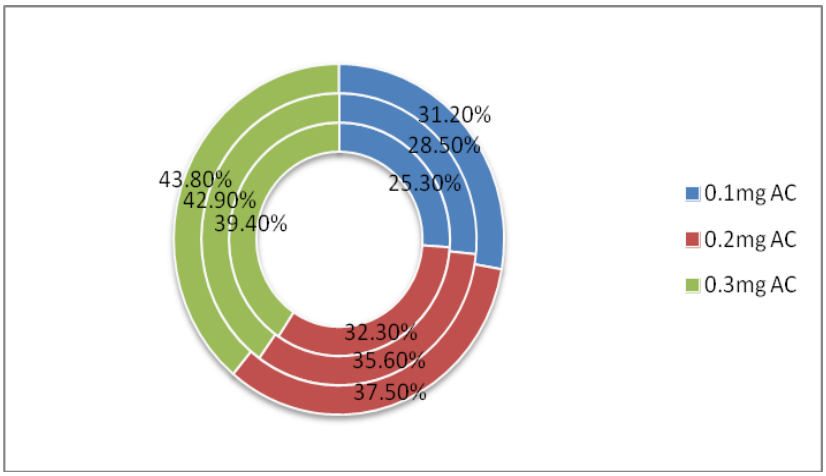

Fig 5: Doughnut representation of MDA levels percentage of embryo treated with Acrylamide with differrent concentrations for different time intervals Lower circle represents 96hrs, Middle circle 120hrs and outer circle 144hrs of Acrylamide treatment.

To make the conclusive decisions the arithmetic area studies of triangle and quadrangle were calculated and used.

Further the MDA levels were raised in 0.1mg to 0.3mg from the range of 28.33% to 42.04%. Hence increased continuous acrylamide administration found to contain increased production of MDA from 28.33% to 42.04%.

After knowing the influence of acrylamide and GSH, separately on MDA levels and embryo growth rate, the mixed effect of both acrylamide and GSH were tested upon their administration as mentioned in methodology. The results are represented in Table 6-7 and

Figure 6-7. The combined molecules effect also showed an increase in the values of MDA in liver. However these values were found to be less when compared to Table 4.

Table 6: Effect of GSH on Acrylamide induced Lipid peroxidation in Liver of 14th day old Chick Embryo.

Treatment	96hrs	120hrs	144hrs
Control	2.38±0.18	2.38±0.18	2.38±0.18
0.1mg	2.46±0.22	2.59±0.28	2.73±0.15
0.2mg	3.07±0.24	3.16±0.11	3.52±0.17
0.3mg	3.78±0.22	3.9±0.17	3.94±0.20

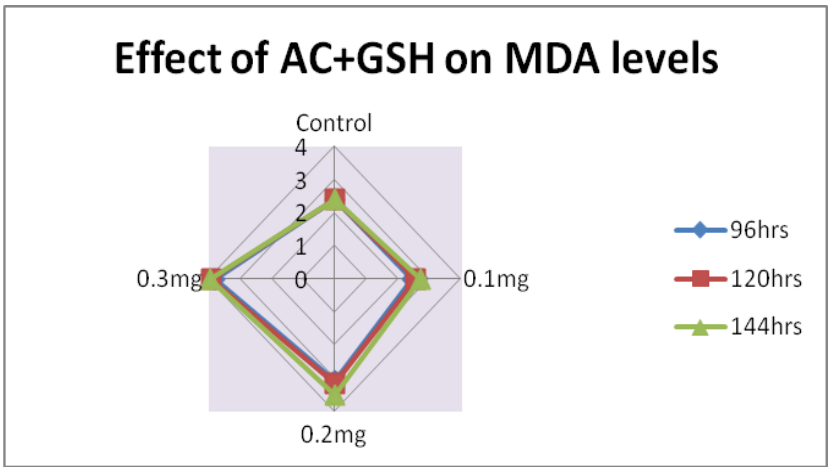

Fig 6: Radar distribution data comparison on MDA levels of AC+GSH treated Chick embryo liver at different time intervals.

To make the conclusive decisions the arithmetic area studies of triangle and quadrangle were calculated and used.

The combination of Acrylamide and GSH, in low concentration AC was produced 33% of MDA than the increased concentration of AC, which showed about 66%of MDA.

Table 7: Effect of Acrylamide+GSH on MDA levels.

Conc of AC+GSH	96hrs	120hrs	144hrs
0.1mg AC+0.1mg	3.25%	8.10%	9.50%
0.2mg AC+0.1mg	15.00%	19.50%	22.40%
0.3mg AC+0.1mg	25.30%	27.80%	31.00%

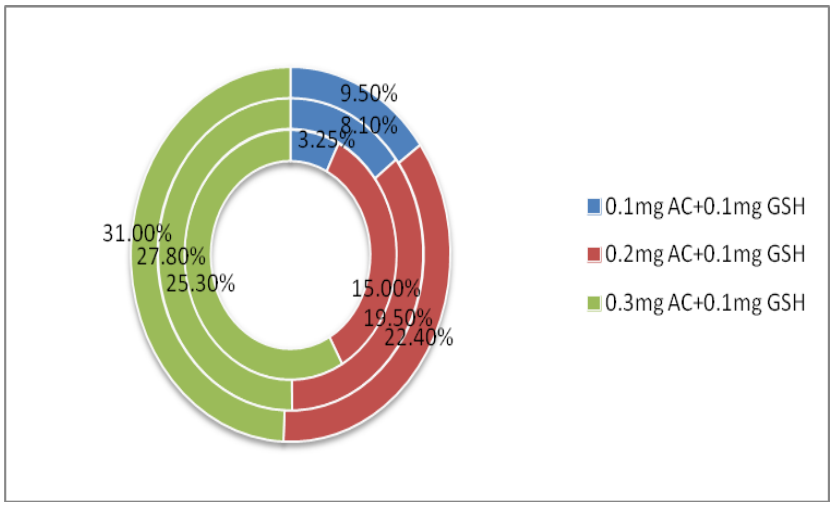

Fig 7: Doughnut representation of MDA levels percentage of embryo treated with Acrylamide+GSH with differrent concentrations for different time intervals Lower circle represents 96hrs, Middle circle 120hrs and outer circle 144hrs of Acrylamide+GSH treatment.

To make the conclusive decisions the arithmetic area studies of triangle and quadrangle were calculated and used.

DISCUSSION

Acrylamide, a synthetic chemical widely used as a water treatment agent and in the manufacture of adhesives, dyes and fabrics, has recently been shown to occur naturally in an increasing number of foods ranging from French fries to coffee. Carbohydrate rich food when cooked at high temperature can lead to the formation of acrylamide due to chemical reaction. Acrylamide is also used in laboratories for separation of macromolecules in electrophoresis. Acrylamide has been extensively studied and there is a large database on its toxicity and pharmokinetics indicating that this compound is carcinogenic. Although the genotoxic effects of AC and its reactive metabolite, GA, are well established in the liver of BB mice. [7] it is a multisite carcinogen, in studies examining only the lung and skin, acrylamide induced lung and skin tumors. This study focused mainly on the acrylamide toxicity and the influence of glutathione on the acrylamide effect for long time intervals of incubation.

Lipid peroxidation is one of the main manifestations of oxidative damage and has been found to play an important role in the toxicity of many xenobiotics. [8] The prevention of lipid peroxidation is essential for all living organisms and so the organisms are well equipped with antioxidant systems that directly or indirectly protect cells against the adverse effects of

xenobiotics, carcinogens and toxic radicals. [9] Reactive oxygen species (ROS) formed by lipid peroxidation are in fact required for cell functions if produced in physiological concentrations. Acrylamide induced free radical production in hepatocytes have been suggested to be responsible for the oxidative damage. The data obtained in our laboratory confirms statistically significant increase in the MDA levels of AC treated developing chick embryonic liver and somewhat decrease in the level of MDA was observed on Glutathione and Acrylamide combined treatment. 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. [10] Free radicals are continuously produced *in vivo* and there are number of protective antioxidants (Superoxide dismutase, catalase, glutathione S-transferase, glutathione peroxidase, glutathione reductase and reduced glutathione) for dealing with these toxic substances. The delicate balance between the anobolism and catabolism of oxidants is crucial for maintenance of the biological function. [11]

The data represented (Table 4 & 6) confirm that AC and AC & GSH mixture cause a significant increase of lipid peroxides in liver of chick embryo, Yousef and Demerdash et al, [12] and Veenapani et al. [13] have reported induction in the lipid peroxides in rat treated with AC. Srivastava et al. [14] suggested that enhancement of lipid peroxidation is a consequence of depletion of glutathione to certain critical levels. Venkatswamy et al, [15] have reported the depletion of vitamin C in chick embryo due to acrylamide treatment. Therefore acrylamide due to its oxidation to glycidamide, a reactive epoxide and undergoes adduct formation with DNA and creates new sites for DNA degradation because of depletion of antioxidants such as GSH and Vit C. Hence sufficient quantity off GSH is necessary to deplete th effect of GA upo conjugation process. Increased GST activity with the increase of acrylamide concentration could be due to increased formation of S-conjugates between GA and GSH [12] to form mercapturic acid was considered as a major pathway for AC metabolism in chick embryos. Chick embryos have been used in the past for several years to investigate the effect of environmental chemicals and radiations on developmental effects, morphogenesis, etc. Ruxana et al. [16] reported on the effect of acrylamide on lipid peroxidation in chick liver about increased MDA levels in a dose dependent manner. The results of the present study revealed significant decrease in MDA levels due to glutathione effect in chick embryo liver and role of GSH content in the reduction of MDA formation in Acrylamide treated chick embryo liver.

CONCLUSION

On the basis of results obtained in the present study concludes that acrylamide caused disturbances in the oxidative status and pronounced effect with the low doses for long time intervals (96hr, 120hr and 144hr). The results also have indicated a risk of organ damage during exposure to acrylamide. The supplementation of glutathione to chick embryo has showed a decrease in MDA levels of acrylamide treated chick embryonic liver. Therefore in in vivo the GSH may serve as potential scavenger in the removal of free radical mediated damage from formed glycidamide from acrylamide in chick embryo liver.

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