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Research Article

FLUORIDE-INDUCED OXIDATIVE STRESS IN ADRENAL GLAND OF RAT

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ABSTRACT
Fluorosis is a dreaded condition caused due to high exposure of sodium fluoride in drinking water. The aim of this study was to use sensitive biomarkers to evaluate damage to adrenal gland after fluoride exposure. Recently, weaned Wistar rats were exposed to 100, 200 and 300 ppm fluoride concentrations in drinking water for a period of 20 and 40 days. At the end of exposure period, oxidative stress parameters and activity of antioxidant enzymes were measured in adrenal tissue. The fluoride content in the adrenal tissue increased significantly ($p<0.001$) in all rats after 20 and 40 days of fluoride intoxication in comparison to control. The level of malondialdehyde in adrenal gland of test rats were also increased significantly ($p<0.001$) while antioxidant enzymes such as superoxide dismutase and catalase were reduced after sodium fluoride treatment. Glutathione concentration was significantly ($p<0.001$) decreased.

Keywords: *Adrenal Gland, Catalase, Malondialdehyde, Reduced Glutathione, Sodium Fluoride, Superoxide Dismutase*

INTRODUCTION
Fluorosis is both endemic and global, spanning several continents. Natural geological sources and more recently the advent of increased industrialization and environmental pollution have contributed greatly to the increasing incidences of fluoride related issues. High levels of fluoride in drinking water have become a potential health hazard all over the world.

There are multiple effects of fluoride on human health. Some are characterized by mineralization changes in the calcified tissues resulting in dental fluorosis (Shashi and Bhardwaj, 2011), skeletal deformities (Shashi *et al.,* 2008; Arlappa *et al.*, 2013) and metabolic changes in soft tissues such as brain (Shashi and Kumar, 2016), liver (Shashi *et al*., 2001; Ekambaram *et al.*, 2010), kidney (Nabavi *et al.,* 2012), reproductive organs (Rao *et al.*, 2012) and endocrine glands viz; thyroid (Shashi and Kumar, 2016), parathyroid (Shashi and Singla, 2013), pancreas (Shashi *et al.*, 2010) and adrenal gland.

Experimental evidences have indicated that exposure to fluoride results in oxidative stress both in vitro and in vivo in soft tissues (Barbier *et al.*, 2010). Various studies demonstrated the adverse effects of fluoride on adrenal in different animals. Fluoride has been known to inhibit or activate many enzymes of adrenal function. It plays an important role in the maintenance of the internal environment through its multiple and diverse functions. Adrenal gland is a multifunctional steroidogenic organ. Adrenal insufficiency as a result of organic solvent exposure cause severe change in electrolyte and carbohydrate metabolism. Adrenal gland have many features which render them susceptible to toxicological insult including high membrane content of unsaturated fatty acid, high rate of blood flow, free radicals generation during steroid biosynthesis and mechanism for uptake and storage of lipoprotein. Adrenal insufficiency as a result of organic solvent exposure cause severe change in electrolyte and carbohydrate metabolism, coma and sometimes death.

The aim of the present investigation was to evaluate the fluoride-induced oxidative stress and level of antioxidant enzymes in the adrenal gland of rat.

MATERIALS AND METHODS
Research Methodology
Male and female Wistar albino rats, weighing between 100-150 g were housed in polypropylene cages with stainless steel grill tops and fed with standard rat pellet diet (Hindustan Lever Limited, India). The

water was given *ad libitum*. The animals were acclimatized for two weeks before starting the experiments.

The experiments were performed under the approval of the animal ethical committee of Punjabi University, Patiala (Animal maintenance and Registration No.107/99/CPCSEA-2013-42).

Experimental Protocol

The animals were divided into eight groups containing six rats in each. The animals of control group received 1 ppm deionized water/kg body weight/day and the remaining groups were given sodium fluoride at the dose of 100, 200, 300 ppm/kg body weight for 20 and 40 days. The control and experimental animals were sacrificed under ether anaesthesia. The adrenal gland was dissected out, washed in normal saline, and processed for assay of fluoride, malondialdehyde, reduced glutathione and antioxidant enzymes.

Biochemical Analysis

The fluoride content in adrenal tissue of control and fluoride treated rats was estimated by the method of Hardwood (1969). The level of malondialdehyde was determined by the method of Ohkawa *et al*., (1979). The activity of superoxide dismutase was measured by the method of Das *et al.*, (2000) and catalase was determined by the method of Aebi (1984). The level of reduced glutathione was estimated by the method of Moron *et al*., (1979).

Statistical Analysis

The data was analyzed using statistical package SPSS version 17 for windows Inc. Chicago, II. One-way analysis of variance (ANOVA) was used to compare the means. The Tukey-Kramer post-hoc test was applied to identify significance among groups. Multiple pair-wise comparisons among all treatment groups were performed by Bonferroni post-hoc. The value of $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Results

Fluoride

The content of fluoride in adrenal gland increased in all fluoride treated rats significantly ($p<0.001$) after 20 days ($F=123.235$) and 40 days ($F=1177.135$) of fluoride intoxication in comparison to control (Figure 1). The maximum increase was noticed in group VI (245.79%).

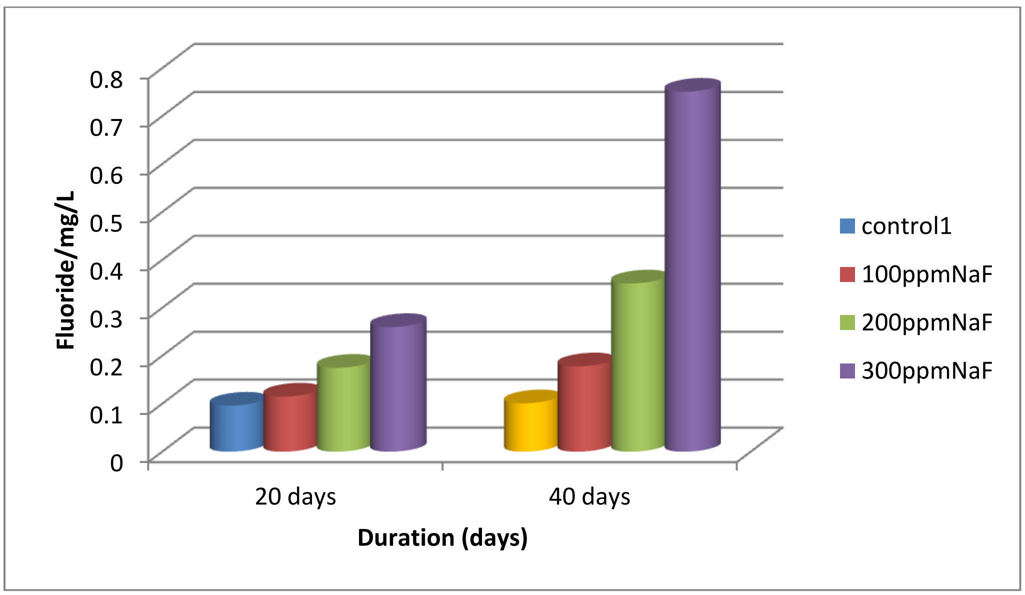

Figure 1: Changes in the Level of Fluoride in Adrenal Gland of Experimental Rats after 20 and 40 Days of Fluoride Treatment

Bonferroni multiple comparison test after ANOVA (F=123.235, p<0.001) showed a significant increase in the level of fluoride in adrenal gland (95%CI=-0.046 to -0.057) with mean difference of -0.018 to -0.085 among all fluoride treated groups as well as compared to control after 20 days and more significant increase (F=1177.135, p<0.001, 95%CI=-0.109 to -0.330) with mean difference -0.076 to -0.363 after 40 days of fluoride treatment.

Malondialdehyde

The level of malondialdehyde (MDA) in adrenal gland of test rat showed a significant (F=60.815, p<0.001) increase after 20 days of fluoride treatment but more significant (F=130.694, p<0.001) increase was observed after 40 days (Figure 2). Most prominent increase was registered in highest dose group (300 ppm fluoride/kg b.w./day).

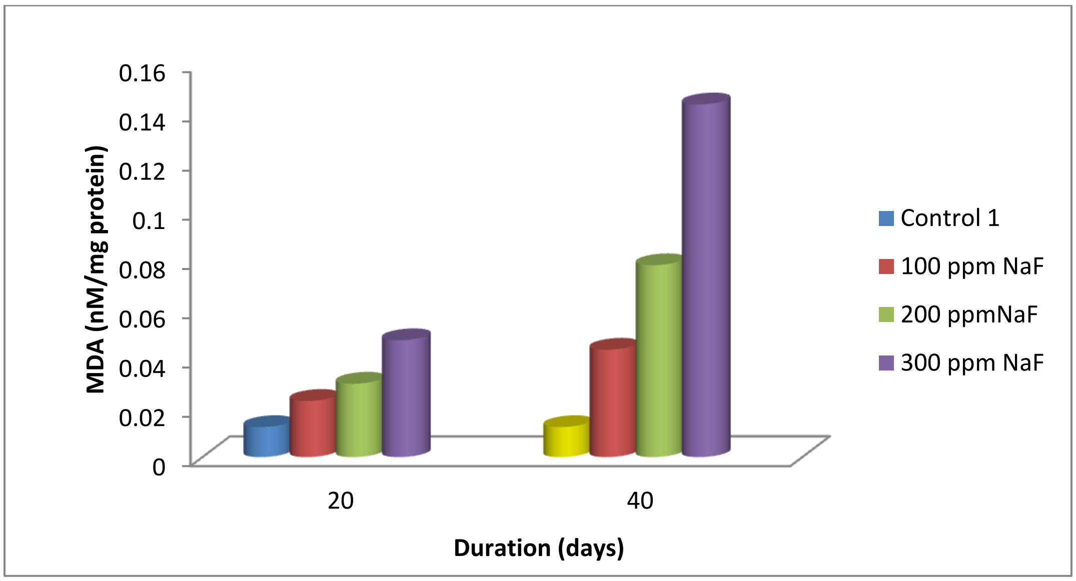

Figure 2: Changes in the Level of Malondialdehyde in Adrenal Gland of Experimental Rats after 20 and 40 Days of Fluoride Treatment

Bonferroni multiple comparison test after ANOVA (F=60.815, p<0.001) showed a significant increase in the level of MDA in adrenal (95%CI=-0.018 to -0.009) with mean difference of -0.010 to -0.017 among all fluoride treated groups as well as compared to control after 20 days and more significant increase (F=130.694, p<0.001, 95%CI=-0.051 to -0.045) with mean difference -0.031 to -0.065 was noted after 40 days of fluoride treatment.

Pearson's bivariate correlation analysis revealed a significant (r =0.905, p<0.001) positive relationship between concentration of fluoride and level of MDA in adrenal of test rat after 20 days. Further regression analysis (Y=0.010+0.129X, R^2=0.82) showed that as the level of fluoride increases in the adrenal gland, the level of MDA also increased (Figure 3).

Pearson's bivariate correlation analysis also revealed a significant (r=0.948) positive relationship between level of fluoride and MDA in adrenal of test rat after 40 days of fluoride treatment. Further regression analysis (Y=0.012+0.05X, R^2= 0.898) showed that as the level of fluoride increases in tissue, the activity of MDA was increased in adrenal of test rats (Figure 4).

Superoxide Dismutase

The activity of superoxide dismutase (SOD) in adrenal gland of test rat showed a significant (F=5.489, p<0.01) decrease after 20 days of fluoride treatment, but more significant (F=31.318, p<0.001) decrease was observed after 40 days (Figure 5). Maximum decrease was registered in highest dose group (300 ppm fluoride/kg b.w./day).

Bonferroni multiple comparison test after ANOVA (F=5.489, p<0.01) showed a significant decrease in the level of SOD in adrenal (95%CI= 0.026 to 0.135) with mean difference of 0.108 to 0.053 among all fluoride treated groups as well as compared to control after 20 days and more significant decrease in the level of SOD in adrenal gland was noted (F=31.318, p<0.001, 95%Cl=0.003 to 0,056) with mean difference 0.04 to 0.015 after 40 days fluoride treatment.

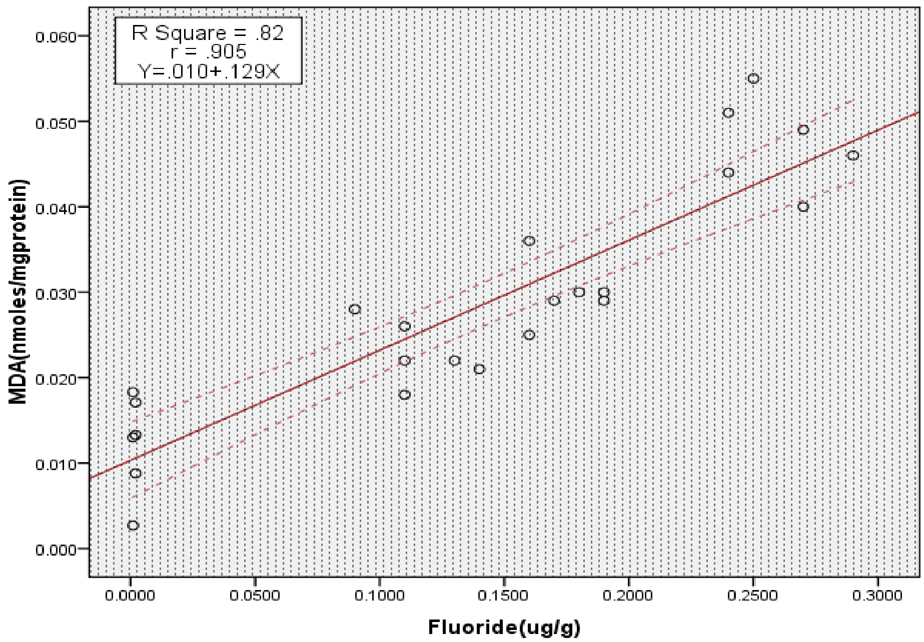

Figure 3: Scatterplot Showing Correlation between Fluoride and Level of MDA in Test Rats after 20 Days of Fluoride Treatment

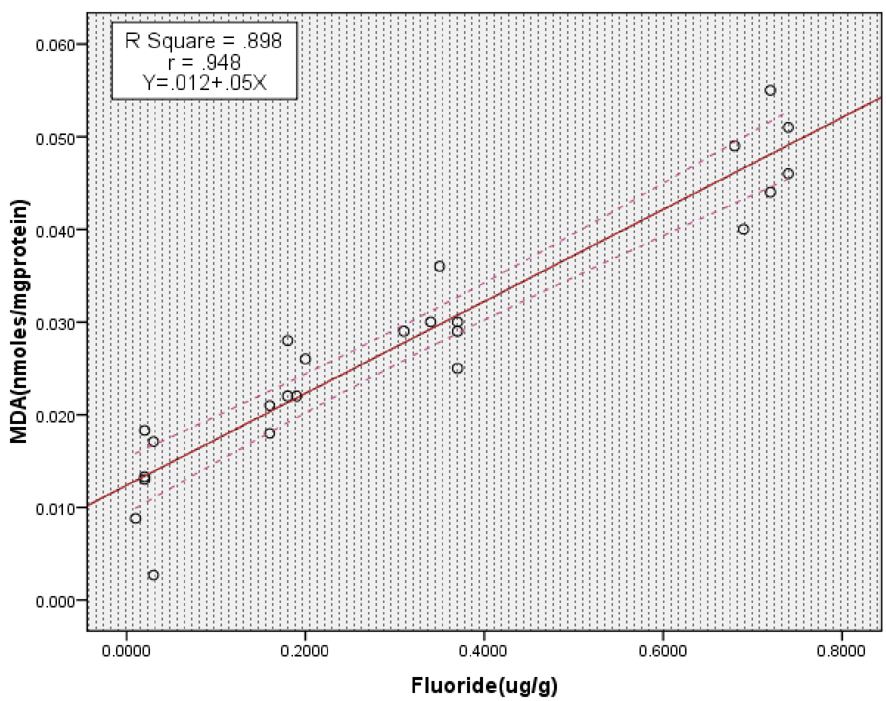

Figure 4: Scatterplot Showing Correlation between Fluoride and Level of MDA in Adrenal of Test Rats after 40 Days of Fluoride Toxicity

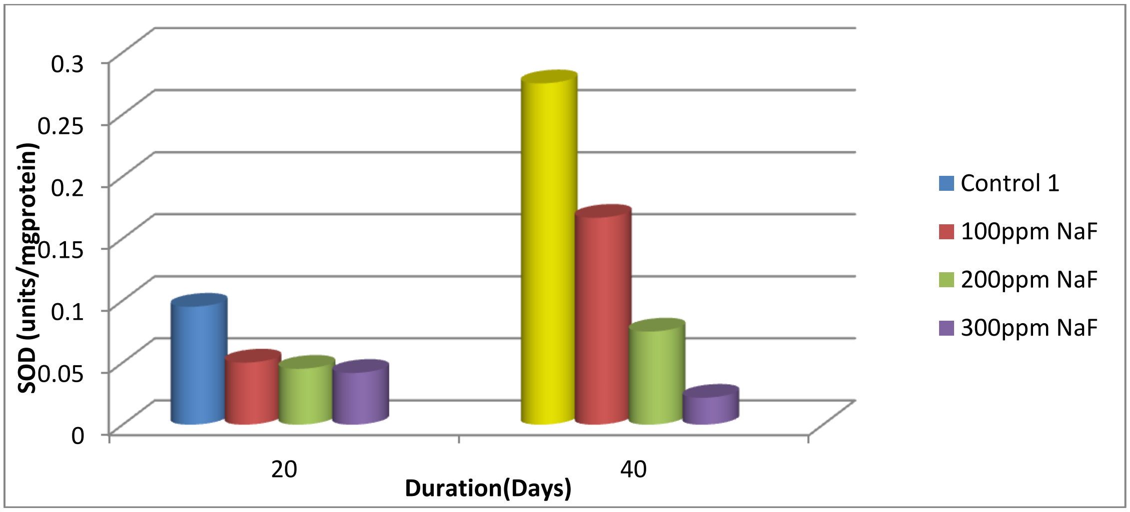

Figure 5: Changes in the Activity of SOD in the Adrenal of Experimental Rats after 20 and 40 Days of Fluoride Treatment

Pearson's bivariate correlation analysis revealed a significant (r =-0.624, p<0.01) positive relationship between concentration of fluoride and level of SOD in adrenal of test rat after 20 days of fluoride intoxication. Further regression analysis (Y=0.093-0.413X+0.877X, R^2=0.39) showed that as the level of fluoride was elevated in the adrenal tissue, the level of SOD also decreased subsequently (Figure 6).

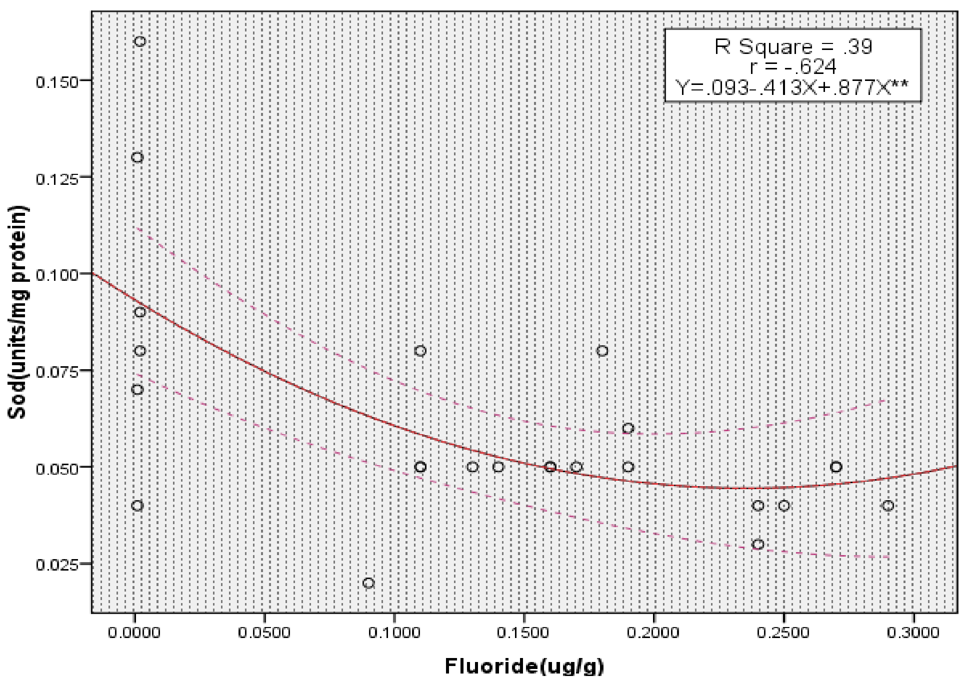

Figure 6: Scatterplot Showing Correlation between Fluoride and Activity of SOD in Test Rats after 20 Days of Fluoride Treatment

Pearson's bivariate correlation analysis revealed a significant (r =-0.90, p<0.001) positive relationship between level of fluoride and activity of SOD in adrenal of test rat after 40 days of fluoride treatment. Further regression analysis (Y=0.291-0.826X+627X, R^2=0.81) showed that as the level of fluoride decreases in tissue, the activity of SOD decrease in adrenal of test rats (Figure 7).

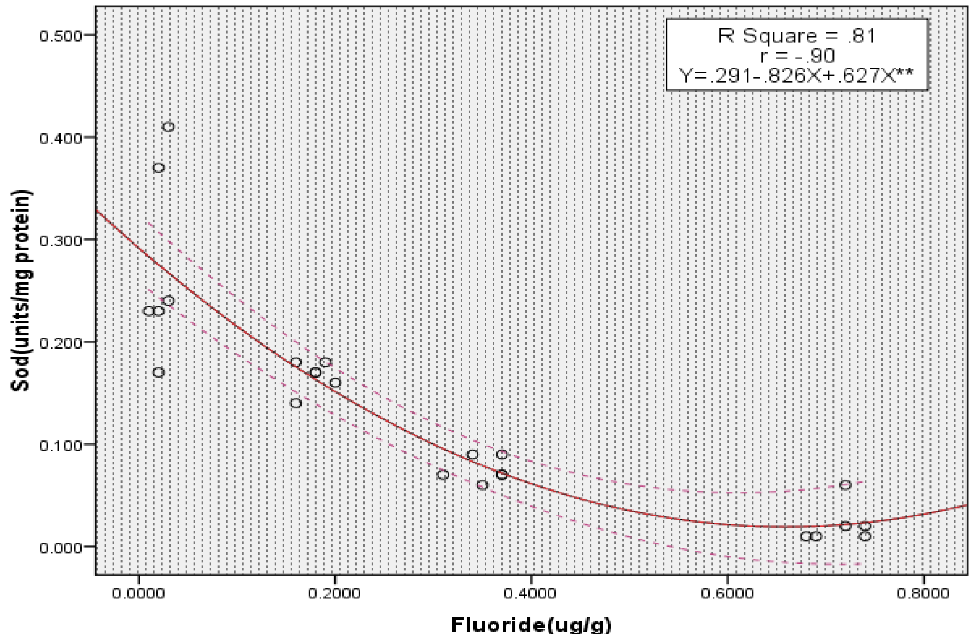

Figure 7: Scatter Plot Showing Correlation between Fluoride and Activity of SOD in Test Rats after 40 Days of Fluoride Treatment

Catalase

The activity of catalase in adrenal gland of test rat showed a significant (F=3.622, $p<0.05$) fall after 20 and 40 days (F=3.928, $p<0.05$). More prominent decrease was registered in highest dose group (300 ppm fluoride/kg b.w./day) (Figure 8).

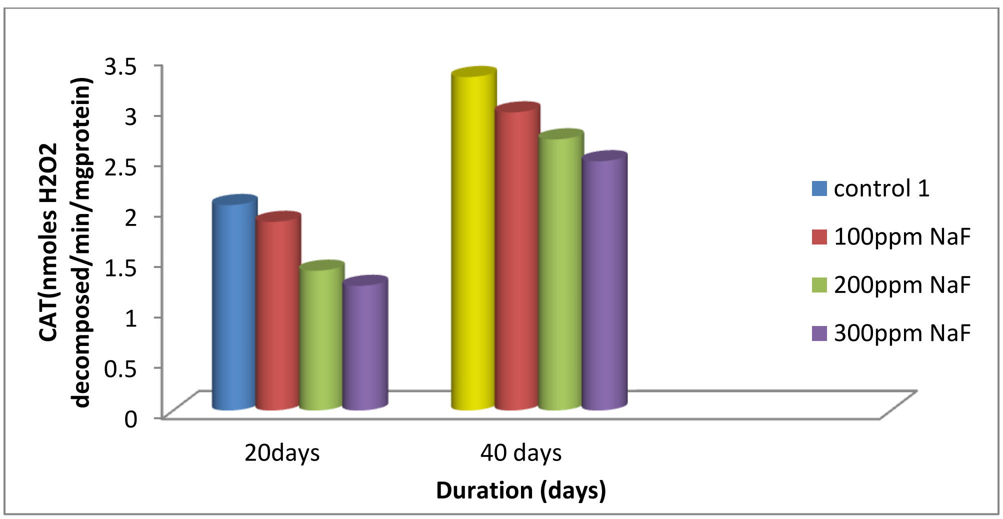

Figure 8: Changes in the Activity of CAT in Adrenal Gland of Experimental Rats after 20 and 40 Days of Fluoride Treatment

Bonferroni multiple comparison test after ANOVA (F=3.622, $p<0.05$) showed a significant decrease in the activity of CAT in adrenal gland (95%CI= -0.431 to 0.998) with mean difference of 0.350 to 0.216 among all fluoride treated groups as well as compared to control after 20 days and most significant decrease in the activity of CAT in adrenal gland was noted (F=3.928, $p<0.05$) 95%CI=-0.630 to 0.947) with mean difference 0.166 to 0.150 after 40 days of fluoride treatment.

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Pearson's bivariate correlation analysis revealed a significant (r =-0.563, p<0.01) negative relationship between concentration of fluoride and activity of CAT in adrenal gland of test rat after 20 days. Further regression analysis (Y=3.278-3.105X, R^2= 0.317) showed that as the level of fluoride decreases in the adrenal tissue, the activity of CAT also decreases (Figure 9).

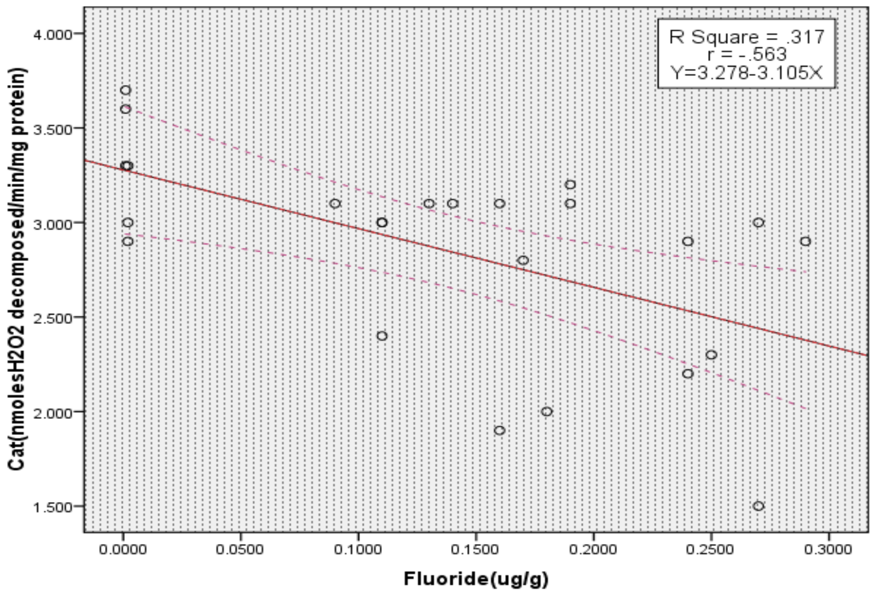

Figure 9: Scatter Plot Showing Correlation between Fluoride and Activity of CAT in Test Rats after 20 Days of Fluoride Treatment

Pearson's bivariate correlation analysis showed a significant (r=0.588, p<0.01) negative relationship between level of fluoride and activity of CAT in adrenal gland of test rat after 40 days of fluoride treatment. Further, regression analysis (Y=2.02-1.234X, R^2=0.345) showed that as the level of fluoride decreases in tissue, the activity of CAT decrease in adrenal gland of test rats (Figure 10).

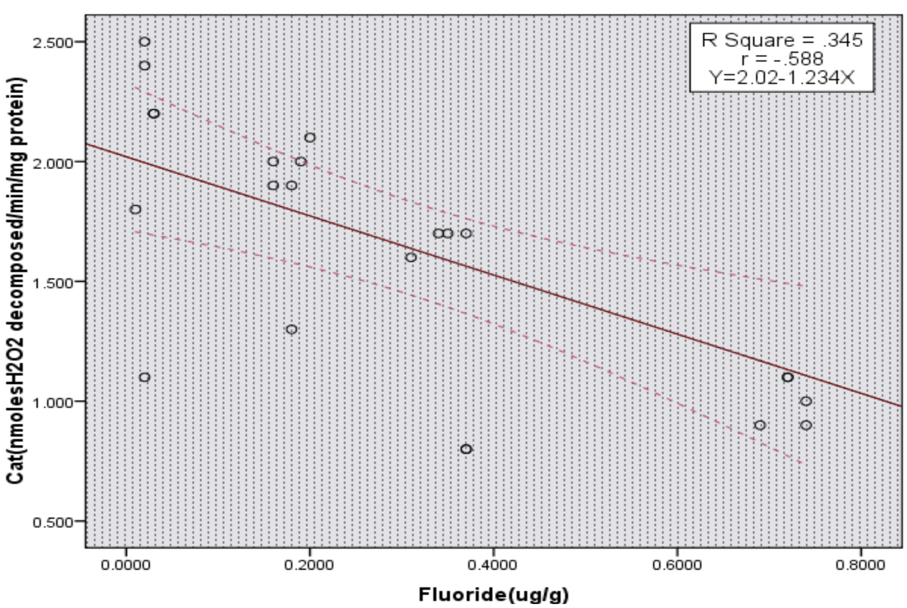

Figure 10: Scatter Plot Showing Correlation between Fluoride and Activity of CAT in Test Rats after 40 Days of Fluoride Treatment

Glutathione

The level of reduced glutathione (GSH) in adrenal gland of test rat showed a significant (F=167.780, p<0.001) decrease after 20 days and 40 days. (F=378.878, p<0.001) of fluoride treatment (Figure 11). Most prominent decrease was registered in highest dose group (300 ppm fluoride/kg b.w./day).

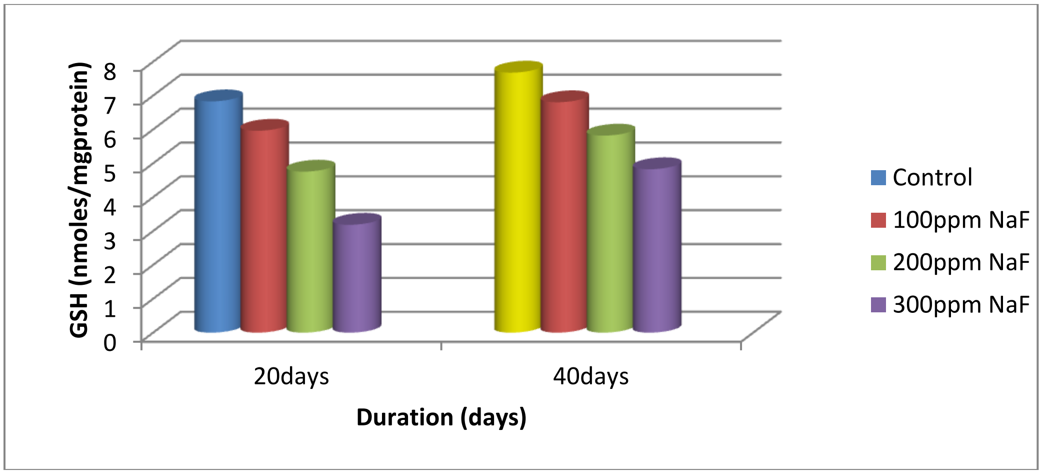

Figure 11: Changes in the Activity of GSH in Adrenal Gland of Experimental Rats after 20 and 40 Days of Fluoride Treatment

Bonferroni multiple comparison test after ANOVA (F=167.780, p<0.001) showed a significant decrease in the level of GSH in adrenal gland (95%CI= 0.481to1.385) with mean difference of 0.875 to 0.991 among all fluoride treated groups as well as compared to control after 20 days and more significant decrease in the level of GSH in adrenal gland was noted (F=378.878, p<0.001, 95%Cl=0.532 to1.906) with mean difference 0.868 to 1.570 after 40 days of fluoride treatment.

Pearson's bivariate correlation analysis revealed a significant (r=0.959,p<0.001) negative relationship between level of fluoride and activity of GSH in adrenal gland of test rat after 20 days of fluoride treatment. Further regression analysis (Y =7.794-10.972X, R^2= 0.919) showed that as the level of fluoride increased in adrenal gland, the content of GSH showed significant decline (Figure 12).

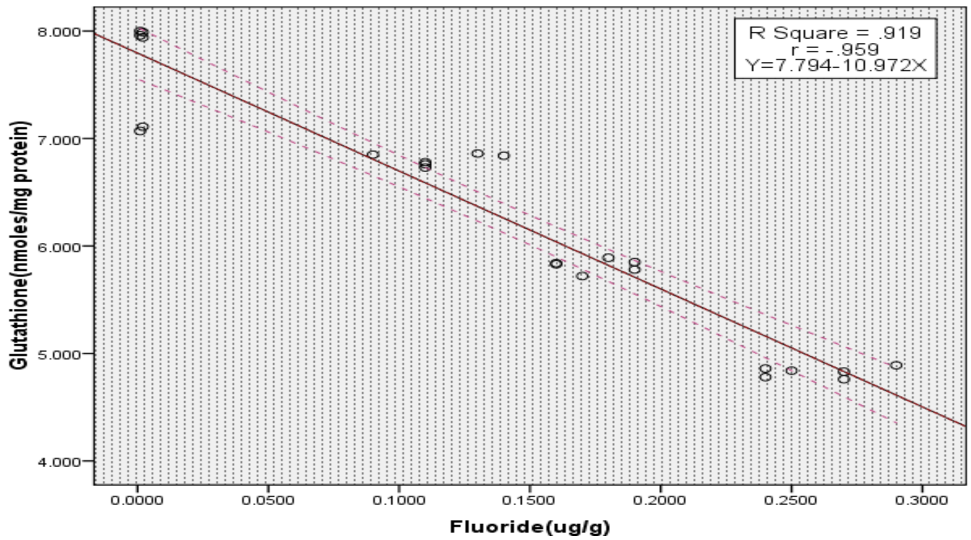

Figure 12: Scatter Plot Showing Correlation between Fluoride and Level of GSH in Test Rats after 20 Days of Fluoride Treatment

Pearson's bivariate correlation analysis revealed a significant (r=-0.985, p<0.001) positive relationship between levels of fluoride and GSH in adrenal gland of test rat after 40 days of fluoride treatment. Further regression analysis (Y=6.849+5.262X, R^2= 0.971) showed that as the level of fluoride decreases in tissue, the of GSH content was decreased in adrenal of test rats (Figure 13).

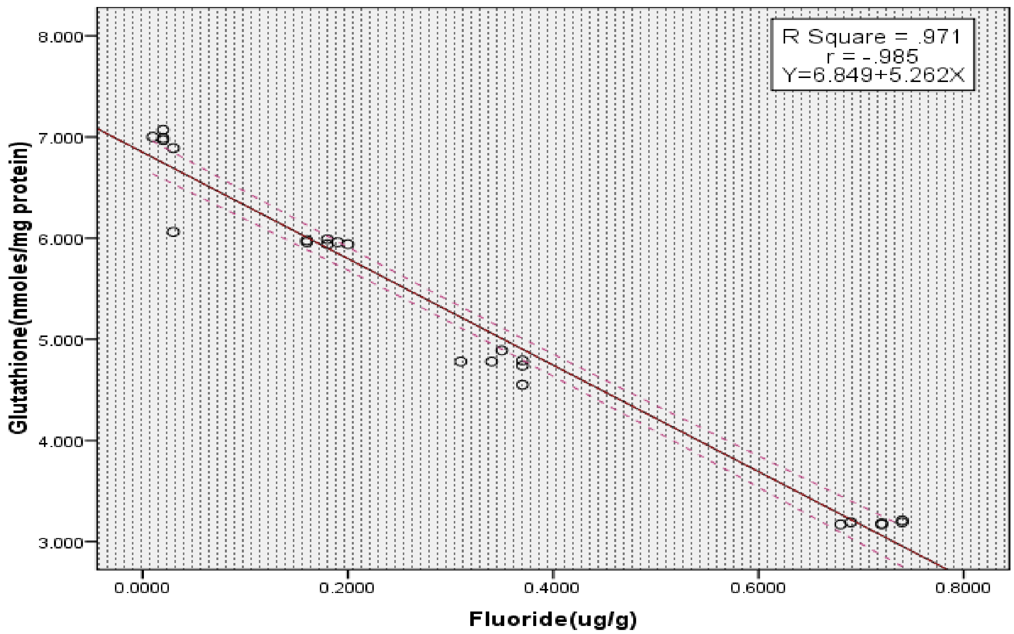

Figure 13: Scatter Plot Showing Correlation between Fluoride and Level of GSH in Test Rats after 40 Days of Fluoride Treatment

Discussion

The present study revealed that intake of water containing 100, 200 and 300 ppm sodium fluoride for 40 days resulted in a marked increase in concentration of fluoride in the adrenal gland of rat. As a potent protoplasmic poison, fluoride is toxic to any living cell and is known to cause various biochemical alterations, including oxidative stress when certain limited levels are exceeded (Shanthakumari *et al.*, 2004).

Malondialdehyde is considered to be the most significant indicator of membrane lipid peroxidation arising from the interaction of reactive oxygen type with cellular membranes. Increased lipid peroxidation from fluoride toxicity may be due to the generation of reactive oxygen species by high levels of H_2O_2 being formed in cells by controlled pathways. H_2O_2 at high concentration is deleterious to cells, and its accumulation cause oxidation of cellular targets such as protein, lipid, and DNA leading to mutagenesis and cell death.

The present study demonstrated that intoxication of rats with fluoride significantly increases malondialdehyde level in adrenal when compared with control. The level of malondialdehyde was more significantly increased after 40 days treatment as compared to 20 days intoxication, which was consistent with previous investigations (Zhan *et al.*, 2005). This increased level of lipid peroxidation is known to be mediated by iron ions that generate hydroxyl radical via the fenton type reactions (Kokilavani *et al.*, 2005).

Many studies have proposed that fluoride in varying concentration induces increased reactive oxygen species generation, enhanced lipid peroxidation and impaired antioxidant enzyme defense system in blood and tissue of experimental animals by interfering with the major metabolic pathway of living system (Mittal and Flora, 2006).

This study showed that the enhanced lipid peroxidation and decreased activities of antioxidant enzymes play crucial roles in membrane structure lesion, systemic dysfunctions and cell apoptosis. Interestingly, excessive fluoride is highly related to the increased superoxide free radicals and lipid peroxidation. The increased malondialdehyde content and the decreased activities of antioxidant enzymes in this study suggested that the balance between the oxidative system and antioxidant system was broken during fluoride exposure as also suggested by Guo *et al.*, (2003).

Previous studies have reported that activities of antioxidant enzymes including superoxide dismutase and glutathione peroxidase were significantly decreased in animals exposed to fluoride (Ghosh *et al*., 2008; Burgstahle, 2009). It has been reported that fluoride consumption leads to excessive formation of free radical and other reactive substances and diminishes the activity of antioxidant enzymes (Nabavi *et al.*, 2012).

In the present study, a marked decrease in the activities of the antioxidant enzymes such as superoxide dismutase, catalase and reduced glutathione suggest that the pro oxidant-antioxidant balance was disturbed leading to oxidative damage. Prominent decrease was observed after intoxication with fluoride for 40 days compared to 20 days treatment. The loss of superoxide dismutase activity may be explained due to the fact that fluoride ions are among competitive inhibitors of superoxide dismutase activity and the reaction rate for fluoride binding to the active site reaches an equillibrium within a very short period of time (Lawson and Yu, 2003).

Superoxide dismutase converts the dismutase of superoxide anion (o_2-) to a less reactive non radical species, H_2O_2, in the presence of metal ions. It has been reported that fluoride in varying concentration impaired superoxide dismutase activity in liver, kidney, brain, thyroid, and cultured cells (Zhan *et al.*, 2005; Blaszczyk *et al.,* 2008; Nabavi *et al*., 2012).

In our study, the adrenal glutathione level in fluoride treated groups was significantly decreased as compared to control. It has been reported that the GSH redox cycle consist of GSH, GPx, GR and GST, which are the major components of the antioxidant defense system. Coordinated activities of these enzymes maintain intracellular thiol status. GSH play a role in the detoxication of a variety of electrophilic compounds and peroxides via catalysis by glutathione S-transferase and glutathione peroxidase (Awasthi *et al.,* 1995).

Glutathione is involved in several defense processes against oxidative damage protects cells against free radical, peroxides and other toxic compound. Indeed, glutathione depletion increases the sensitivity of cells to various aggression and also has several metabolic effects. It is widely known that a deficiency of GSH within living organisms can lead to tissue disorder and injury (Pacheco *et al.,* 2007).

As a water soluble tripeptide, glutathione is the most abundant intracellular small thiol molecules and a predominant defense against reactive oxygen species in tissues. Glutathione react directly with reactive oxygen species and electrophilic metabolite, protect essential thiol groups from oxidation, promotes the regeneration of α-tocopherol and serves as a substrate for glutathione -related enzymes, such as glutathione peroxidase and glutathiones-transferase (Townsend *et al.*, 2003).

The glutathione play an important role in the maintenance of the intracellular redox state and in the cellular defense against oxidative damage. Our finding the level of glutathione is decreased in the adrenal of fluorotic rats is consistent with the study of Ganie *et al.,* (2010).

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