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ANALYSIS OF RELATIVE GENE EXPRESSION OF ANTIOXIDANT ENZYMES IN ADRENAL GLAND USING REAL TIME QUANTITATIVE PCR IN EXPERIMENTAL FLUOROSIS AND MODULATORY POTENTIAL OF CURCUMIN ADMINISTRATION

A. Shashi* and Manisha Tikka

Department of Zoology and Environmental SciencesPunjabi University, Patiala -147002, India.

***Corresponding Author: A. Shashi**

Department of Zoology and Environmental SciencesPunjabi University, Patiala -147002, India.

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ABSTRACT

The present study was conducted to investigate the efficatory effect of Curcumin on oxidative stress, antioxidant enzymes gene expression and pathological alterations in adrenal gland of rat in sodium fluoride (NaF) intoxication. A total of 36 young Wistar albino rats were randomly divided into six groups. Each group contained six rats. The group I was given deionized water orally for 40 days. The groups II and III were administered with 300 and 600 mg of NaF/kg b.w./day for same period. The group IV was given 200 mg/kg b.w./day of Curcumin only for 20 days. The groups V and VI exposed to NaF toxicity of 300 and 600 mg/kg b.w./day for 40 days respectively were post-treated with 200 mg/kg b.w./day of Curcumin for 20 days. At the end of experimental period, animals were sacrificed under anaesthesia and adrenal gland was removed and processed for biochemical, molecular and histopathological analysis. The levels of malondialdehyde (MDA), reduced glutathione (GSH) and the activities of antioxidant enzymes such as mitochondrial superoxide dismutase 2 (MnSOD or SOD 2), glutathione peroxide (GPx), and catalase (CAT) were determined. The gene expression of mitochondrial SOD 2 and GPx was evaluated by using real time RT-PCR.The histopathological alterations in adrenal gland tissue were examined using hematoxylin and eosin staining technique. Results revealed that NaF treatment induced significant ($P<0.0001$) elevation in lipid peroxidation and SOD 2 activity while significant ($P<0.0001$) reduction in the activities of GPx, CAT and GSH content was observed. The relative gene expression of SOD 2 was significantly ($P<0.05$-0.01) upregulated, where as GPx was significantly ($P<0.05$-0.01) downregulated on fluoride exposure. Histopathological evaluation also revealed that NaF intoxication resulted into loss of cellular architecture and distorted cell behaviours including ruptured cell membrane, pyknotic nuclei, and necrosis in the adrenal gland cells. Interestingly, Curcumin supplementation markedly minimized the biochemical and molecular changes and also normalized the cellular adversities caused by the NaF exposure in adrenal gland of rat and, therefore, showed ameliorative effect by eliminating the ill effects.

KEYWORDS: Adrenal gland; Antioxidant enzymes; Curcumin; Gene expression; Oxidative stress, Fluorosis.

Abbreviations

ACTH (adrenocorticotropic hormone), B.W. (body weight), CAT (catalase), g (gram), GPx (glutathione peroxidase), GSH (reduced glutathione, GSSG (oxidized glutathione), g (gram), Kg (kilogram), mg (milligram), NaF (sodium fluoride), ROS (reactive oxygen species), SOD 2 (mitochondrial superoxide dismutase), ZG (zona glomerulosa), ZF (zona fasciculata), ZR (zona reticularis).

INTRODUCTION

Fluorosis is one of the environmental health issue related with excess fluoride in ground water. According to World Health organization and Bureau of Indian Standard, the permissible limit of fluoride in drinking water is 1.5 mg/L and 1.0 mg/L respectively (Arlappa *et al.*, 2013; Shyam *et al.*, 2021). Excessive intake of fluoride than optimum level can lead to dental and skeletal fluorosis (Shashi and Bhardwaj, 2011; Shashi *et al.*, 2008) and non skeletal fluorosis (Shashi, 2003). The researchers demonstrated that fluoride is toxic to all of different tissues both in virto and in vivo which was responsible for cell death, necrosis and apoptosis (Barbier *et al.*, 2010; Agalakova and Gusev, 2012).

The adrenal gland is one of the most important endocrine gland affected by chemically induced abrasions. It is necessary to understand the structure and function of the adrenal gland to know the affect and mechanism of drug induced changes. It consists of adrenal cortex and

medulla. The adrenal cortex secretes the steroid hormones and medulla contains chromaffin cells which secretes epinephrine and norepinephrine hormones (Rosol *et al.,* 2001). It is well known that fluoride stimulates the increase in the production of free radicals and also causes decrease in the activity of antioxidant enzymes, and ultimately plays a vital role in inducing fluorosis (Barbier *et al.,* 2010; Bouaziz *et al.,* 2007).

Superoxide dismutase 2 is a mitochondrial enzyme, also known as manganese- dependent superoxide dismutase (MnSOD). It is an enzyme coded by SOD 2 gene in human on chromosome number 6. This gene is a mitochondrial member of the iron/manganese superoxide dismutase family. The mitochondrial protein encoded by SOD 2 gene forms a homotetramer and binds one manganese ion per subunit. Superoxide is a toxic byproduct of the mitochondrial electron transport chain and SOD 2 convert it into hydrogen peroxide and diatomic oxygen. Thus, plays important role in removal of mitochondrial reactive oxygen species (ROS) and provide protection against cell death, oxidative stress, ionizing radiation, and inflammatory cytokines (Pias *et al.,* 2003; Becuwe *et al.,* 2014). Glutahione peroxidase is a member of enzyme family which has peroxidase activity. The important role of this enzyme is to protect the organism from oxidative damage (Muthukumar and Nachiappan, 2010). Catalase is an enzyme present in all living organisms which when exposed to oxygen, catalyzes the decomposition of hydrogen peroxide to water and oxygen. It plays an important role in protecting the cell from oxidative damage formed by reactive oxygen species (ROS) (Chelikani *et al.,* 2004).

The adrenal gland expresses the enzymes mitochondrial superoxide dismutase 2 (MnSOD; also known as SOD 2) and glutathione peroxide (GPx) to detoxify superoxide eventually to H2O. The over expression of SOD 2 was shown to decrease the mitochondrial superoxide in hippocampal neurons and life span of mice was extended (Hu *et al.,* 2007). SOD 2 is the most important antioxidant enzyme which scavenges superoxide radicals formed by electron transport chain in mitochondria. Electrons departed from the respiratory chain react with oxygen molecule and form superoxide and peroxynitritre. Superoxide and its metabolites such as ROS targets the protein, lipid and nucleic acid and finally results into altered structure and function of the cell (Turrens, 1997).

Superoxide is converted into H_2O_2 by superoxide dismutase enzyme which is neutralized by GPx. If this neutralization is not proper, excess of H_2O_2 is transformed into toxic hydroxyl radicals via Fenton reaction (Halliwell and Gutteridge, 1990). Hence, SOD 2 and GPx constitutes the two step process which exists in mitochondria to scavenge free radicals. The reduction in the level of mRNA is related with the elevated level of SOD 2 mRNA, when induced by adrenocorticotropic hormone (ACTH). Thus, indicates imbalance between SOD 2 and GPx ratio. This resulted in the H_2O_2 accumulation for the Fenton reaction and lead to generation of toxic hydroxyl radicals. Such mechanism was also reported in other condition (Haan *et al.,* 1997).

Curcumin is an active ingredient of *Curcuma longa* and widely used as traditional medicine in India, China and Thailand. Curcumin is also known to possess many pharmacological effects due to its anti-carcinogenic, anti-inflammatory and antioxidant properties (Beevers and Huang, 2011). Additionally, it has also been efficiently used as an important factor in alleviating the oxidative stress caused by fluoride induced toxicity in heart, brain and liver (Nabavi *et al.,* 2011; Sharma *et al.,* 2014; Moghadaam *et al.,* 2015). However, whether Curcumin has the ability to ameliorate fluoride induced toxic effects in the adrenal glands of fluoridated rats or not, is not known yet so far. To our knowledge, there are no scientific reports which elucidate the ameliorative effects of Curcumin against fluoride-intoxication. Therefore, to address this issue, present study was carried out to evaluate the ameliorative effect of Curcumin against sodium fluoride toxicity in rat adrenal gland.

MATERIALS AND METHODS

Sodium fluoride and Curcumin were purchased from Loba Chemie Pvt. Ltd, Mumbai, India. All chemicals used in the experiment were analytic grades.

Animals and treatment

A total of 36 young Wistar albino rats weighing 150-200 g were housed in polypropylene cages with stainless still grill tops and fed standard rat pellet diet (Hindustan Lever Limited, India) and water was given *ad libitum*. After one week of quarantine period, rats were randomly divided into six groups and each group consisted of six rats. The group I was given deionized water orally for 40 days, groups II and III were administered with 300 mg and 600 mg of NaF/kg b.w./day, respectively, for the same period. Group IV was given 200 mg/kg b.w./day of Curcumin for 20 days. However, groups V and VI were initially treated with 300 mg and 600 mg of NaF/kg b.w./day, respectively, for 40 days followed by post-treatment with 200 mg/kg b.w./day of Curcumin for 20 days. At the end of experimental period, the overnight fasting rats were sacrificed under anaesthesia. The adrenal tissues were removed, washed in 0.9% normal saline and processed further for biochemical, molecular and histopathological analysis.

Biochemical analysis

For biochemical evaluation, adrenal tissue from all the experimental groups was homogenized in 0.1 M phosphate buffer (pH 7.4) and centrifuged at 3000 rpm for 10 minutes.The supernatant was used for the assay.

Detection of fluoride

The fluoride in adrenal tissue of control and experimental rats was extracted by method of

Inkielewicz *et al.* (2003). The concentration of fluoride in adrenal tissue was determined by method of Harwood, 1969.

Detection of oxidative stress parameters

The level of MDA in adrenal tissue of rat was measured by the method of Ohkawa *et al*.(1979). The GSH level was determined by using method of Moron *et al.* (1979).

Assay of antioxidants enzymes

The activity of SOD 2 enzyme was measured by using ELISA kit (Elabscience, Hubei) following method given in respective datasheets on ELISA Reader (Rayto, RT-2100C microplate reader, Shenzhen, China). The GPx activity was determined by method of Rotruck *et al.* (1973). The catalase activity was measured by method of Aebi (1983).

Molecular analysis

For evaluating the effect of NaF induced toxicity and Curcumin at molecular level, adrenal tissue from all the experimental groups was homogenized in phosphate buffer saline (pH 7.4) and stored in -80 ºC until further use.

RNA extraction and reverse transcriptase quantitative PCR (RT-qPCR)

Total RNA was extracted from adrenal gland using Trizol reagent (Sigma) by method of Chomczynski and Sacchi (2006). The total RNA concentration was determined by measuring the optical density at 260 nm and a 260 nm/280 nm ratio, respectively. The mRNA isolation was done using dT column. For the expression detection of SOD 2 and GPx and GAPDH mRNA was reversely transcribed into cDNA using Revert Aid^{TM} Synthesis Kit (Fermentas) in a thermocycler (MWG Biotech). A set of specific forward and reverse primers for the amplification of GAPDH, GPx and SOD 2 was selected from published paper (Suwa *et al.*, 2000). The forward and reverse primers used in real time RT-PCR with product length and accession number are given in Table 1. The amplified PCR products were stained with ethidium bromide, run in 1% agarose gel and visualized in Gel Documentation system and photographed (VWR International).

The real time quantitative PCR analysis was conducted using SYBR green mix (Applied biosystem). GAPDH served as the housekeeping gene to normalize the expression of SOD 2 and GPx. RT-qPCR was performed in RT-qPCR instrument (ABI- Prism SDS 7000). The cycling parameter for Real time RT-PCR reaction was as follows: denaturation at 95 ºC for 20 seconds, annealing at 50 ºC for 20 seconds and extension at 72 ºC for 40 seconds. A total of 40 cycles were repeated. The cycles were followed by 72 ºC for 3 minutes. The threshold cycle (Ct) value was obtained using 7000 system software. Each Ct value of the target gene was normalized with the corresponding Ct values of the GAPDH gene. The relative gene expression of target gene was determined by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The gene expression values of the control group-I (1 mL deionized water) of experiment was used for the gene expression calibration, where the value of control was observed as 1. Therefore, the value higher or lower than control (i.e.1) was presented as overexpressed and downregulated respectively. Each reaction was conducted in triplicate and repeated at least three times.

Histopathological evaluation

Adrenal glands from control, fluoridated and fluorotic rats post-treated with Curcumin were removed, fixed in Bouin's fluid for 24 hours, washed in 70% alcohol, dehydrated in 80%, and 90% alcohol, tertiary butyl alcohol for 6 hours, cleared in amyl acetate, and embedded in Paraffin wax. Serial sections were cut at 7 µm and stained with haematoxylin and eosin (Drury and Wallington, 1967). Histopathological changes were studied under research binocular microscope (Leica microsystem) and subsequently microphotographed.

Data analysis

Results were expressed as mean ±SD. All analysis was performed using SPSS 20.0 statistical software (IBM). Data was analyzed using one-way analysis of variance (ANOVA) followed by Post hoc Tukey's HSD and pair wise comparison by Bonferroni multiple comparison test. The results were considered significant at $P<0.05$. The relationships between concentration of fluoride and biochemical parameters were determined by Pearson's bivariate correlation and simple linear regression test.

RESULTS

NaF induction resulted into enhanced fluoride levels

A significant ($P<0.0001$) increase in the level of fluoride was observed in group IIand III in comparison to control group I. The fluoridated groups IV and V post-treated with 200 mg Curcumin showed significant ($P<0.0001$) decrease in fluoride (Table 2, Fig. 1).

Curcumin administration displayed curative effects by relieving NaF induced oxidative stress: Biochemical evaluation

Lipid peroxidation

The result of lipid peroxidation (MDA) as shown in Table 2 revealed that administration of NaF showed significant increase ($P<0.0001$) in the level of MDA in groups II and III as compared to control-1 (Fig. 2A).Pearson's bivariate correlation and simple linear regression analysis demonstrated significant ($P<0.0001$) positive relationship between levels of fluoride and MDA in adrenal gland (Pearson $r = 0.974$, $R^2 = 0.948$, $Y = 1.609+3.911X$; Fig. 2B) after 40 days of fluoride treatment.

Furthermore, after the post-treatment with Curcumin the level of MDA was significantly ($P<0.0001$) decreased in group V and VI in comparison to group II and III

respectively (Fig. 2C). These results advocated the anti-oxidative properties of Curcumin in fluoridated rats.

Reduced glutathione

There was significant (P<0.0001) reduction in the glutathione content in adrenal gland of both fluoridated groups of rats. (Table 2; Fig. 3A). Pearson's bivariate correlation and simple linear regression analysis demonstrated significant (P<0.0001) negative relationship between levels of fluoride and GSH in adrenal gland (Pearson r = -0.979, R^2 = 0.958, Y = 10.343-3.553X; Fig. 3B) after fluoride exposure of 40 days. However, post-treatment with Curcumin restored the GSH content in rat adrenal (Table 2, Fig. 3C) and hence showed curative effects.

Antioxidant enzymes

To determine the effect of various conditions (i.e. NaF induction and post-treatment with Curcumin), activity of antioxidant enzymes was evaluated. The effect of Curcumin on the activities of SOD 2, GPX and CAT in NaF intoxicated rat are shown in Table 3. The activity of SOD 2 enzyme significantly (P<0.0001) elevated in group II and III in comparison to control group I (Fig. 4A). Pearson's bivariate correlation and simple linear regression analysis demonstrated significant (P<0.0001) positive relationship between levels of fluoride and SOD 2 activity in adrenal gland of rats (Pearson r = 0.954, R^2 = 0.909, Y = 0.185+0.099X; Fig. 4B) after 40 days of fluoride intoxication. However, after post-treatment with Curcumin, the activity of SOD 2 decreased significantly (P<0.05) in group V and VI in comparison to group II and III (Fig. 4C).On the other hand, the activities of GPx and CAT were significantly (P<0.0001) decreased in group II and III as compared to group I. Pearson's bivariate correlation and simple linear regression analysis demonstrated significant (P<0.0001) negative relationship between levels of fluoride and activity of GPx (Pearson r = -0.967, R^2 = 0.935, Y = 10.456-3.090X; Fig. 5B) and CAT in adrenal gland (Pearson r = -0.966, R^2 = 0.933, Y = 5.574-1.986X; Fig. 6B) after 40 days of fluoride treatment. Expectedly, the fluoridated rats when post-treated with Curcumin showed significantly upregulated activity of both the GPx and CAT enzymes in group V (P<0.01) and VI (P<0.0001) (Table 3; Fig. 5C and 6C).

Curcumin possessed restorative effects and normalized the gene expression similar to control group: Molecular evaluation.

All the experimental groups were evaluated for the expression of anti-oxidant genes using RT-qPCR. The relative gene expressions of the targeted genes i.e. SOD 2 and GPx were normalized with the housekeeping gene GAPDH and further confirmed for primer specificity with the help of gel electrophoresis images showing specific band sizes of appropriate lengths i.e. 351, 387 and 424 bp in case of GAPDH, SOD 2 and GPx gene respectively (Fig. 7A, 7B and 8A).

The relative gene expression of targeted gene i.e. SOD 2 was significantly upregulated in NaF treated groups with value 1.524 ± 0.074 in 300 mg NaF group (P<0.05) and 2.377 ± 0.097 in 600 mg NaF group (P<0.01) in comparison to control-1 (Fig. 7C). In consistence with other results, post-treatment with Curcumin resulted into significant (P<0.05) decrease in the values i.e. 1.200 ± 0.038 in 300 mg NaF+200 mg curcumin group and 1.632 ± 0.146 in 600 mg NaF+200 mg Curcumin treated group in comparison to respective 300 mg NaF and 600 mg NaF group. However, a significant downregulation (P<0.05) in the gene expression with value 0.845 was observed in Curcumin (200 mg) only treatment group i.e. control-2 when compared to control-1 (Fig. 7D)

Homogenized tissue samples from all the experimental groups were also used for evaluating the expression of another anti-oxidative marker gene i.e. GPx (Fig. 8). The relative gene expression of GPx was significantly downregulated with the values 0.820 ± 0.023 in 300 mg NaF group (P<0.05) and 0.599 ± 0.044 in 600 mg NaF group (P<0.01) in comparisonto control-1 (Fig. 8B). Like previously mentioned results, in comparison to 300 mg NaF treatment (0.820 ± 0.023), a significant (P<0.01) increase in the relative gene expression of GPx with the value 1.110±0.067 was observed when Curcumin was administered (300 mg NaF + 200 mg Curcumin). On the other hand, similar significant (P<0.05) increase in the expression of targeted gene i.e. GPx with the values 0.935 ± 0.044 was observed under Curcumin administration (600 mg NaF + 200 mg Curcumin) when compared to 600 mg NaF treated group (0.599 ± 0.044). However, control group (C2) which was given 200 mg Curcumin only was shown to have highest expression for GPx (Table 4, Fig 8C). These results revealed that Curcumin possess curative effects due to its anti-oxidative properties and helps in relieving oxidative stress by regulating the expression of anti-oxidative genes in adrenal gland of fluoridated rat.

Curcumin treatment normalized the NaF induced adversities in adrenal gland: Histopathological lesions

In the adrenal gland of control group, both of the adrenal cortex and adrenal medulla were evaluated for the histological examinations. The cortex contained three zones, zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) (Fig. 9A). In the rats treated with 300 mg/kg b.w./day NaF for 40 days, there was loss of architecture in all the three zones. The blood capillaries of ZR were markedly congested (Fig. 9B). Some cells partially lost their cell membranes and a cytoplasmic syncytium was formed between them. The blood sinusoids appeared collapsed between the cells (Fig. 9C). The ZR showed inflammatory cells infiltration and cellular debris. The numerous apoptotic cells were also found (Fig. 9D).

In rats treated with 600 mg/kg b.w./day NaF for 40 days, increased capillary densities that were dilated and

hyperemic, were observed (Fig. 9E). The adrenal gland showed irregular capsule, the ZG and ZF cells lost their familiar organization and pyknotic nuclei (Fig. 9F). The ZF contained vacuolated cytoplasm and disrupted cell boundaries and increase in lipid droplets (Fig. 9G). The ZR showed irregular anastomosing cords separated by wide blood capillaries (Fig. 9H). The cells of adrenal medulla were irregular and damaged. Chromaffin cells of adrenal medulla showed granular depletion. Apoptotic changes were also observed (Fig. 9I).

In the rat treated with 200 mg/kg b.w,/day of Curcumin showed normal architecture as that of control (Fig. 10A). The rats post-treated with 200 mg/kg b.w./day of Curcumin for 20 days after 300 mg/kg b.w./day NaF for 40 days revealed nearly normal structure of adrenal gland ZG and ZF cells were more or less similar to those of control. They appeared more compact with moderately vacuolated cytoplasm (Fig. 10B). Most of the chromaffin cells were normal in clusters with few vacuolar degeneration (Fig. 10C).

In the rats post-treated with 200 mg/kg b.w./day of Curcumin after 600 mg/kg b.w./day of NaF demonstrated ZG with few vacuolated cells, most of the cells of ZF were shown to have rounded vesicular nuclei, however, some displayed vacuolated faintly stained cytoplasm and few pyknotic nuclei (Fig. 10D). The network of ZR was almost preserved. Most of the cells exhibited large rounded nuclei with few vacuoles in the cytoplasm (Fig. 10E).The adrenal medulla was restored (Fig. 10F). These results displayed restorative effects of Curcumin against NaF induced toxicity in adrenal gland of rat.

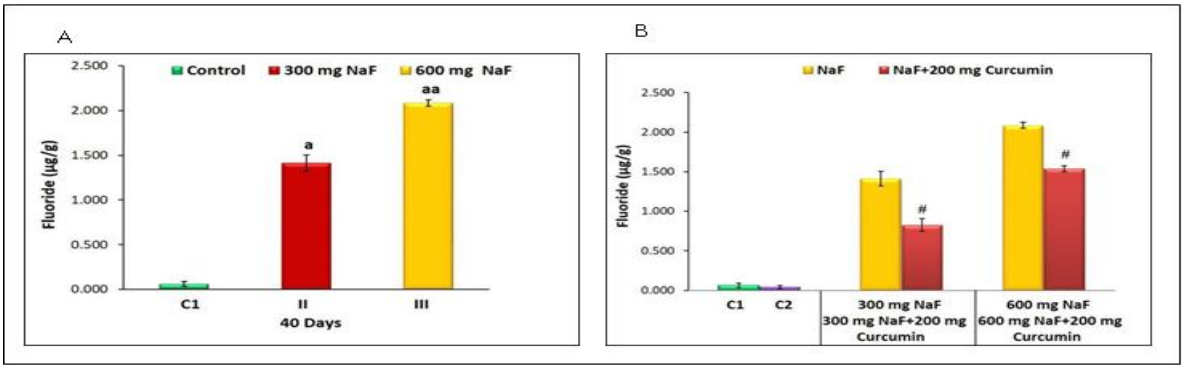

Fig. 1: A. Mean levels of fluoride (µg/g) in adrenal gland of control and fluoridated rats. [a]P<0.0001 Group II-III compared with control-1 [aa]P<0.0001 Group II compared with group III. B. Mean levels of fluoride (µg/g) in adrenal gland of fluoridated rats post-treated with Curcumin. [#]P<0.0001 values were significantly different as compared to respective NaF treated groups.

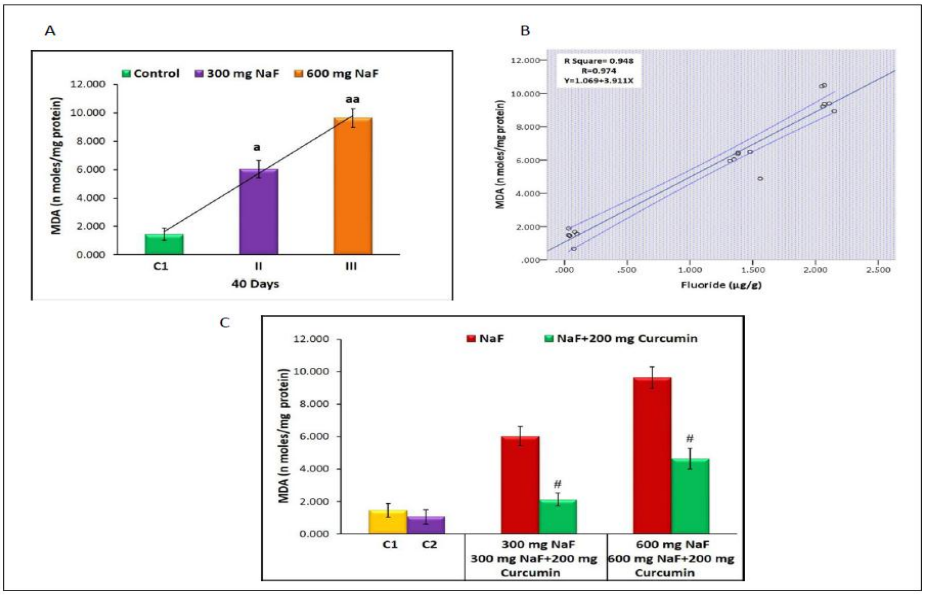

Fig. 2 A. Mean levels of MDA (n moles /mg protein) adrenal gland of control and fluoride treated rats. P<0.0001 Group II-III compared with control-1. [aa]P<0.0001 Group II compared with group III. B. Scatterplot showing Pearson's bivariate correlation and simple linear regression between level of fluoride (µg/g) and MDA (n moles/mg protein) in adrenal glandin test rats after 40 days of fluoride exposure. C. Mean levels of MDA (n moles/mg protein) in adrenal gland of fluoridated rats post-treated with Curcumin. [#]P<0.0001 values were significantly different as compared to respective NaF treated group.

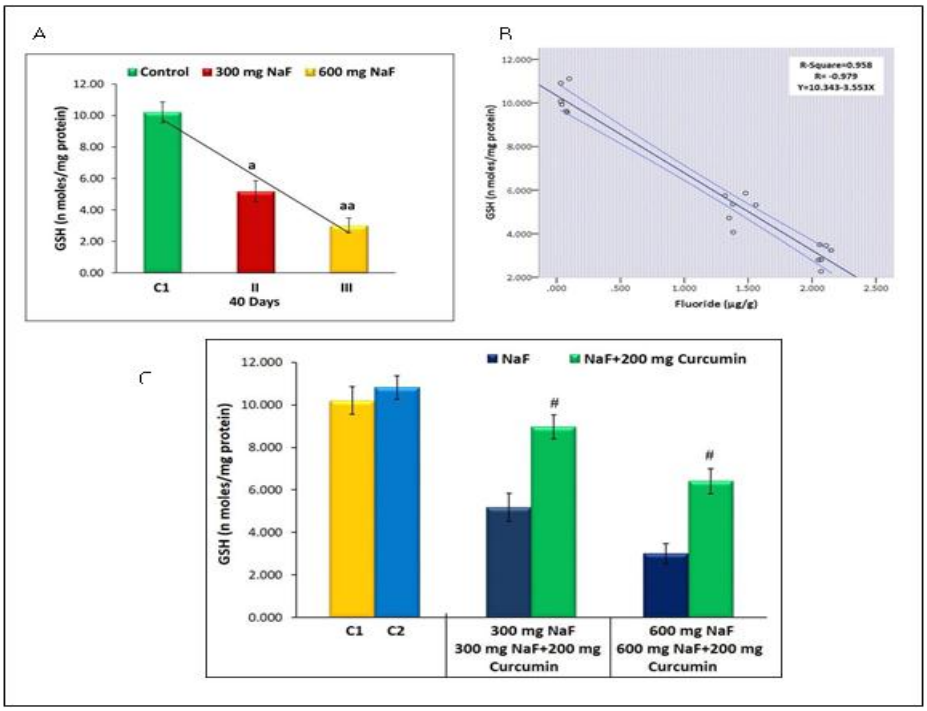

Fig. 3 A. Mean levels of GSH (n moles/mg protein) in adrenal gland of control and fluoride treated rats for 40 days. [a]P<0.0001 Group II-III compared with control-1. [aa]P<0.0001 Group II compared with group III. B. Scatterplot showing Pearson's bivariate correlation and simple linear regression between level of fluoride (µg/g) and GSH (n moles/mg protein) in adrenal gland in experimental rats after 40 days of fluoride intoxication. C. Mean levels of GSH (n moles/mg protein) in adrenal gland of fluoride treated rats after post-treatment with Curcumin. [#]P<0.0001 values are significantly different as compared to respective NaF treatedgroups.

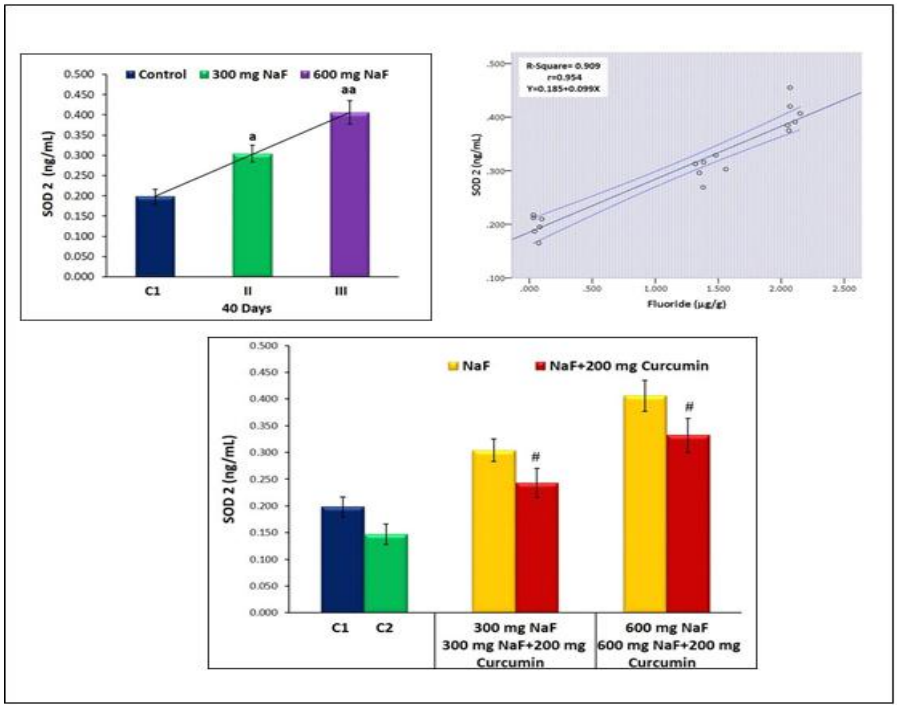

Fig. 4 A. Mean activity of mitochondrial superoxide dismutase (SOD 2) (ng/mL) in adrenal gland of control and fluoride treated rats for 40 days. [a]P<0.0001 Group II-III compared with control-1 .[aa]P<0.0001 Group II compared with group III. B. Scatterplot showing Pearson's bivariate correlation and simple linear regression between level of fluoride (µg/g) and activity of mitochondrial superoxide dismutase 2 (SOD 2) (ng/mL) in adrenal gland of test rats after 40 days of fluoride exposure. C. Mean activity of SOD 2 (ng/mL) in adrenal gland of fluoride treated rats post-treated with Curcumin. [#]P<0.05 values were significantly different as compared to respective NaF treated group.

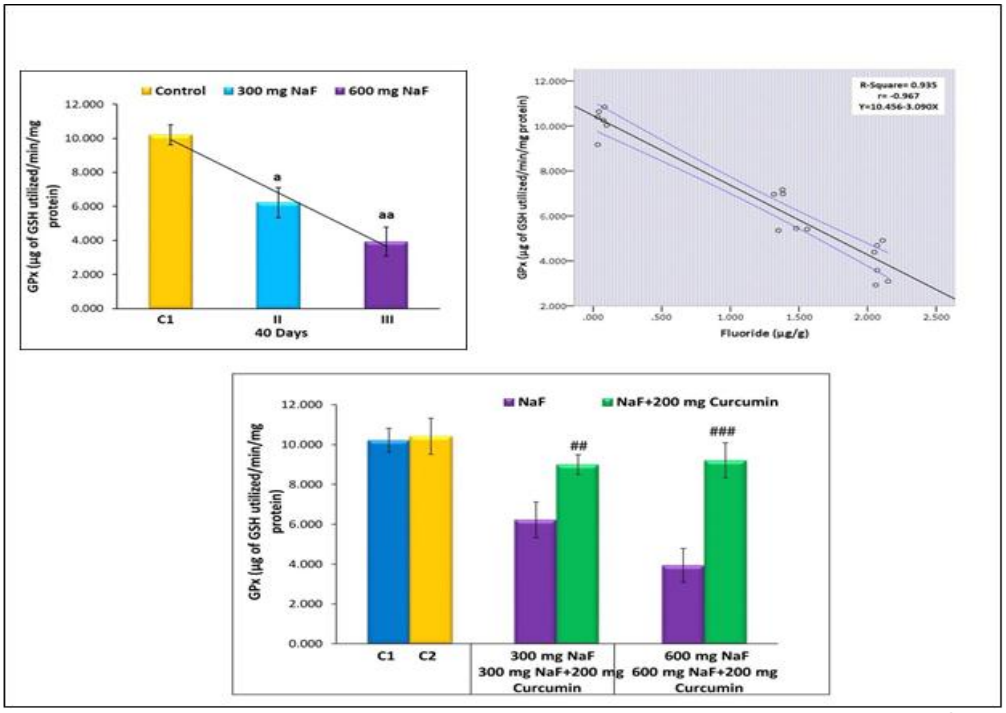

Fig. 5 A. Mean activity of GPx in adrenal gland of control and fluoride exposed rats. [a]P<0.0001 Group II-III compared with control-1. [aa]P<0.0001 Group II compared with group III. B. Scatterplot showing Pearson's bivariate correlation and simple linear regression between level of fluoride (µg/g) and GPx activity (µg of GSH utilized/min/mg protein) in adrenal gland of rats after 40 days of fluoride treatment. C. Mean activity of GPx (µg of GSH utilized/min/mg protein) in adrenal gland of fluoridated rats post-treated with Curcumin. [##]P<0.01, [###]P<0.0001 values were significantly different as compared to respective NaF treated group.

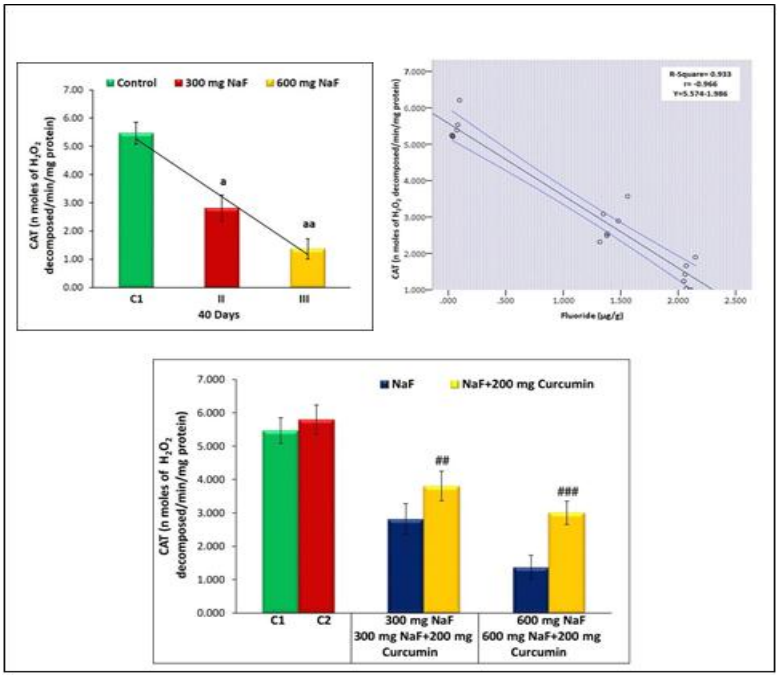

Fig. 6 A. Mean activity of catalase (n moles H2O2 decomposed/min/mg protein) in adrenal gland of control and fluoride exposed rats. [a]P<0.0001 Group II-III compared with control-1. [a,a]P<0.0001 Group II compared with group III. B. Scatterplot showing Pearson's bivariate correlation and simple linear regression between level of fluoride (µg/g) and activity of catalase (n molesH2O2 decomposed/min/mg protein) in adrenal gland of fluoridated rats. C. Mean activity of GPx (µg of GSH utilized/min/mg protein) in adrenal gland of fluorotic rats post-treated with Curcumin. [##]P<0.01, [###]P<0.0001 values were significantly different as compared to respective NaF treated group.

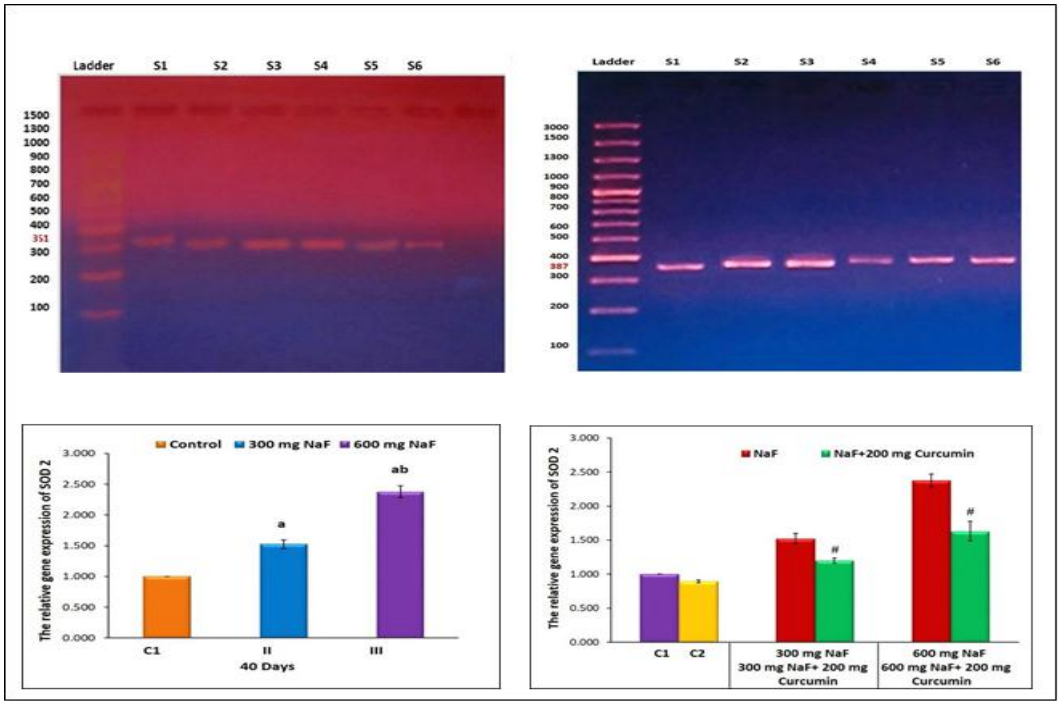

Fig. 7. Gel Electrophoresis showing PCR products of A. GAPDH gene. B. SOD 2 gene. Lane S1-Control-1, Lane S2-300 mg NaF, Lane S3-600 mg NaF, Lane S4-Control-2, Lane S5-300 mg NaF + 200 mg Curcumin, Lane S6-600 mg NaF+ 200 mg Curcumin. Control group I was observed as 1.0. C. The relative gene expression of SOD 2 gene in adrenal gland of experimental rats. One way ANOVA followed by Post hoc Tukey's HSD test. D. The relative gene expression of SOD 2 gene of fluoridated rat after post-treatment with Curcumin. The pair wise comparison was done by Bonferroni multiple comparison test. $^{a}P<0.05$, $^{b}P<0.01$, Group II-III compared with control-1. $^{ab}P<0.001$ Group II compared with group III. $^{\#}P<0.05$, values were significantly different as compared to respective NaF treated group.

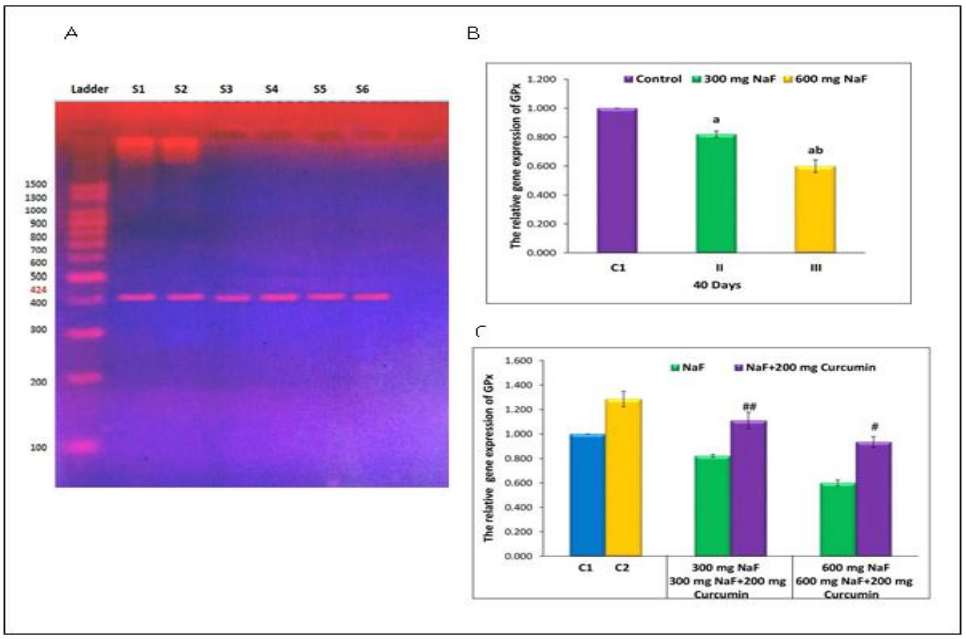

Fig. 8.A. Gel Electrophoresis showing PCR products of GPx. Lane S1-Control-1, Lane S2- 300 mg NaF, Lane S3-600 mg NaF, Lane S4-Control-2, Lane S5-300 mg NaF + 200 mg Curcumin, Lane S6- 600 mg NaF+ 200 mg Curcumin. Control group I was observed as 1.0. The relative gene expression of GPx gene in adrenal gland of experimental rats. One way ANOVA followed by Post hoc Tukey's HSD test. C. The relative gene expression of GPx gene of fluoridated rat after post-treatment with Curcumin. The pair wise comparison was done by Bonferroni multiple comparison test. $^{a}P<0.05$, $^{b}P<0.01$, Group II-III compared with control-1. $^{a,b}P<0.05$ Group II compared with group III. $^{\#\#}P<0.01$, $^{\#}P<0.05$ values were significantly different as compared to respective NaF treated group.

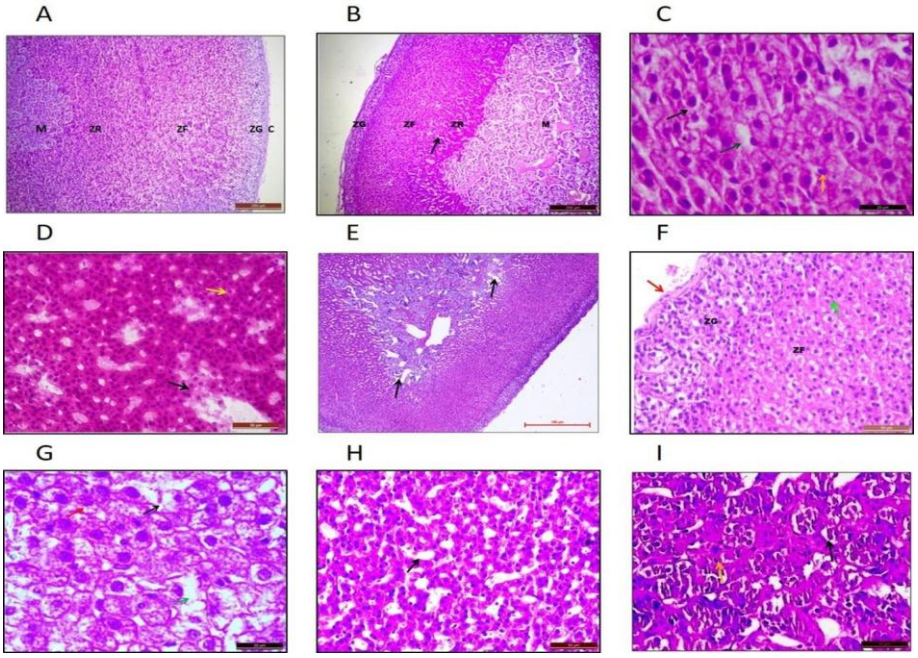

Fig. 9. T.S. of the adrenal gland stained with Haematoxylin and Eosin. A. Control group showing adrenal cortex, capsule (C), zona glomerulosa (ZG), zona fasciculata (ZF), zona reticularis (ZR) and adrenal medulla (M) X100. The adrenal gland of rat treated with 300 mg/kg b.w./day of NaF showing B. Loss of normal architecture of ZG, ZF, ZR and M congested blood capillaries (↑) in ZR.X100. C. ZF cells having cytoplasmic syncytium(↑), collapsed sinusoid (↑) and pyknotic nuclei (↑). X1000. D. ZR contained infiltration of inflammatory cell (↑) and apoptotic cells (↑).X400. The adrenal gland of rat treated with 600 mg/kg b.w./day of NaF showing E. increase in capillary densities that are dilated and hyperemic (↑).X100. F. Irregular capsule (↑), ZG, ZF, cells lost their familiar organization and pyknotic nuclei (↑).X400. G. ZF Cells showing cytoplasmic vacuolation (↑), lipid droplets (↑) and disrupted cell boundaries (↑). H. Zona reticularis of adrenal gland of rat showed irregular anastomosing cords separated by wide blood capillaries (↑).X400. I. Adrenal medulla showing shrunken clusters of chromaffin cells (↑) due to depletion of granules and chromaffin cells apoptosis (↑). X400.

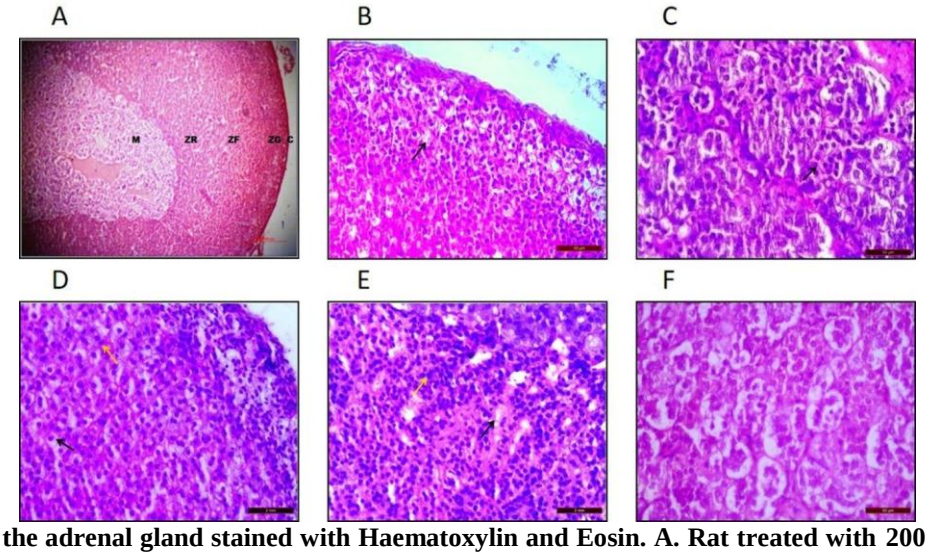

Fig. 10. T.S. of the adrenal gland stained with Haematoxylin and Eosin. A. Rat treated with 200 mg/kg b.w./day of Curcumin for 20 days group showing normal architecture of adrenal cortex, capsule (C), zona glomerulosa (ZG), zona fasciculata (ZF),zona reticularis (ZR) and adrenal medulla (M).X100. Rat treated with 300 mg/kg b.w./day of NaF post-treated with 200 mg/kg b.w./day of Curcumin showing B. compact ZG and ZF cells (↑)

and moderately vacuolated cytoplasm.X400. C. Adrenal medulla showing clusters of chromaffin cells (↑) similar to control.X400. D. Rat treated with 600 mg/kg b.w./day of NaF post-treated with 200 mg/kg b.w./day of Curcumin showed ZF cells has rounded vesicular nuclei, (↑) and fewpyknotic nuclei (↑). X400. E. Cells of ZR with large rounded nuclei (↑) and few vacuole in cytoplasm X400. F. Adrenal medulla showed restoration of chromaffin granules.X400.

Table 1: The list of Primers used in RT-qPCR analyis of gene expression.

Gene	Primer Sequence (5'-3')		Product Size	Accession No.
	Forward primer	Reverse primer		
GAPDH	GCCAAGGTCATCCATGACAAC	AGTGTAGCCCAGGATGCCCTT	351 bp	NM_017008.4
SOD 2	TGACCTGCCTTACGACTATG	CGACCTTGCTCCTTATTGAA	387 bp	NM_017051.2
GPx	CCACCGTGTATGCCTTCTCG	ACCGGGGACCAAATGATGTA	424 bp	NM_030826.4

Table 1: showed the forward and reverse primer with product length and accession number ofGAPDH, SOD 2 and GPx genes.

Table 2: Mean levels of adrenal fluoride, malondialdehyde, glutathione in control and experimental groups.

Treatment Group	Fluoride(µg/g)	MDA (nmoles/mgprotein	GSH(nmoles/mgprotein
I Control -1	0.060±0.028	1.455±0.419	10.203±0.655
II 300 mg NaF	1.412±0.091[a]	6.026±0.604[a]	5.178±0.674[a]
III 600 mg NaF	2.085±0.038[aa]	9.637±0.657[aa]	3.012±0.475[aa]
IV Control-2	0.043±0.019	1.057±0.460	10.814±0.555
V 300 mg NaF+200 mg Curcumin	0.825±0.082[#]	2.130±0.393[#]	8.966±0.578[#]
VI VI 600 mg NaF+200 mg Curcumi	1.537±0.037[#]	4.643±0.643[#]	6.416±0.589[#]

Table 2 showed values expressed as Mean±SD. [a]P<0.0001 Group II-III compared withcontrol-1. [aa]P<0.0001 Group II compared with group III . [#]P<0.0001values were significantly different as compared to respective NaF treated groups II and III.

Table 3: Mean levels of mitochondrial superoxide dismutase 2, glutathione peroxidase and catalase in control and experimental groups.

TreatmentGroup	SOD2 (ng/mL)	GPx (µg of GSH utilized/min/mg protein	Catalase (nmoles H_2O_2decomposed/ min/mg protein)
I Control-1	0.198±0.019	10.220±0.588	5.468±0.384
II 300 mg NaF	0.304±0.021[a]	6.222±0.896[a]	2.813±0.464[a]
III 600 mg NaF	0.406±0.029[aa]	3.933±0.845[aa]	1.375±0.355[aa]
IV Conrol-2	0.147±.0192	10.418±0.090	5.799±0.433
V 300 mg NaF+200 mg Curcumin	0.243±0.027[#]	8.997±0.492[##]	3.807±0.444[##]
VI 600 mg NaF+200 mg Curcumin	0.332±0.032[#]	9.208±0.879[###]	3.004±0.352[###]

Table 3 showed values expressed as Mean±SD. [a]P<0.0001 Group II-III compared withcontrol-1.[aa]P<0.0001 Group II compared with group III. [#]P<0.05values are significantly different as compared to respective NaF treated groups II and III. [##]P<0.01, [###]P<0.0001 values were significantly different as compared to respective NaF treated groups II and III.

Table 4: Relative gene expression of mitochondrial superoxide dismutase 2 and glutathione peroxidase in control and experimental groups.

Treatment Group	SOD 2	GPx
I Control-1	1.000	1.000
II 300 mg NaF	1.524±0.074[a]	0.820±0.023[a]
III 600 mg NaF	2.377±0.097[ab]	0.599±0.044[ab]
IV Control -2	0.845±0.019	1.286±0.063
V 300 mg NaF+200 mg Curcumin	1.200±0.038[#]	1.110±0.067[##]
VI 600 mg NaF+200 mg Curcumin	1.632±0.146 [#]	0.935±0.044[#]

Table 4 showed values expressed in Mean±SD. Control group I was observed as 1.0. In case of SOD 2 [a]P<0.05, [b]P<0.01 Group II-III compared with control-1. [ab]P<0.001 Group II compared with group III . [#]P<0.05 values were significantly different as compared to respective NaF treated groups II and III in. In case of GPx [a]P<0.05, [b]P<0.01 Group II-III compared with control. [ab]P<0.05 Group II compared with group III.[##]P<0.01, [#]P<0.05 values were significantly different as compared to respective NaF treated groups II and III

DISCUSSION

Fluoride causes oxidative stress which leads to the production of excessive ROS, lipid peroxidation, reduction in GSH/GSSH ratio and changes in the activities of antioxidant enzymes thus, inhibits the glycolysis and stimulates the depletion of cellular ATPs and disturbs the cellular metabolism (Agalakova and Gusev, 2012). It was hypothesized that all such disturbed activities under fluoride intoxication can be minimized and controlled to normal levels by using suitable and safer therapeutic agents having anti-oxidative properties.

Lipid peroxidation causes disturbances in the integrity of cellular membranes and thus leads to leakage of cytoplasmic enzymes (Salam and Agha, 2006). Lipid peroxidation can be evaluated by knowing the values of MDA, a lipid peroxidation specific marker whose levels are increased under adverse conditions including fluorosis. It was demonstrated that lipid peroxidation following oxidative stress caused disturbances in the membrane integrity and cell degeneration (Tsou *et al.,* 2004). Furthermore, ROS induced changes in the protein properties. Therefore, it affects the receptor function, enzymes, antibodies and transport proteins and results into changes in the DNA. In the present study, rats when induced with different concentrations of NaF, showed increased MDA levels in the adrenal gland. However, when fluoridated rats were post-treated with Curcumin, MDA levels were decreased and hence curative effects were implicated.

In accordance with the present study results, similar findings were reported in a study which demonstrated that level of MDA was significantly ($P<0.05$) increased by 1.9, 5, 3.5 and 3.1 fold at days 1, 2, 3, 5 respectively in adrenal gland of male rats after administration of 2,3,7,8-tetracholotrodibenzo-p-dioxin (Bestervelt *et al.,* 1994). More recently, Khalaf *et al.* noticed similar observations in their study focused on nicotine induced toxicity on adrenal zona fasciculata (Khalaf *et al.,* 2017).

GSH play role in several defense mechanisms against oxidative damage, conserve the cell against free radicals, peroxide and other toxic compounds. Reduction in the glutathione level increases the sensitivity of cells to various aggressions and also causes several metabolic effects. Deficiency of GSH within the living organisms can cause tissue disorder and injury (Limon-Pacheco *et al.,* 2007). These findings are similar with Fattah *et al.* who noticed that administration of nicotine caused 56% decrease in GSH content and increase in the MDA level (Fattah *et al.,* 2019). Another researcher also reported significant enhanced levels of MDA in the adrenal tissue of endotoxemic mice. However, the resveratrol treatment showed restoration of elevated level of MDA ($P<0.01$) in adrenal gland of endotoxemic mice (Duan *et al.,* 2016).

NaF induced toxicity has been related to increase in the ROS and free radicals and depletes the antioxidant system in body. Likewise, reduced GPx, SOD 2 and CAT activity was observed in the liver, kidney, testis and brain tissues of NaF treated rats (Hamza *et al.,* 2015). Researchers have also observed adrenal insufficiency, elevation in MDA and lipid peroxidation levels while reduction in the activity of GPx, glutathione reductase, and GSH in the rats treated with toxicants such as carbon tetrachloride and tramadol (Fakunle *et al.,* 2013; Abdelaleem *et al.,* 2017).

The present study demonstrated that the activity of mitochondrial SOD 2 was increased in adrenal gland of fluoridated rats which was further reduced under the effect of Curcumin. Additionally, the present study revealed similar adrenal disturbances when rats were intoxicated with NaF. Furthermore, Curcumin treatment exhibited its curative effects by normalizing the adrenal insufficiencies like their normal counterparts (control group).

Similar observations were reported by Klivenyi *et al.* who demonstrated that the 1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine toxicity increased the MnSOD activity in brain tissue (Klivenyi *et al.,* 1998). Raza *et al.* documented that pretreatment with ACTH caused increase in the activity of MnSOD in the adrenal gland (Raza *et al.,* 2005). The similar increase in the MnSOD activity was also noticed in the animals fed with a low sodium diet. ACTH stimulated the imbalance between MnSOD and GPx and resulted in the production of excess hydrogen peroxide, which caused downregulation of CYP11B12 and aldosterone synthesis in the glomerulosa (Suwa *et al.,* 2000). The stress stimulates various pathways which leads to increase in the free radicals generation and ultimately causes pathological conditions (Ganesan *et al.,* 2011).

Popovic *et al.* investigated that chronic restraint stress rats showed increase in the SOD2 gene expression. The increase in the activity of SOD2 in stress regime is a sign of relative increase in the generation of superoxide radicals, which could trigger the second line defense involving catalase (Popovic *et al.,* 2017). Jang *et al.* also demonstrated that the activity of MnSOD was significantly ($P<0.05$) increased approximately two fold in kidney, liver and skeletal muscles of young and old transgenic mice (Jang *et al.,* 2009). In accordance with the above mentioned studies, similar abnormalities were displayed by the adrenal gland of NaF intoxicated rats of the present study. The present results revealed that there was significant ($P<0.0001$) decrease in the gene expression of GPx and significant ($P<0.0001$) increase in the gene expression of SOD 2 in fluoride induced toxicity in adrenal gland while Curcumin restored the altered gene expression of SOD 2 and GPx as shown by real time RT-PCR.

The inhibitory effect of fluoride on activity of CAT was observed in people living in endemic fluorosis areas as

well as in animal models exposed to fluoride (Barbier *et al.*2010; Kalyanalakshmi *et al.,* 2007). Ranjan *et al.* also reported same inhibitory effects of NaF on CAT activity within erythrocytes, liver, and kidney in a rabbit model (Ranjan *et al.,* 2009). This effect of fluoride on CAT in our experiment as well as in other studies could be explained by the interaction of fluoride with di- or trivalent metals present in the active site ofantioxidative enzymes, which would lead to the inhibition of the enzyme (Ravula *et al.,* 2012;Yamaguti *et al.,* 2013). Rats administered with high concentration of fluoride caused significant increase in the levels of lipid peroxidation and decrease in the activities of CAT, GPx in the brain (Basha *et al.,* 2011). Fluoride exposure raised the level of MDA, reduce the level of reduced GSH and decrease the activities of CAT, SOD in heart, brain and liver of rat exposed to NaF (Nabavi *et al.,* 2011; Sharma *et al.,* 2014; Moghadaam *et al.,* 2015). Present study revealed similar adverse effects on the CAT activity under NaF induction which were further eliminated and brought to improved levels when Curcumin was administered.

In another study, Althnaian *et al.* demonstrated that Alfatoxin B1 significantly ($P<0.05$) reduced the expression of GPx in the kidney tissue of rat in comparison to the control (Althnaian *et al.*. 2016). The agarose gel electrophoresis of RT-PCR products showed decrease in the GPx expression in tramadol group while the expression was increased in the tramadol withdrawal group (Abdelaleem et al., 2017). Zhou *et al.* reported that the relative mRNA expression levels were significantly down regulated after 70 days of fluoride treatment in the liver (Zhou *et al.,* 2015). The mRNA expression levels of GPx enzyme were down regulated in the ACTH treated group (Weng *et al.,* 2019). In consistence with these studies, present study also displayed reduction in the expression of GPx mRNA by adrenal gland tissue samples under the effect of NaF induction.

In a study by Suwa *et al*. it was demonstrated that transcription of MnSOD gene might be used by corticotropic stimulation, which supports the concept of physiological role. The ACTH treated rat showed significant ($P<0.05$) fivefold increase in MnSOD mRNA content than control and was upregulated by ACTH while significantly reduced to one third mRNA content than control. These changes were recovered by co-administration of vitamin-C or dimethyl sulfoxidewith ACTH (Suwa *et al*., 2000). Similar recovery was shown under Curcumin administration which indicates that antioxidative properties of the therapeutic agent contributes in major therapeutic effects in ameliorating the disturbances caused by any toxicant.

The present finding also revealed histopathological alterations in the adrenal gland of NaF treated rats in a dose dependent manner. A correlation between severities of alterations with increased concentration of NaF was evaluated. Similar finding were reported by Abass *et al.* who demonstrated that atrazine administration resulted in structural and ultrastructural alteration in the adrenal cortex of rats. The increase in vacuolation in the cells ZG, ZF and accumulation of lipid droplets especially in ZF were recorded (Abass *et al.*, 2016). Lipid droplet accumulation specifically in ZF and ZR was also seen in dexamethasone treated rats (Almeida *et al.,* 2006). Other studies have also displayed accumulation of abnormally large quantity of lipids in ZF cells, destroying the cells structure and impair its function (Rosol *et al.,* 2008; Evan, 2009). Khalil also demonstrated marked morphological changes in the adrenal cortex of ketanozole treated rats in the form of enlarged outer cortex, disturbed cytological architecture with shrinkage in glomerulosa and enlarged fasciculata region. These observations indicated that under the effect of toxicant, severe abnormalities in the cellular architecture occurs which further results into functional impairment of the concerned tissue or organ (Khalil, 2015). Present study also revealed similar cytological disturbances under NaF induced cytotoxicity. These changes in the present study which resulted into the functional impairment and injury in the adrenal gland may be due to the oxidative stress as exposure to stress has been found to stimulate various pathways leading to increased productions of free radicals, occurrence of pathological condition and oxidative damage by imbalance between oxidants and antioxidant factors (Ganesan *et al.,* 2011). Interestingly, all such adversities were eradicated and normalized to the levels of healthy rats when Curcumin was administered. Anti-oxidative properties might have played a major role in alleviating NaF associated all ill effects.

Curcumin is a biphenolic, natural, and active component of Curcuma longa. It has antidepressant, antioxidative, anti-inflammatory anti-carcinogenic, antiviral, and anti-infectious activities (Joe *et al.,* 2004). Curcumin showed mechanism of action at various

levels. Curcumin has a potential to change the epigenetic modification and regulate the gene expression and molecular target which is responsible for the tumorigenesis. Furthermore, Curcumin can stimulate the modification of histone by acetylation and deacetylation which are most important epigenetic changes that causes alterations in expression of genes that create a risk of cancer (Hassan *et al.,* 2019). Above results suggested that Curcumin showed recovery against fluoride induced toxicity in oxidative stress, activities of antioxidative enzymes, SOD2 and GPx genes expression.

CONCLUSION

In conclusion, the present results suggest that adrenal gland of rats exposed to NaF toxicity increases the oxidative stress. The administration of Curcumin showed the ameliorative effects on the adrenal gland against fluoride induced oxidative stress. This is cleared by the reduced levels of MDA which is a marker of lipid peroxidation by Curcumin post-treatment. The Curcumin treatment also restored the level of GSH, activities of

GPx and CAT and decreases the activity of SOD 2. The undesired changes in expression of SOD 2 andGPx genes were also normalized by Curcumin administration. Curcumin administration also resulted in improving the abnormal histopathological conditions to the normal status. Overall results revealed that, Curcumin helps in curing the NaF induced cytotoxic effects in adrenal gland of rat.

Ethical approval

The experiments were performed under the approval of Institutional Animal Ethics Committee of Punjabi University, Patiala, India (Animal maintenance and Registration No.107/GO/ReBi/S/99/CPCSEA 2017-19).

Conflict of interest statement

The Authors declare that there is no conflict of interest

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REFERENCES

1. Abass, M.A., Elkhateeb, S.A., Abd El-Baset, S.A., Kattaia, A.A., Mohamed, E.M., Atteia, H.H. Lycopene ameliorates atrazine-induced oxidative damage in adrenal cortex of male rats by activation of the Nrf2/HO-1 pathway. Environ. Sci. Pollut. Res. Int., 2016; 23: 15262-15274. 10.1007/s11356-016-6637-x.
2. Abdelaleem, S.A, Hassan, O.A., Ahmed, R.F., Zenhom, N.M., Rifaai, R.A., El-Tahawy, N.F.,2017. Tramadol induced adrenal insufficiency: histological, immunohistochemical, ultrastructural, and biochemical genetic experimental Study. J. Toxicol, 2017; 9815853. 10.1155/2017/9815853.
3. Aebi, H. Catalase in Vitro. Methods Enzymol, 1983; 105: 121-126. http://dx.doi.org/10.1016/S0076-6879(84)05016-3.
4. Agalakova, N.I., Gusev, G.P. Molecular Mechanisms of cytotoxicity and apoptosis induced by inorganic fluoride. ISRN Cell Biol., 2012; 1-16. https://doi.org/10.5402/2012/403835.
5. Almeida, H., Matos, L., Ferreira, J., Neves, D. Age-related effects of dexamethasone administration in adrenal zona reticularis. Ann. NY Acad, Sci., 2006; 1067: 354-360. 10.1196/annals.1354.050.
6. Althnaian, T. Gene expression and activities of antioxidant enzymes in kidneys of rat intoxicated with alfatoxin B1. J. Biol. Sci., 2016; 16: 65-71. 10.3923/jbs.2016.65.71.
7. Arlappa, N., Qureshi, A.I., Srinivas, R. Fluorosis in India: An overview. Int. J. Res. Dev. Health, 2013; 1: 97‑102.
8. Barbier, O., Arreola-Mendoza, L., Razo, L.M.D. Molecular mechanisms of fluoride toxicity. Chem. Biol. Interact, 2010; 188: 319-333. 10.1016/j.cbi.2010.07.011.
9. Basha, P.M., Rai, P., Begum, S. Evaluation of fluoride induced oxidative stress in rat brain: a multigeneration study. Biol. Trace. Elem. Res., 2011; 142: 623–637. https://doi.org/10.1007/s12011-010-8780-4.
10. Becuwe, P., Ennen, M., Klotz, R., Barbieux, C., Grandemange, S. Manganese superoxide dismutase in breast cancer: From molecular mechanisms of gene regulation to biological and clinical significance. Free Radic. Biol. Med, 2014; 77: 139-151.10.1016/j.freeradbiomed.2014.08.026.
11. Beevers, C.S., Huang, S. Pharmacological and clinical properties of curcumin. Botanics: Targets and Therapy, 2011; 1: 5-18. 10.2147/BTAT.S17244.
12. Bestervelt, L.L., Piper, D.W., Pitt, J.A., Piper, W.N. Lipid peroxidation in the adrenal glands of male rats exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Toxicol. Lett., 1994; 70: 139-145. https://doi.org/10.1016/0378-4274(94)90157-0.
13. Bouaziz, H., Croute, F., Boudawara, T., Soleilhavoup, J.P., Zeghal, N. Oxidative stress induced by fluoride in adult mice and their suckling pups. Exp. Toxicol. Pathol, 2007; 58: 339-349. 10.1016/j.etp.2006.11.004.
14. Chelikani, P., Fita, I., Loewen, P.C. Diversity of structures and properties among catalases. Cell Mol. Life Sci., 2004; 61: 192-208. 10.1007/s00018-003-3206-5.
15. Chomczynski, P., Sacchi, N. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. Nat. Protoc, 2006; 1: 581-585. 10.1038/nprot.2006.83.
16. Drury, A.R., Wallington, E.A. Carleton's histological techniques. 5th edn. Oxford University Press. London, 1967; 140.
17. Duan, G.L., Wang, C.N., Liu, Y.J., Yu, Q., Tang, X.L., Ni, X., Zhu, X.Y. Resveratrol alleviates endotoxemia-associated adrenal insufficiency by suppressing oxidative/nitrative stress. Endocr. J., 2016; 63: 569-580. 10.1507/endocrj.EJ15-0610.
18. Evans, G.O. Animal clinical chemistry: A practical handbook for toxicologists and biomedical researchers. CRC Press. https://doi.org/10.1201/9781420080124, 2009.
19. Fakunle, P.B., Ajibade, A.J., Oyewo, E.B., Olojede, S.O. Some protective effects of ginger against CCl4 induced toxicity in the adrenal cortex of adult Wistar rats (*Rattus norvegicus*). Int. J. Curr. Res., 2013; 5: 551-555.
20. Fattah, S.A., Rizk, A.A.E.E., Motawie, A.G., El-Galil, T.I.A., Sebaie, M.E. Effects of nicotine on rat adrenal gland: crosstalk between oxidative and inflammatory markers, and amelioration by melatonin. Biotech. Histochem, 2019; 94: 234-243. 10.1080/10520295.2018.1545159.

21. Ganesan, B., Anandan, R., Lakshmanan, P.T., Studies on the protective effects of betaine against oxidative damage during experimentally induced restraint stress in Wistar albino rats. Cell Stress Chaperones, 2011; 16: 641-52. 10.1007/s12192-011-0276-4.
22. Haan, J.B.D., Wolvetang, E.J., Cristiano, F., Iannello, R., Bladier, C,, Kelner, M.J., Kola, I. Reactive oxygen species and their contribution to pathology in Down syndrome. Adv Pharmacol, 1997; 38: 379-402. 10.1016/s1054-3589(08)60992-8.
23. Halliwell, B., Gutteridge, J.M. Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol, 1990; 186: 1-85. 10.1016/0076-6879(90)86093-b.
24. Hamza, R.Z., El-Shenawy, N.S., Ismail, H.A. Protective effects of blackberry and quercetin on sodium fluoride-induced oxidative stress and histological changes in the hepatic, renal, testis and brain tissue of male rat. J. Basic Clin. Physiol. Pharmacol, 2015; 26: 237-251. 10.1515/jbcpp-2014-0065.
25. Harwood, J.E. The use of an ion selective electrode for routine fluoride analysis on water sample. Water Res., 1969; 3: 273-80. https://doi.org/10.1016/00431354(69)90024-4.
26. Hassan, F. U., Rehman, M. S., Khan, M. S., Ali, M. A., Javed, A., Nawaz, A., Yang, C. Curcumin as an alternative epigenetic modulator: mechanism of action and potential effects. Front. Genet., 2019; 10: 514. 10.3389/fgene.2019.00514.
27. Hu, D., Cao, P., Thiels, E., Chu, C. T., Wu, G. Y., Oury, T. D., Klann, E., 2007.
28. Hippocampal long-term potentiation, memory, and longevity in mice that overexpress mitochondrial superoxide dismutase. Neurobiol. Learn Mem, 87: 372-384. 10.1016/j.nlm.2006.10.003.
29. Inkielewicz, I., Krechniaka, J. Fluoride content in soft tissues and urine of rats exposed to sodium fluoride in drinking water. Fluoride, 2003; 36: 263-266.
30. Jang, Y.C., Perez, V.I., Song, W., Lustgarten, M.S., Salmon, A.B., Mele, J., Qi, W., Liu, Y., Liang, H., Chaudhuri, A. and Ikeno, Y. Overexpression of Mn superoxide dismutase does not increase life span in mice. J. Gerontol. A. Biol. Sci. Med. Sci., 2009; 64: 1114-1125. 10.1093/gerona/glp100.
31. Joe, B., Vijaykumar, M., Lokesh, B.R. Biological properties of curcumin-cellular and molecular mechanisms of action. Crit. Rev. Food Sci. Nutr., 2004; 44: 97-111. 10.1080/10408690490424702.
32. Kalyanalakshmi, P., Vijayabhaskar, M., Dhananjaya, Naidu, M. Lipid peroxidation and antioxidant enzyme status of adult males with skeletal fluorosis in Andhra Pradesh, India. Fluoride, 2007; 40: 42-45.
33. Khalaf, H.A., Ghoneim, F.M., Arafat, E.A., Mahmoud, E.M. Histological effect of nicotine on adrenal zona fasciculata and the effect of grape seed extract with or without withdrawal of nicotine. J. Microsc. Ultrastruct, 2017; 5: 123-131. https://doi.org/10.1016/j.jmau.2016.11.001.
34. Khalil, M.S. Vitamin D3 may ameliorate the ketoconazole induced adrenal injury: histological and immunohistochemical studies on albino rats. Acta Histochem. Cytochem, 2015; 48: 103-113. 10.1267/ahc.14062.
35. Klivenyi, P., St Clair, D., Wermer, M., Yen, H.C., Oberley, T., Yang, L., Beal M.F. Manganese superoxide dismutase overexpression attenuates MPTP toxicity. Neurobiol. Dis., 1998; 5: 253-258. 10.1006/nbdi.1998.0191.
36. Limon-Pacheco, J.H., Hernandez, N.A., Fanjul-Moles, M.L., Gonsebatt, M.E. Glutathione depletion activates mitogen-activated protein kinase (MAPK) pathways that display organ-specific responses and brain protection in mice. Free Radic. Biol. Med., 2007; 43: 1335-1347. 10.1016/j.freeradbiomed.2007.06.028.
37. Livak, K.J., Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 2001; 25: 402-408. 10.1006/meth.2001.1262.
38. Moghaddam, A.H., Nabavi, S.F., Nabavi, S.M., Loizzo, M.R., Roohbakhsh, A., Setzer, W.N. Ameliorative effects of curcumin against sodium fluoride- induced hepatotoxicity. Progr. in Nutr, 2015; 17: 324-330.
39. Moron, M.S., Depierre, J.W., Mannervik, B. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochim. Biophys. Acta, 1979; 582: 67-78. 10.1016/0304-4165(79)90289-7.
40. Muthukumar, K., Nachiappan, V. Cadmium-induced oxidative stress in Saccharomyces cerevisiae. Indian J Biochem Biophys, 2010; 47: 383-387.
41. Nabavi, S.F., Nabavi, S.M., Ebrahimzadeh, M.A., Eslami, S.H., Jafari, N., Moghaddam, A.H. The protective effect of curcumin against sodium fluoride-induced oxidative stress in rat heart. Arch. Biol. Sci., 2011; 63: 563-569. 10.2298/ABS1103563N.
42. Ohkawa, H., Ohishi, N., Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem, 1979; 95: 351-358. 10.1016/00032697(79)90738-3.
43. Pias, E.K., Ekshyyan, O.Y., Rhoads, C.A., Fuseler, J., Harrison, L., Aw, T.Y. Differential effects of superoxide dismutase isoform expression on hydroperoxide- induced apoptosis in PC-12 cells. J. Biol. Chem, 2003; 278: 13294-13301. 10.1074/jbc.M208670200.
44. Popovic, N., Pajovic, S.B., Stojiljkovic, V., Pejic, S., Todorovic, I.V., Gavrilovic, L. Prefrontal catecholaminergic turnover and antioxidant defense system of chronically stressed rats. Folia Biol., 2017; 65: 43-53. https://doi.org/10.3409/fb65_1.43.
45. Ranjan, R., Swarup, D., Patra, R.C. Oxidative stress indices in erythrocytes, liver and kidneys in fluoride-

exposed rabbits. Fluoride, 2009; 42: 88-93.
46. Ravula, S., Harinarayan, C.V., Prasad, U.V., Ramalakshmi, T., Rupungudi, A., Madrol, V. Effect of fluoride on reactive oxygen species and bone metabolism in postmenopausal women. Fluoride, 2012; 45: 108-815.
47. Raza, F.S., Okamoto, M., Takemori, H., Vinson, G.P. Manganese superoxide dismutase activity in the rat adrenal. J. Endocrinol, 2005; 184: 77-84. 10.1677/joe.1.05755.
48. Rosol, T.J., Yarrington, J.T., Latendresse, J., Capen, C.C. Adrenal gland: structure, function, and mechanisms of toxicity. Toxicol. Pathol, 2001; 29: 41-48. 10.1080/019262301301418847.
49. Rotruck, J.T., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D.G., Hoekstra, W.G. Selenium: biochemical role as a component of glutathione peroxidase. Science, 1973; 179: 588-590. 10.1126/science. 179.4073.588.
50. Salam, Z., Agha, A. Histological, histochemical and ultrastructural studies on the kidney of rats after administration of monosodium glutamate. Al-Aqsa UniversityGaza Palestine, 2006; 10: 20-40.
51. Sharma, C., Suhalka, P., Sukhwal, P., Jaiswal, N., Bhatnagar, M. Curcumin attenuates neurotoxicity induced by fluoride: An in vivo evidence. Pharmacogn Mag, 2014; 10: 61-65. 10.4103/0973-1296.126663.
52. Shashi, A., Bhardwaj, M. Prevalence of dental fluorosis in endemic fluoride areas of Punjab, India. Biosci. Biotech Res. Comm, 2011; 4: 155-63.
53. Shashi, A., Kumar, M., Bhardwaj, M. Incidence of skeletal deformities in endemic fluorosis. Trop. Doct., 2008; 38: 231-233. 10.1258/td.2008.070379.
54. Shashi, A., Fluoride and adrenal functions in rabbits. Fluoride, 2003; 36: 241-251.
55. Shyam, R., Manjunath, B.C., Kumar, A., Narang, R., Rani, G., Singh, S. Prevalence of dental fluorosis and treatment needs among 11-14 years old school children in endemic fluoride areas of Haryana, India. Indian J. Dent. Res., 2021; 32: 110-114. 10.4103/ijdr.IJDR_835_18.
56. Suwa, T., Mune, T., Morita, H., Daido, H., Saio, M., Yasuda, K. Role of rat adrenal antioxidant defense systems in the aldosterone turn-off phenomenon. J. Steroid Biochem. Mol. Biol., 2000; 73: 71-78. 10.1016/s0960-0760(00)00049-2.
57. Tsou, T.C., Yeh, S.C., Tsai, F.Y. and Change, L. W. The protective role of intracellularGSH status in the arsenite-induced vascular endothelial dysfunction. Chem. Res. Toxicol, 2004; 17: 208 – 217. 10.1021/tx034202v.
58. Turrens, J.F. Superoxide production by the mitochondrial respiratory chain. Biosci. Rep., 1997; 17: 3-8. 10.1023/a:1027374931887.
59. Weng, Q., Li, B., Liu, Z., Liu, K., Wu, W., Liu, H. ACTH induces oxidative stress and decreases DNA methylation of Fkbp5 in Suhuai Sow. Pak. Vet. J., 2019; 39: 401-405.10.29261/pakvetj/2019.083.
60. Yamaguti, P.M., Simoes, A., Ganzerla, E., Souza, D.N., Nogueira, F.N., Nicolau, J. Effects of single exposure of sodium fluoride on lipid peroxidation and antioxidant enzymes in salivary glands of rats. Oxid. Med. Cell Longev. 2013:674593. 10.1155/2013/674593, 2013.
61. Zhou, B.H., Zhao, J., Liu, J., Zhang, J.L., Li, J., Wang, H.W. Fluoride-induced oxidative stress is involved in the morphological damage and dysfunction of liver in female mice. Chemosphere, 2015; 139: 504-511. 10.1016/j.chemosphere.2015.08.030.