EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES

http://www.ejbps.com

ISSN 2349-8870
Volume: 8
Issue: 12
408-424
Year: 2021

FLUORIDE-INDUCED ADRENAL INSUFFICIENCY: ADRENOCORTICOTROPIN ENDOCRINE DISRUPTION AND ULTRASTRUCTURAL EXPERIMENTAL STUDY. THE POSSIBLE AMELIORATION EFFECT OF CURCUMIN SUPPLEMENTATION

A. Shashi* and Manisha Tikka

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

Corresponding Author:* **A. Shashi

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

Article Received on 20/10/2021 **Article Revised on 10/11/2021** **Article Accepted on 30/11/2021**

ABSTRACT

The present study was designed to evaluate the curative effect of Curcumin on fluoride induced alterations in the adrenocorticotropic hormone (ACTH), steroid hormones, catecholamines and ultrastructural changes. Wistar albino rats were administered with 300 and 600 mg/kg b.w./day of sodium fluoride (NaF) daily for 40 days. The control group was given 1 ml of deionized water/kg b.w./day for the same period. The fluoride treated animals were post-treated with 200 mg/ kg b.w./ day of Curcumin for 20 days. After the treatment period, the rats were sacrificed, adrenal tissue was taken out and blood was collected so as to process further for plasma samples. The levels of fluoride, ACTH, aldosterone, cortisol, epinephrine and norepinephrine in plasma were measured by using diagnostic kits. There was significant ($P<0.0001$) increase in the plasma levels of ACTH, and catecholamines while aldosterone and cortisol levels were significantly ($P<0.0001$) declined after 40 days of fluoride exposure. The ultrastructural examination of the adrenal gland in fluoridated rats showed irregular shaped nucleus, vacuoles of variable sizes and scarce cytoplasmic organelles, swollen mitochondria with disrupted cristae, large lipid droplets, numerous ribosomes and dilated smooth endoplasmic reticulum (SER). The adrenal medulla showed degeneration, damaged mitochondria, irregular nucleus and depletion of secretory granules. All the changes caused by NaF toxicity were reversed by Curcumin. The results suggested that Curcumin was effective in alleviating the toxic effects of NaF on the hypothalamic– pituitary–adrenal (HPA) axis, catecholamines and ultrastructural changes.

KEYWORDS: Adrenocorticotropic hormone, Aldosterone, Catecholamines, Cortisol, Fluorosis, Transmission

Abbreviations

ACTH (adrenocorticotropic hormone), B.W. (body weight), HPA (Hypthamamic– pituitary–adrenal axis), Kg (kilogram), mg (milligram), NaF (sodium fluoride), SER (smooth endoplasmic reticulum), ZG (zona glomerulosa), ZF (zona fasciculata), ZR (zona reticularis).

1. INTRODUCTION

Fluorosis is a disease caused by consumption of fluoride through water, food and use of fluoride containing products. Excessive intake of fluorides can lead to disturbances in enamel development and bone homeostasis, caused dental and skeletal fluorosis respectively (Shashi *et al.*, 2008; Shashi and Bhardwaj, 2011). Fluoride above the threshold value in body causes ultrastructural pathological, and physiological changes in adrenal gland (Shashi, 2003), steroidal hormones (Shashi and Kaushal, 2020) and gonadal hormones in rats (Shashi and Khan, 2016).

Adrenal gland is a vital effector organ of stress. It is divided into two parts-the outer cortex and inner medulla. It is surrounded by a connective tissue layer called capsule. The adrenal cortex is subdivided into 3 concentric biochemically and morphologically different zones of steroid synthesizing cells. The outer zona glomerulosa (ZG) synthesize mineralocorticoid, the middle zona fasciculata (ZF) synthesize glucocorticoid and inner zona reticularis (ZR) produces androgens (Wotus *et al.*, 1998). Adrenal cortical hormones play important role in a number of physiological processes, electrolyte and fluid balance, cardiovascular homeostasis, and carbohydrate metabolism (Hart and Barton, 2011). The catecholamines released from the medullary part of adrenal gland and steroid hormones from the adrenal cortex contributes in fast and long term adaptation role in organism against any stressful conditions (Raber, 1998). Epinephrine is one of the catecholamine produced by adrenal medulla which stimulates the glycogenolysis in liver and increases the free fatty acid concentration in plasma. Norepinephrine is another catecholamine which increases systolic and

diastolic blood pressure, also stimulates glycogenolysis, however, shows stronger activity to release free fatty acids (Brook and Marshall, 1996). Adrenal hormones also help to regain stress-disturbed homeostasis (McQuade and Young, 2000).

From previous scientific literature, it has been revealed that various toxicants and endocrine disrupting chemicals such as aminoglutethimide, etomidate and Ketoconazole caused endocrine disruption and hence affects the adrenal gland function (Rosol *et al.*, 2001; Hinson and Raven, 2006; Harvey, 2016). These toxicants can interfere with the endocrine system and disturb the production and secretion of hormones, metabolism and transport (De Falco *et al.*, 2014; Giulivo *et al.*, 2016). Therefore, there is need to study more about adrenal gland and its role in endocrine homeostasis, an important topic to be considered for investigating the link between toxicants and activation/dysregulation of the hypothalamic- adrenal-axis in relation to adrenal gland function

Efforts have been made by researchers regarding selection of various natural compounds which exhibit important biological activities and also possess effective response against toxicants-induced damage (Navabi *et al.*, 2012; Shashi and Khan. 2017, Shashi and Kaushal, 2020). Curcumin is a polyphenolic antioxidant, isolated from the rhizome of *Curcuma longa* L. It has been used as medicinal food all over the world from ancient times. Numerous studies have demonstrated that Curcumin has many beneficial effects, including anti-inflammatory, antioxidant, anti-mutagen, anti-infectious and anti-tumoral properties (Schaff *et al.*, 2009; Abdel-Aziz and Ahmed, 2011; Elsayed *et al*., 2016; Badawy, 2018). It also normalizes the hyperactivated HPA axis. However, not much is known about the effect of Curcumin as a valuable therapeutic target against NaF induced toxicity and associated hormonal disturbances. Therefore, present study aimed at evaluating the possible curative effect of Curcumin against NaF-induced hormonal impairments and ultrastructural alterations in rat adrenal gland.

2. MATERIALS AND METHODS

Sodium fluoride and Curcumin were purchased from Loba Chemie Pvt. Ltd, Mumbai, India. ELISA kits were procured from Elabscience, Hubei.

2.1. Experimental design

Young Wistar albino rats of both sexes weighing 150-200 g were housed separately 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 acclimatization, rats were randomly divided into six groups as mentioned in the previous study (Shashi and Tikka, 2021). Each group contained six rats. Briefly, group I was given deionized water orally for 40 days. The groups II and III were treated with 300 mg and 600 mg of NaF/kg b.w./day, respectively, for the same period. Group IV was treated with 200 mg/kg b.w./day of Curcumin for 20 days. However, groups V and VI were treated with 300 mg and 600 mg of NaF/kg b.w./day, respectively, for 40 days and post-treated 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 transmission electron microscopy. The blood was taken from each rat and collected into EDTA vials to obtain plasma.

2.2. Preparation of samples

The blood samples from control and experimental rats were collected into EDTA vials, centrifuged at 3000 rpm for 15 minutes to separate plasma and immediately stored at -20 ºC for the determination of fluoride and hormones until further use.

2.3. Plasma fluoride analysis

The fluoride in plasma of control and experimental rats was extracted by using method of Inkielewicz *et al.* (2003). The level of fluoride in control and experimental rats was estimated with fluoride ion selective electrode (ELIT 8221) (Harwood, 1969).

2.4. Hormone analysis

Plasma samples from control and experimental rats were used for analyzing the hormonal content. Briefly, the plasma levels of ACTH, aldosterone, cortisol, epinephrine and norepinephrine of control and experimental rats were determined using diagnostic ELISA kits (Elabscience, Hubei) by following instructions given by the manufacturers on ELISA Reader (Rayto, RT-2100C microplate reader, Shenzhen, China).

2.5. Transmission electron microscopy

The adrenal tissues were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 4-6 hours at 4 °C by the method of Karnovsky (1965). After washing in buffer, tissues were post-fixed in 1% osmium tetraoxide for 1 hour, dehydrated in acetone, infiltrated and embedded in araldite CY 212 (TAAB, UK), cut with an ultramicrotome (Leica Ultracut UC7, Austria), and stained with aqueous toluidine blue under a light microscope for gross observation of the area and quality of the tissue fixation. The ultra-thin sections of grey-silver colour interference (70-80 nm) were cut and mounted on 300 mesh-copper grids, stained with 8% uranyl acetate and lead citrate. All the studies were performed under a Tecnai G2 20 high resolution transmission electron microscope (Fei Company, The Netherlands) at an operating voltage of 200 kV. A CCD camera (Megaview III, Fei Company) using TIA software attached to the microscope for digitally acquired images at All India Institute of Medical Sciences, New Delhi, India.

2.6. Data analysis

Results were expressed as mean ±SD. All analysis were 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 plasma fluoride and hormones were determined by Pearson's bivariate correlation and simple linear regression test.

3. RESULTS

3.1. Evaluation of Fluoride level

The plasma fluoride levels were significantly ($P<0.0001$) raised in groups II and III when compared to control group I. However, post-treatment with 200 mg Curcumin showed significant fall in the level of plasma fluoride in group IV ($P<0.01$) and group V ($P<0.05$) in comparison to groups II and III, respectively (Table 1; Fig. 1).

3.2. Curcumin administration displayed curative effects by relieving NaF induced hormonal changes: Hormone evaluation

All the experimental groups were evaluated for their ability to secrete associated hormones by using their blood plasma samples. For this purpose, various corticotropic and steroidal hormone levels were assessed under all conditions including normal (control; group I), fluoridated (group II and III), Curcumin treated only (group IV), and NaF induced followed by post-treated with Curcumin (group V and VI).

3.2.1. Corticotropic hormone secreted by pituitary gland

3.2.1.1. Adrenocorticotropic hormone

The results shown in Table 1 revealed that administration of NaF caused significant increase ($P<0.0001$) in the level of plasma ACTH in groups II and III as compared to group I (Fig. 2A). Pearson's bivariate correlation and simple linear regression analysis demonstrated significant ($P<0.0001$) positive relationship between levels of fluoride and ACTH in plasma of rats (Pearson r = 0.968, R^2 = 0.937, Y = 390.698+202.845X; Fig. 2B) after 40 days of fluoride exposure. Furthermore, after the post-treatment with Curcumin the level of plasma ACTH was significantly ($P<0.0001$) declined in group V and VI in comparison to group II and III, respectively (Fig. 2C). These results revealed the efficatory properties of Curcumin in fluoridated rats.

3.2.2.Steroidal hormones secreted by adrenal cortex

3.2.2.1. Aldosterone

NaF treatment induced significant reduction ($P<0.0001$) in the plasma aldosterone level in fluoridated rats of groups II and III in comparison to the control group I (Table 1; Fig. 3A). Pearson's bivariate correlation and simple linear regression analysis demonstrated a significant ($P<0.0001$) negative relationship between levels of plasma fluoride and aldosterone (Pearson r = -0.938, R^2 = 0.880, Y = 80.406-31.605X; Fig. 3B) after fluoride treatment for 40 days. However, post-treatment with Curcumin restored the plasma aldosterone in rat (Table 1; Fig. 3C) and hence showed curative effects.

3.2.2.2. Cortisol

The level of plasma cortisol in fluoridated groups exhibited significant ($P<0.0001$) decrease in comparison to the control (Table 1; Fig.4A). Pearson's bivariate correlation and simple linear regression analysis revealed significant ($P<0.0001$) negative relationship between levels of fluoride and cortisol (Pearson r = -0.967, R^2 = 0.934, Y = 141.807– 52.802X; Fig. 4B) in rats exposed to fluoride for 40 days. However, post-treatment with Curcumin exhibited restoration in the plasma cortisol levels in rats (Table 1; Fig. 4C) and hence showed ameliorative effects.

3.2.3. *Catecholamines secreted by adrenal medulla*

The effect of Curcumin on plasma level of catecholamines i.e. epinephrine and norepinephrine in NaF intoxicated rats are shown in Table 1. The levels of epinephrine and norepinephrine were significantly ($P<0.0001$) increased in group II and III in comparison to group I (Table 1; Fig. 5A; Fig. 6A). Pearson's bivariate correlation and simple linear regression analysis demonstrated a significant ($P<0.0001$) positive relationship between levels of plasma fluoride and epinephrine (Pearson r = 0.968, R^2 = 0.937, Y = 106.427+120.688X; Fig. 5B) and norepinephrine (Pearson r = 0.968, R^2 = 0.937, Y = 4.911+4.648X; Fig. 6B) after 40 days of fluoride exposure. However, after post-treatment with Curcumin, the level of plasma epinephrine was significantly decreased in group V ($P<0.05$) and VI ($P<0.0001$) in comparison to group II and III, respectively (Table 1; Fig. 5C). Moreover, similar results were seen in the level of norepinephrine which was significantly ($P<0.0001$) decreased in group V and VI as compared to respective groups II and III (Table 1; Fig. 6C).

3.3. Transmission electron microscopy

To analyze the ultrastructural changes in the rat's adrenal gland under the effect of NaF induction and post-treatment with Curcumin, transmission electron microscopy was performed.

3.3.1. Adrenal cortex

The transmission electron microscope examination of adrenal cortex of control rat revealed fine structure of ZG cells having nucleus with peripheral clumps of heterochromatin with numerous mitochondria having oval and spherical shapes. In addition, rounded droplets, free ribosomes and SER were also observed (Fig. 7A).The ZF cells also contained euchromatic nucleus with peripheral clumps of heterochromatin, abundant lipid droplet, large and small mitochondria with cristae, and SER (Fig 7B).The ZR cell showed rounded mitochondria, lipid droplets, many free ribosomes, Golgi apparatus, and SER. The nucleus was spherical in shape having condensed heterochromatin on the periphery (Fig. 7C).

The rat treated with 300 mg/kg b.w./day of NaF for 40 days showed irregular nuclei with electron dense heterochromatin in ZG. Additionally, the massive cytoplasmic vacuolation, variable sized vacuoles, and numerous free ribosomes were visible. The widening of cellular junctions between the cells was also prominent (Fig.7D).The cell of ZF had nucleus with dilated perinuclear space. Some mitochondria were swollen while others were degenerated. The fused lipid droplets were noticed in the cells with dilated SER (Fig. 7E). The shrunken nuclei with irregular nuclear envelop, marked increase in lipid droplets, lysosomes and dilation of blood capillaries were observed (Fig. F).The ZR showed damaged mitochondria and have large lipid droplets. Aggregation of numerous free ribosomes and abundant dilated endoplasmic reticulum were recorded (Fig. 7G).

In the rat treated with 600 mg/kg b.w./day of NaF, the adrenal gland showed dilated blood capillary lined by damaged fenestrated endothelium in the cells of ZG. The microvilli of the cells appeared into the pericapillary space. The intercellular space contained collagen fibres. The mitochondria of variable sizes were visible, some of them were swollen with destroyed cristae. The lipid droplets were also seen (Fig. 7H). The nucleus with irregular outline, swollen mitochondria with disruptive cristae was recorded. The cytoplasm was filled with ribosomes arranged in clusters and or rosette form with dilated profile of SER, numerous large lipid droplets coalesced together and also contained autophagic body in ZF (Fig. 7I). The ZR showed indented nucleus with increased amount of dilated endoplasmic reticulum. The mitochondria appeared vacuolated, numerous free lysosomes and large cytoplasmic vacuole were also observed (Fig. 7J). The cytoplasm of the cell was packed with multiple lysosomes and accumulation of many lipid droplets was also seen. The nucleus revealed indentations of nuclear envelop (Fig. 7K).

The rat treated with 200 mg/kg b.w./day of Curcumin for 20 days showed presence of ZG, ZF,ZR in the adrenal cortex and adrenal medulla similar to control group (Fig. 8A, B, C). The rat treated with 300 mg/kg b.w./day of NaF for 40 days followed by post-treatment with 200 mg/kg b.w./day of Curcumin showed rounded nucleus with prominent nuclei in ZG. Cristae reappeared in mitochondria and only comparatively few damaged mitochondria were present in comparison to fluoridated rats treated with 300 mg/kg b.w./day of NaF only (Fig. 8D). The cells of ZF showed rounded nucleus with regular nuclear envelop. Mitochondria with vesicular cristae, few swollen mitochondria and less number of lipid droplets were present (Fig. 8E). The ZR cell exhibited rounded and prominent nucleus but with dilated perinuclear space. The lipid droplets were limited in number along with few dilated endoplasmic reticulum (Fig. 8F). The euchromatic nucleus with irregular nuclear envelop and prominent nucleolus, numerous mitochondria, few mitochondria with disrupted cristae and smooth endoplasmic reticulum were observed (Fig.8G).

The rat treated with 600 mg/kg b.w./day of NaF for 40 days followed by post-treatmentwith 200 mg/kg b.w./day of Curcumin for 20 days showed normal nuclei with clearly visible nucleolus and lipid droplets in ZG (Fig. 8H). The ZF had normal euchromatic nucleus, numerous mitochondria where few mitochondria were normal and numerous lipid droplets (Fig. 8I). ZR showed normal nucleus with prominent nucleolus, large lipid droplets, and SER (Fig. 8J). The cells of ZR contained mitochondria with vesicular cristae and also exhibited damaged mitochondria (Fig. 8K).

3.3.2. Adrenal medulla

The adrenal medulla of control rat showed nuclei with moderate amount of chromatin. The electron dense granules were distributed in the cell. Lipid droplets of variable sizes were observed (Fig. 9A). In the rat treated with 300 mg/kg b.w./day of NaF the cytoplasm of chronic cells showed irregular shaped nucleus, edema in cytoplasm vacuolation, and depletion of the secretory granules (Fig. 9B). Apart of nucleus, vacuoles of variable sizes, scarce cytoplasmic organelles and abundant free ribosomes were observed (Fig. 9C). The cytoplasm showed vacuolization and mitochondria with destroyed cristae. The cell junctions between the membranes of catecholamine producing cells were noticed (Fig. 9D).The adrenal medulla showed degeneration. The mitochondria revealed disrupted cristae, some were swollen and degenerated (Fig. 9E). In the rat treated with 600 mg/kg b.w./day of NaF the chromaffin cells having shrunken pyknotic nuclei and one nucleus with marked extended chromatin was visible. The cells filled with vacuoles of different sizes fused together (Fig. 9F). The adrenal medulla exhibited numerous lipid droplets in chromaffin cells. The swollen vacuolated mitochondria with disrupted cristae were prominent (Fig. 9G).

The rat treated with 200 mg/kg b.w./day of curcumin for 20 days showed adrenal medulla similar to control group (Fig. 10A). The adrenal medulla cells from rat treated with 300 mg/kg b.w./day of NaF for 40 days followed by post-treatment with 200 mg/kg b.w./day of Curcumin revealed normal structure compared with NaF toxicity group. The chromaffin cells had irregular shaped nucleus with eccentric nucleolus and numerous dense granules (Fig. 10B). The cells contained rounded nucleus with prominent nucleolus, few dense granules and mitochondria (Fig. 10C). The adrenal medulla from rat treated with 600 mg/kg b.w./day of NaF for 40 days followed by post-treatment with 200 mg/kg b.w./day of Curcumin showed normal mitochondria whereas the cytoplasm was packed with numerous dense granules (Fig. 10 D). The cell had euchromatic nucleus with prominent eccentric nucleolus. The cytoplasm was filled with dense granules and degenerated mitochondria (Fig. 10E).

Table 1: Mean levels of plasma fluoride, ACTH, aldosterone, cortisol, norepinephrine and epinephrine in control and experimental groups.

Treatment group	Plasma fluoride (µg/mL)	ACTH (pg/mL)	Aldosterone (pg/mL)	Cortisol (ng/mL)	Epinephrine (pg/mL)	Norepinephrine (ng/mL)
I Control -1	0.033±0.015	393.000±21.251	83.509±6.481	145.130±8.699	113.967±15.115	4.686±0.424
II 300 mg NaF	0.834±0.048[a]	559.933±27.749[a]	46.374±5.077[a]	88.039±7.869[a]	195.777±17.565 [a]	9.338±0.483 [a]
III 600 mg NaF	1.619±0.190[aa]	723.333±35.930[aa]	32.779±5.931[ab]	61.011±7.609[aa]	309.507±19.941[aa]	12.260±0.523[aa]
IV Control-2	0.026±.0.009	388.525±21.645	84.651±6.356	143.875±8.887	105.458±12.019	4.370±0.410
V 300 mg NaF+200 mg Curcumin	0.624±0.076[#]	424.150±26.908[###]	61.792±6.989[#]	124.233±7.445[###]	148.895±17.548[##]	6.200±0.478[###]
VI 600 mg NaF+200 mg Curcumin	1.316±0.045[##]	524.417±33.467[###]	53.414±5.934[#]	114.542±6.686[###]	204.352±16.328[###]	8.082±0.526[###]

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

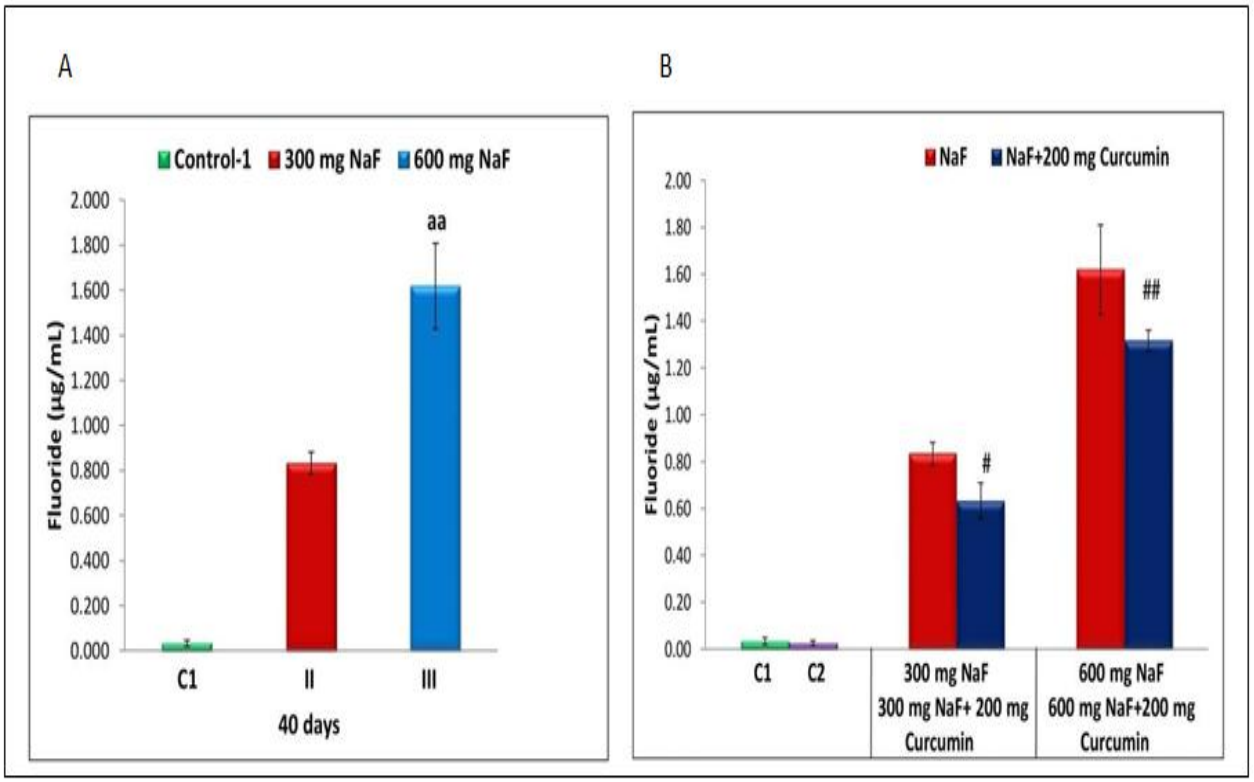

Fig. 1: A. Mean levels of fluoride (µg/mL) in plasma of control and fluoridated rats. [a]P<0.0001 Group II-III compared with control-1 [aa]P<0.0001 Group II compared with group. III. B. The levels of fluoride (µg/mL) in plasma of fluoridated rats post-treated with Curcumin. [#]P<0.01, [##]P<0.05 values were significantly different as compared to respective NaF treated groups.

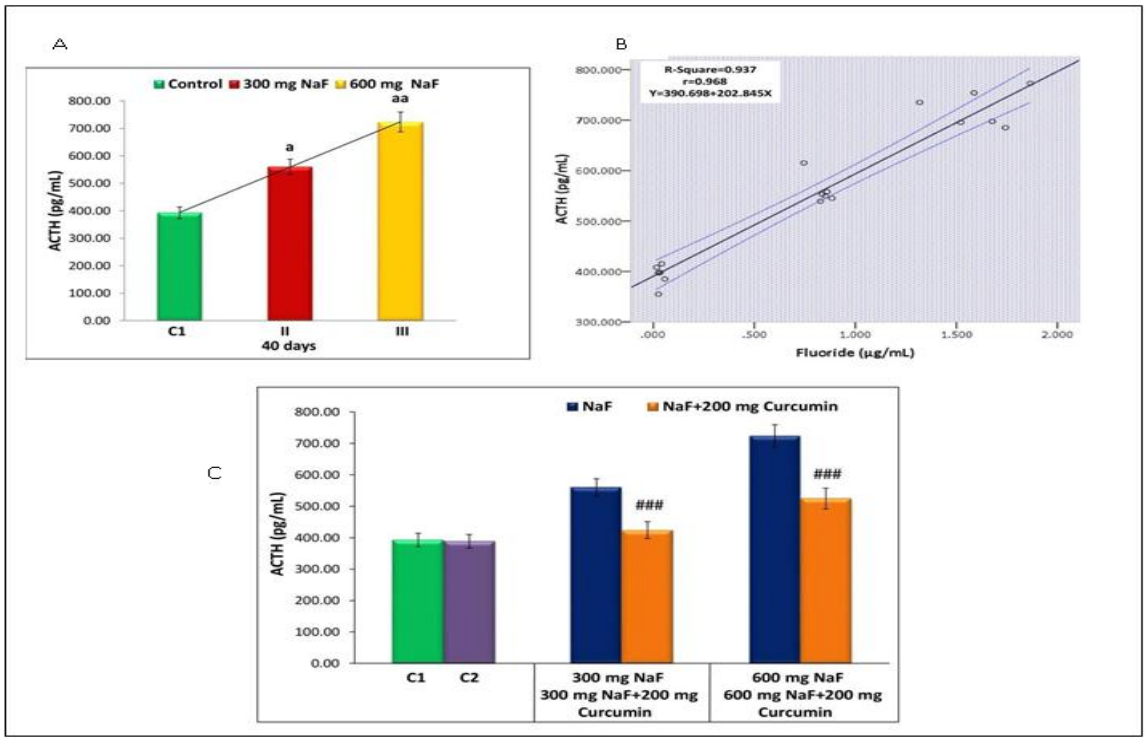

Fig. 2: A. Mean levels of ACTH (pg/mL) in plasma of control and fluoride exposed rats. P<0.0001 Group II-III compared with control-1. [aa]P<0.0001 Group II compared with group III. B. Scatterplot showing Pearson's bivariate correlation and simple linear regression between levels of fluoride (µg/mL) and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

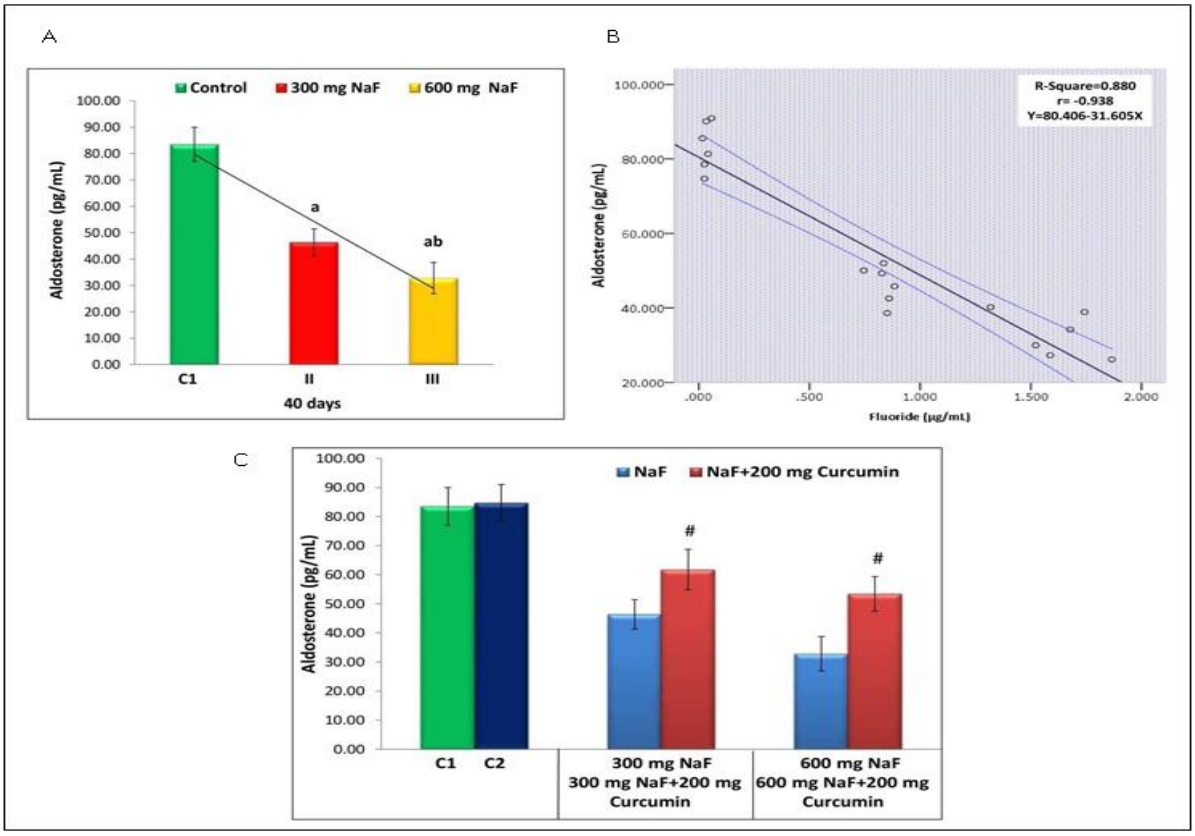

Fig. 3: A. Mean levels of aldosterone (pg/mL) in plasma of control and fluoride treated rats for 40 days. [a]P<0.0001 Group II-III compared with control-1. [ab]P<0.01 Group II compared with group III . B. Scatterplot

showing Pearson's bivariate correlation and simple linear regression between levels of plasma fluoride (µg/mL) and aldosterone (pg/mL) experimental rats after 40 days of fluoride intoxication. C. Mean levels of aldosterone (pg/mL) in plasma of fluoride treated rats after post-treatment with Curcumin. #P<0.01 values were significantly different as compared to respective NaF treated groups.

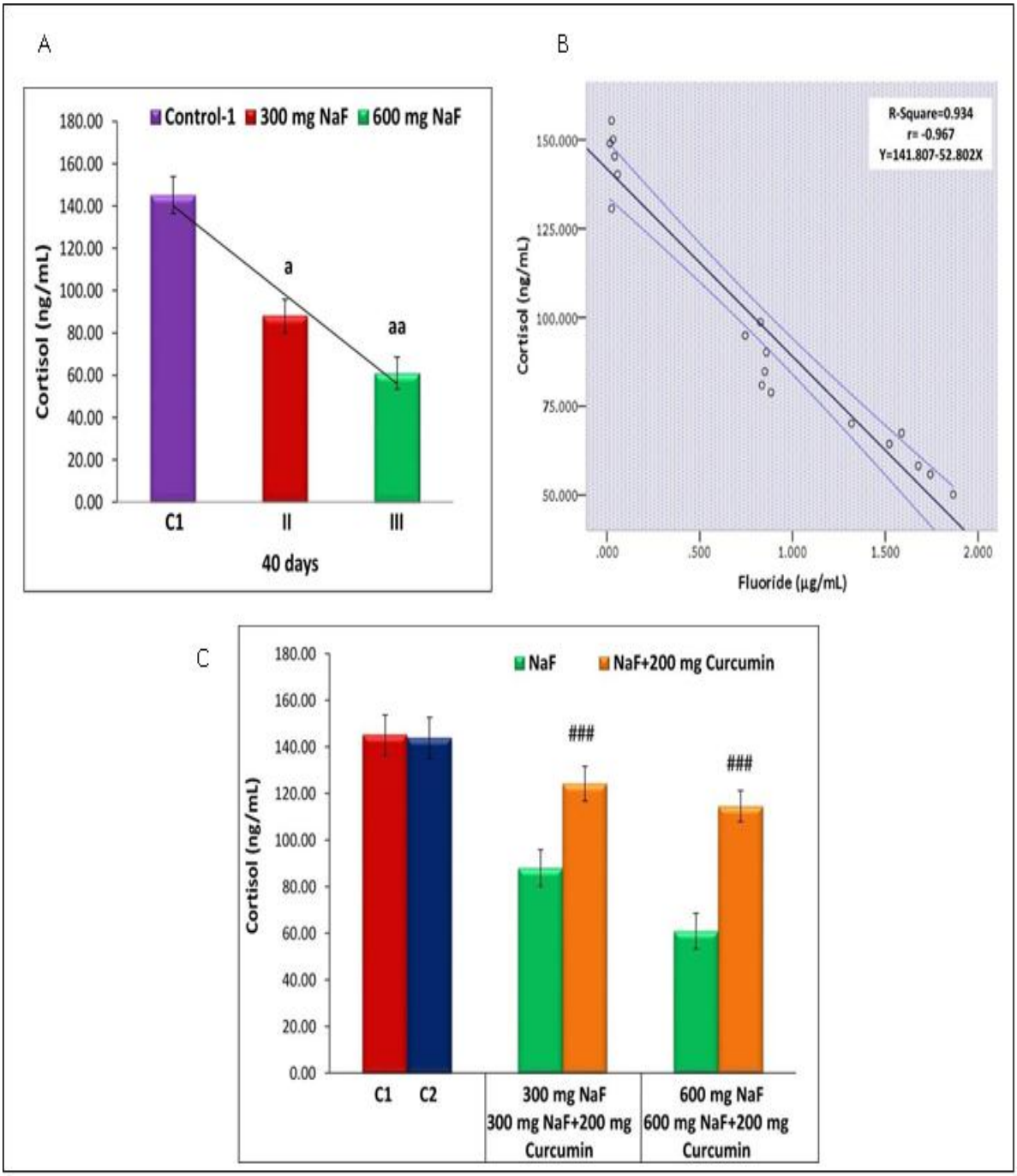

Fig. 4: A. Mean levels of cortisol (ng/mL) in plasma of control and fluoride intoxicated rat for 40 days. [a]P<0.0001 Group II-III compared with control-1. [aa]P<0.0001 Group II compared with group III . B. Scatterplot showing Pearson's bivariate correlation and simple linear regression between levels of plasma fluoride (µg/mL) and cortisol (ng/mL) in experimental rats after 40 days of fluoride exposure. C. Mean levels of cortisol (ng/mL) in plasma of fluoride treated rats after post-treatment with Curcumin. [###]P<0.0001 values were significantly different as compared to respective NaF treated groups.

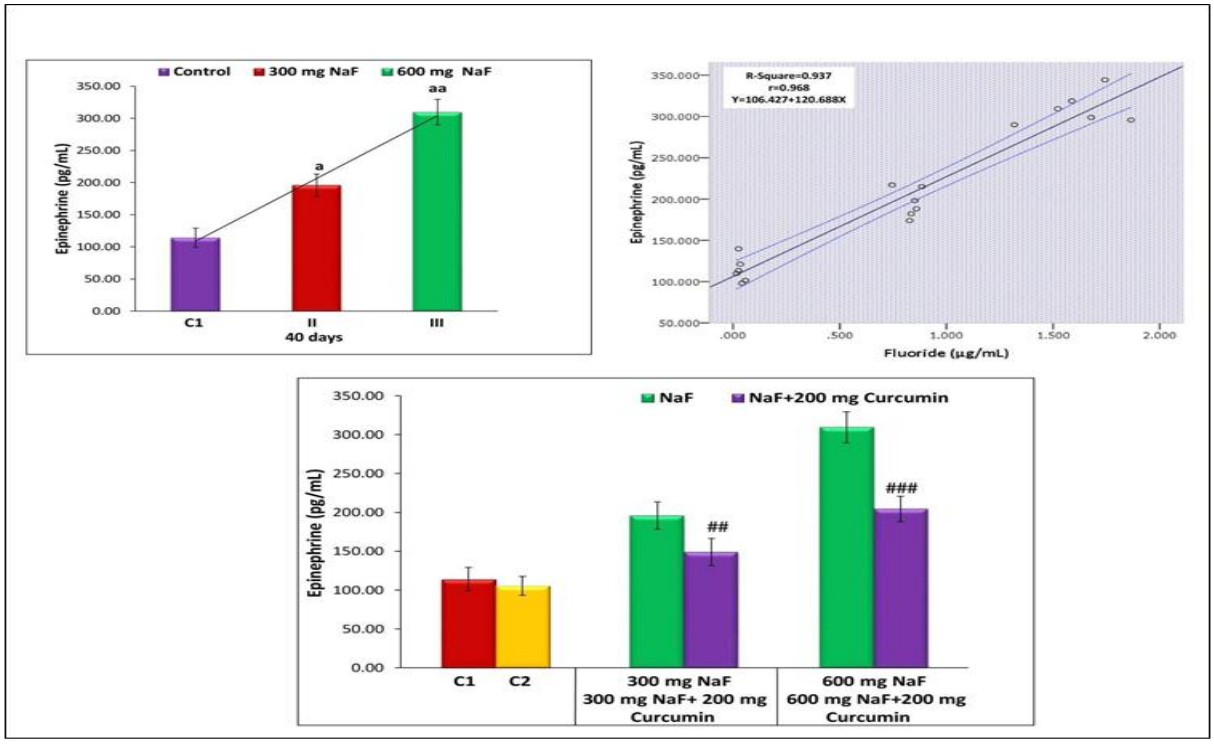

Fig. 5: A. Mean levels of epinephrine (pg/mL) in plasma of control and fluoridated rat for 40 days. [a]P<0.0001 Group II-III compared with control-1. [aa]P<0.0001 Group II compared with group III . B. Scatterplot showing Pearson's bivariate correlation and simple linear regression between levels of plasma fluoride (µg/mL) and epinephrine (pg/mL) in experimental rats after 40 days of fluoride treatment. C. Mean levels of cortisol (ng/mL) in plasma of fluoride treated rats after post-treatment with Curcumin. [##]P<0.05,[###]P<0.0001 values were significantly different as compared to respective NaF treated groups.

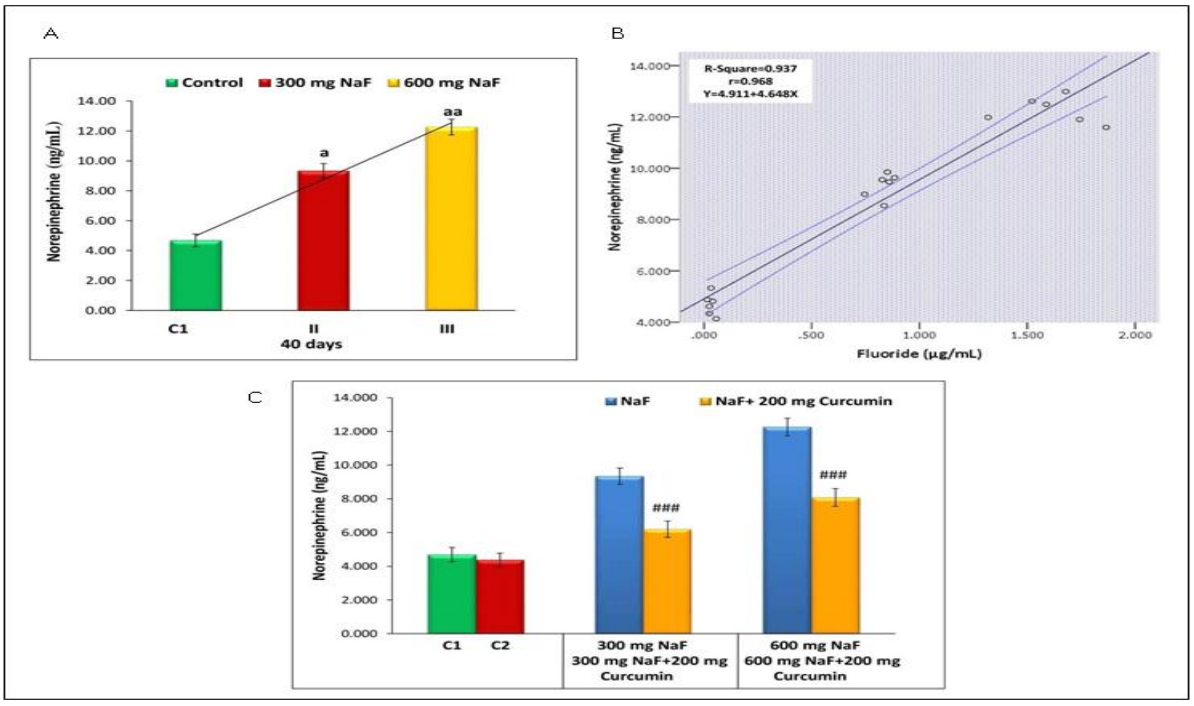

Fig. 6: A. Mean levels of norepinephrine (ng/mL) in plasma of control and fluoride exposed rat for 40 days. [a]P<0.0001 Group II-III compared with control-1. [aa]P<0.0001 Group II compared with group III . B. Scatterplot showing Pearson's bivariate correlation and simple linear regression between levels of plasma fluoride (µg/mL) and norepinephrine (ng/mL) in experimental rats after 40 days of fluoride treatment. C. Mean levels of norepinephrine (ng/mL) in plasma of fluoride treated rats after post-treatment with Curcumin. [###]P<0.0001 values were significantly different as compared to respective NaF treated groups.

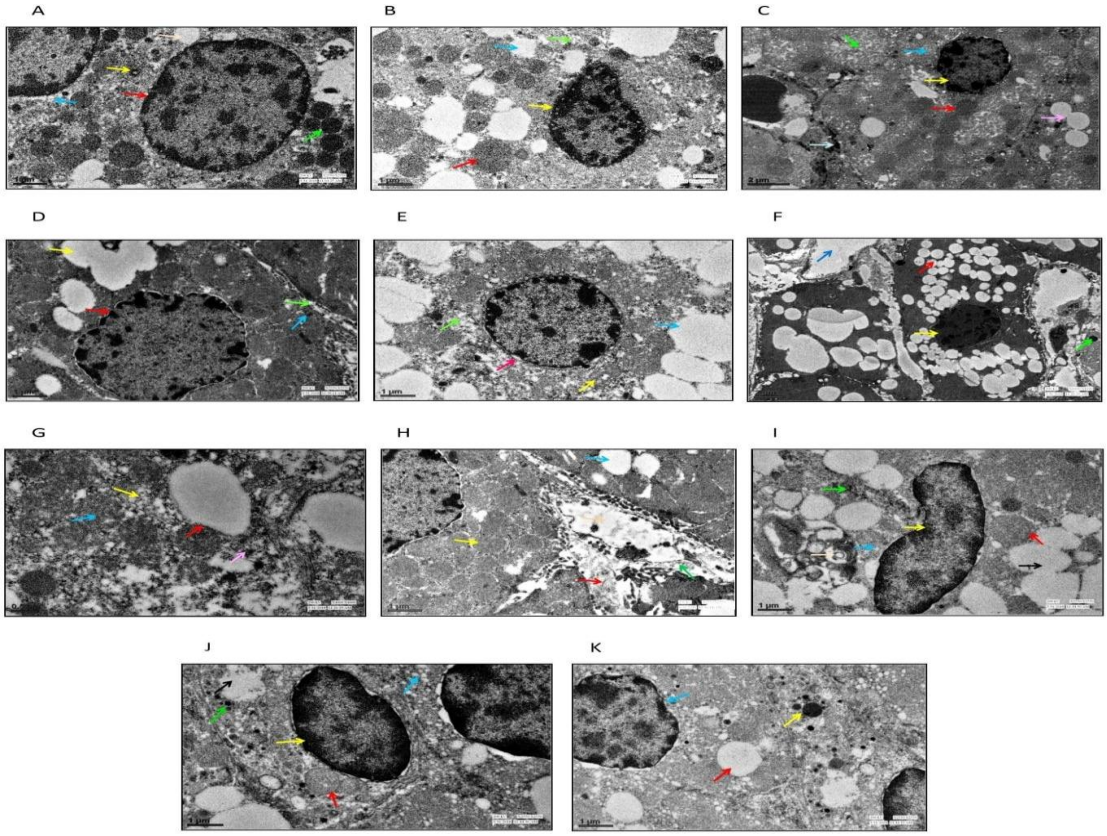

Fig. 7: Transmission electron micrograph of rat adrenal cortex from control group A. Zona glomerulosa cell showing nucleus with peripheral clump of heterochromatin (↑), lipid droplet (↑), numerous mitochondria (↑), free ribosome (↑) and smooth endoplasmic reticulum (↑). X2550. B. Zona fasciculata cell showing euchromatin nucleus with peripheral clumps of heterochromatin (↑), mitochondria (↑) and lipid drpolets (↑) andsmooth endoplasmic reticulum (↑). X2550. C. Zona reticularis showing rounded nucleus(↑), lipid droplets (↑), ribosomes (↑), golgi apparatus and smooth endoplasmicreticulum (↑). X2550. The adrenal cortex of rat treated with 300 mg NaF/kg b.w./day showing D. zona glomerulosa with irregular nucleus (↑) massive cytoplasmic vacuolations(↑), numerous free ribosomes (↑), widening of cellular junctions (↑).X2550. E. Zona fasciculata showing nucleus with perinuclear space (↑), large lipid droplets (↑),numerous mitochondria with destroyed cristae (↑) and dilated smooth endoplasmic reticulum (↑). X2550. F. Zona fasciculata revealing shrunken nucleus (↑), marked increase in lipid droplets (↑), lysosomes (↑) and dilated blood capillary (↑).X1100. G. Zona reticularis revealing damaged mitochondria (↑), large lipid droplets (↑) dilated smooth endoplasmic reticulum (↑) and numerous free ribosomal aggregation (↑) in zona reticularis cell.X4000. The adrenal cortex of rat treated with 600 mg NaF/kg b.w./day showing H. Zona glomerulosa with blood capillary (↑), pericapillary space contained microvilli (↑), and collagen fibres swollen mitochondria with destroyed cristae (↑), lipid droplets (↑). X2550. I. Zona fasciculata cell showing nucleus with irregular outline (↑), swollen mitochondria with destroyed cristae (↑), dilated profile of smooth endoplasmic reticulum (↑), some lipid droplets coalesced together (↑), ribosome arranged in clusters (↑) and autophagic body (↑). X2550. J. Zona reticularis having irregular nucleus (↑), increase amount of dilated smooth endoplasmic reticulum(↑), lysosomes (↑), large cytoplasmic vacuole (↑) and vacuolated mitochondria (↑). X2550. K. Zona reticularis having nucleus with indentation of nuclear envelop (↑) and multiple lysosomes and accumulation of lipid droplets X2550.

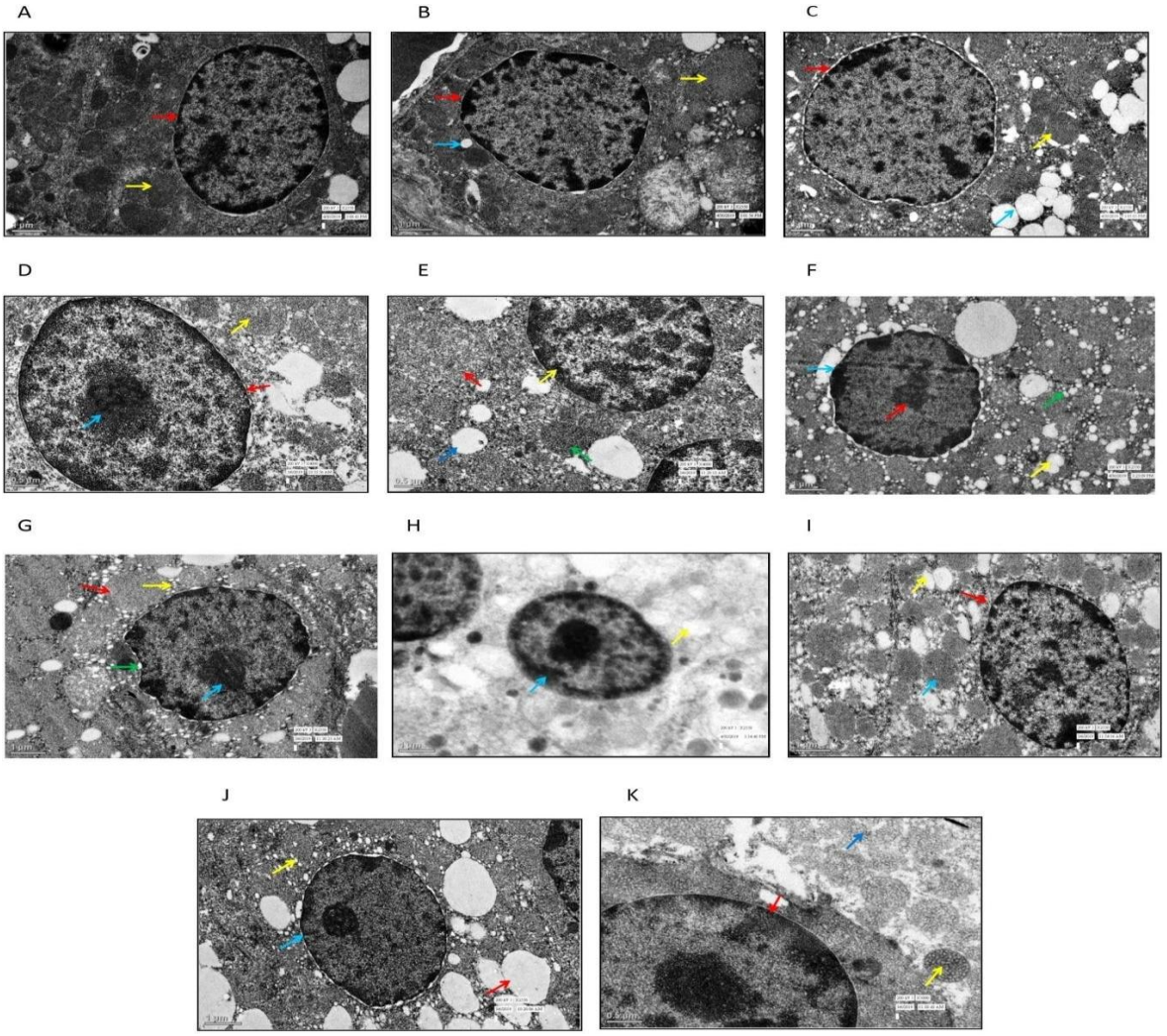

Fig. 8. Transmission electron micrograph of rat adrenal cortex treated with 200 mg/kg b.w./day of Curcumin **A.** Zona glomerulosa having nucleus with prominent nucleolus (↑) and mitochondria (↑). X2550. **B.** Zona fasciculata showing euchromatin nucleus (↑), mitochondria (↑) and lipid droplet (↑).X2550. **C.** Zona reticularis having nucleus (↑), mitochondria (↑) and lipid droplets (↑) similar to control. X2550. The rat adrenal cortex treated with 300 mg NaF/kg b.w./day post-treated with 200 mg of Curcumin showings **D.** Zona glomerulosa having nucleus (↑) with regular outline, nucleolus (↑) and appearance of cristae in mitochondria (↑) with damaged area. X4000. **E.** Zona fasciculata having part of nucleus (↑), mitochondria with cristae (↑), and swollen mitochondria (↑) and lipid droplet (↑). X4000. **F.** Zona reticularis having normal rounded nucleus (↑) with nucleolus (↑) with few lipid droplets (↑) and few dilated smooth endoplasmic reticulum (↑). X2550. **G.** Zona reticularis having euchromatic nuclei with irregular envelop (↑), prominent nucleolus (↑), mitochondria (↑) and smooth endoplasmic reticulum (↑). X2550. The rat adrenal cortex treated with 600 mg NaF/kg b.w./day post-treated with 200 mg of Curcumin showing **H.** Rounded nucleus with prominent nucleolus (↑) and lipid droplets (↑).X2550. **I.** Zona fasciculata having normal nucleus (↑), numerous mitochondria, few are normal (↑) and numerous lipid droplets (↑). X2550. **J.** Zona reticularis having rounded nucleus with prominent nucleolus (↑), smooth endoplasmic reticulum (↑) and lipid droplets (↑). X2550. **K.** Zona reticularis having nucleus with prominent nucleolus (↑) mitochondria showed vesicular cristae (↑) and damaged mitochondria (↑)X2550.

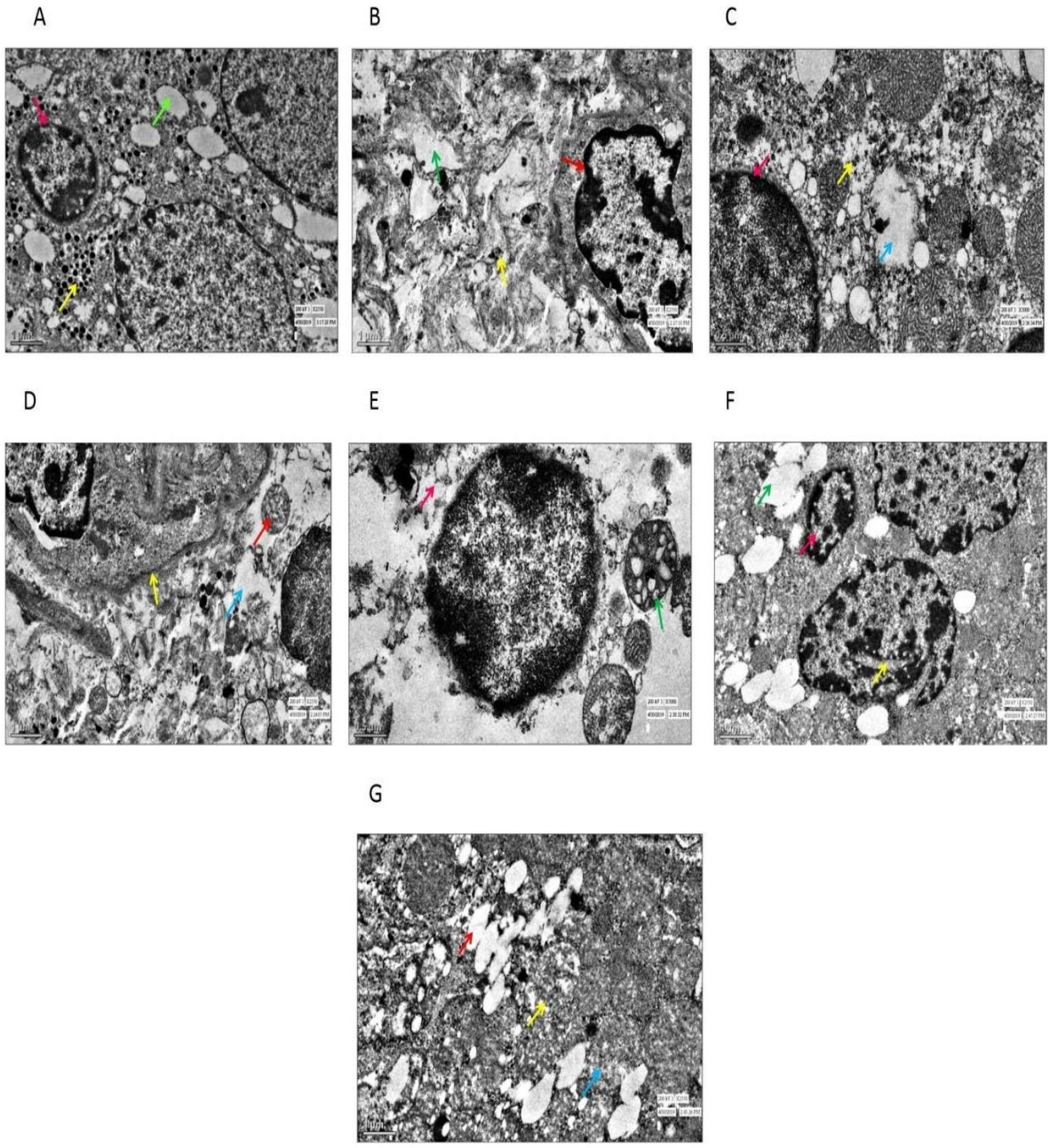

Fig. 9: Transmission electron micrograph of adrenal medulla of control rat showing A. normal nucleus (↑), electron dense granules (↑), lipid droplets (↑).X2550. The adrenal medulla of rat treated with 300 mg NaF/kg b.w./day showing B. Irregular shaped nucleus (↑), edema of cytoplasm and vacuolization (↑) and decreased in secretory granules (↑). X 2550. C. A part of nucleus (↑), vacuole of variable sizes (↑) and scarce cytoplasmic organelles and abundant free ribosomes (↑).X5000. D. Vacuole of variable sizes (↑), mitochondria with destroyed cristae (↑), cell junction between the cell membrane of catecholamine producing cells. (↑). X2550. E.Vacuolar degeneration (↑), swollen mitochondria with damaged cristae (↑). X5000. The adrenal medulla of rat treated with 600 mg NaF/kg b.w./day showing F. Chromatinolysis of nucleus (↑) shrunken pyknotic nucleus (↑) and vacuoles (↑). X2550. G. Numerous lipid droplet (↑), vacuolated mitochondria (↑), swollen mitochondria with disrupted cristae (↑). X2550.

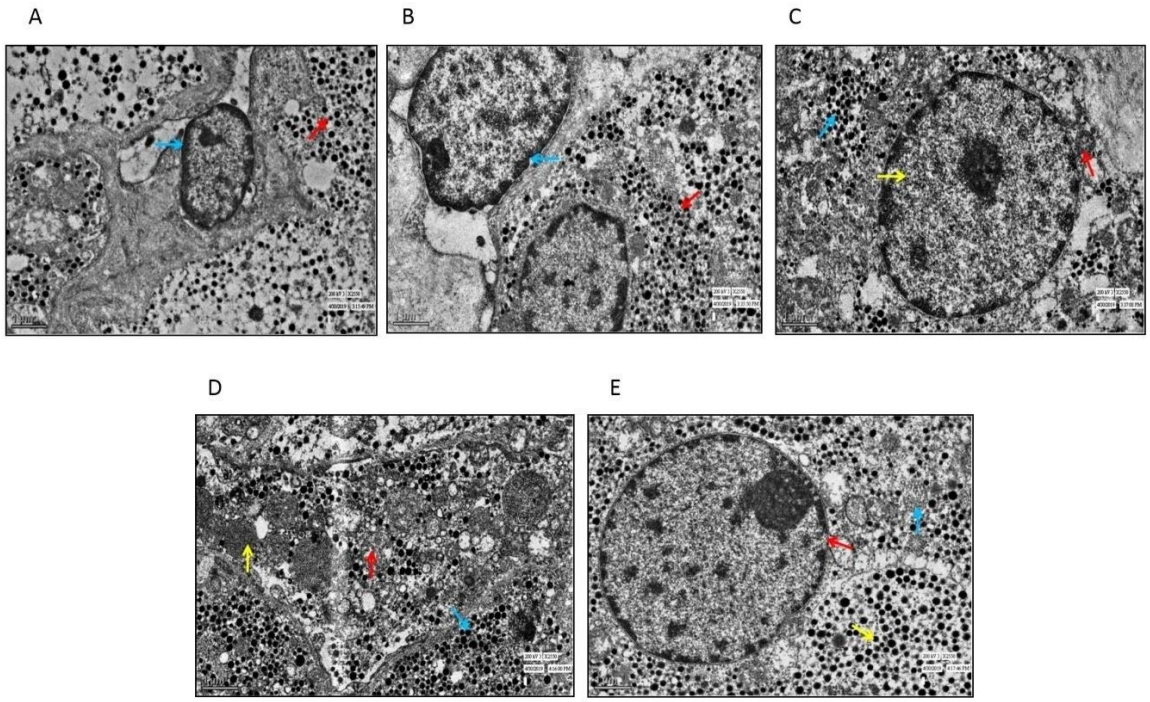

Fig. 10: Transmission electron micrograph of rat adrenal medulla treated with 200 mg/kg b.w./day of Curcumin showing A. nucleus with prominent nucleolus (↑) and dense granules (↑). X2550. The rat adrenal medulla treated with 300 mg NaF/kg b.w./day post-treated with 200 mg/kg b.w./day of Curcumin showing B. Irregular shape nucleus with eccentric nucleolus (↑) and numerous dense granules (↑). X2550. C. Rounded nucleus with prominent nucleolus (↑), few dense granules (↑) and mitochondria (↑). X2550. C The rat adrenal medulla treated with 600 mg NaF/kg b.w./day post-treated with 200 mg/kg b.w./day of Curcumin showing D. Damaged mitochondria (↑) and few normal mitochondria (↑) and dense granules (↑). X2550 E. Nucleus with prominent nuclei (↑) dense granules (↑) and degenerated mitochondria (↑) X2550.

4. DISCUSSION

The present study demonstrated elevation in plasma ACTH in fluoridated rats in comparison to control rats. Furthermore, Curcumin treatment confirmed its role as an effective therapeutic agent by significantly reducing plasma ACTH levels in fluorotic rats. These finding are in agreement with researchers who reported that treatment with various toxicants such as cadmium chloride, atrazine and monosodium glutamate leads to increase in the level of ACTH suggesting stimulation of the HPA (Caride *et al*., 2010; Abass *et al*., 2017;Abdo *et al*., 2018).

The adrenal cortex plays important role in synthesis and secretion of steroid hormones, including mineralocorticoid and glucocorticoid. The HPA axis is responsible for the secretion of steroid hormone by adrenals. The hypothalamus secretes the corticotrophin-releasing hormone which binds to corticotropic receptor factor type 1 receptor in anterior pituitary and results into the formation and secretion of ACTH by anterior pituitary, which in turns is responsible for the induction of adrenal cortex to secrete glucocorticoids and mineralocorticoids (Buford *et al.*, 2017; Minnetti *et al.*, 2020). The ACTH helps in the regulation of ZG function and angiotensin-II governs the regulation of aldosterone secretion. The ZG, however, is under the control of the angiotensin-II that binds to a specific receptor present on their cells (Morgan *et al.*, 2001).

The results of present study also displayed significantly ($P<0.0001$) inhibited aldosterone secretion under NaF intoxication which was further restored by Curcumin. ACTH administration in rats for 5 days showed decrease in plasma aldosterone concentration to 22% as compared to that of control rats. The co-administration of ACTH and vitamin E showed significant increase ($P<0.005$) in the plasma aldosterone (Suwa *et al.* 2000). The decrease in the aldosterone synthesis after ACTH treatment is due to decreased aldosterone synthase activity which is accompanied by the downregulation of CYP11B2 mRNA (Hollandand Carr, 1993; Lehoux *et al.*, 1998).

Recently, a significant ($P<0.001$) decrease in the level of aldosterone was seen in streptozotocin treated group of rats as compared to their untreated counterparts (Soliman and Noya, 2019). Similarly, other studies have also reported significantly increased plasma ACTH levels as well as significantly decreased aldosterone levels in ketoconazole treated rats (Deuschle *et al*., 2003; Khalil,

2015). These changes were mitigated by administering vitamin D3 in targeted rats. The ketoconazole induction resulted into the dilation and engorged sinusoids mainly in ZF and ZR. This finding showed that ketoconazole decreases the steroidogenesis and consequently increases the production of ACTH (Hinson *et al*., 1991).

Cortisol is secreted by ZF cells of adrenal cortex. It activates the catabolism in muscle and fatty tissue, also stimulates the gluconeogenesis in liver and inhibits the cytokine synthesis (Liyanarachchi, 2017). In present study, a significant decrease in the level of plasma cortisol ($P<0.0001$) was recorded in fluoridated rats and after post-treatment with Curcumin, the plasma level of cortisol was increased. These results implicate that NaF intoxication caused damage to the ZF cells which resulted into impaired functioning and ultimately decreased synthesis as well as secretion of cortisol hormone. However, post-treatment with Curcumin neutralized the NaF induced ill-effects and restored normal functioning which resulted into increase in the cortisol levels.

Although, glucocorticoids are very important calcium regulating hormones, they have gained a valuable attention in fluorosis research. Rao and Susheela (1979) investigated decline in the activity of delta 5-3-β hydroxysteroid dehydrogenase by suggesting impaired steroid synthesis in chronic fluoride toxicity. A significant reduction ($P<0.05$) in the level of cortisol was noticed in fluoride–toxicated rats while in exercise regimens, the cortisol levels were elevated at different temperatures (Basha and Sujitha, 2012). Similar decrease in the cortisol levels were observed in the tramadol treated group when compared to the control (Abdelaleem *et al*., 2017). The present findings are in accordance with Schaff *et al.* (2009) who observed that Curcumin showed a hormone suppressing activity, it significantly reduced the ACTH secreted by corticotropic AtT20 cells. Curcumin triggered the cortisol production by adrenal ZF cells and its stimulation also resulted into the increase in cortisol production in a concentration dependent manner, which continued for many hours. This increase in cortisol secretion was high during the 6 hours but persisted for up to 24 hours (Enyeart *et al.*, 2008).

In a study by Abel-Aziz (2010), it was revealed that treatment with nicotine showed deposition of lipid droplets and appearance of cytoplasmic vacuolation in the ZF cells which may be due to impairment in the synthesis and secretion of glucocorticoids. As the ZF is responsible for the synthesis and secretion of glucocorticoids, the toxicity of adrenal cortex plays a vital role in disruption of steroidogenesis. This may happen as a result of disturbance of cytochrome P450 enzymes; which further resulted into inhibition of cholesterol biosynthesis (Eleshennawy and Aboelwafa, 2011).

Similarly, in a study by Soliman *et al.* (2015), the bile duct resection (BDR) group exhibited a significant decrease in the cortisol and aldosterone levels in association with significantly high elevated serum bilirubin and alkaline phosphatase levels. Present study demonstrated similar observations.

Catecholamines play an important role in stress and their levels are increased under stressful conditions. They are produced by chromaffin cells of adrenal medulla (Carbone *etal.* 2019). Researchers have shown that NaF treatment resulted into increase in the level of epinephrine and norepinephrine in blood and hippocampus of rats (Bagmut *et al*. 2018; Kumar *et al.*, 2020). More recently, similar observations were recorded by Costa *et al.* (2021)who demonstrated that the chronic mild predictable stress group showed significant ($P<0.05$) higher levels of plasma epinephrine and norepinephrine as compared to control and environment rich group. In consistence with these findings, present study also demonstrated that NaF induced stress is one of the reason behind increased plasma epinephrine and norepinephrine levels in the fluorotic rats in comparison to control rats which were free from any stress causing stimulus like NaF. This increase could be associated with increase in catecholamine synthesis due to stress caused by intake of fluoride. The increased levels of catecholamine would have a stimulatory action on the sympathetic nervous system and might influence the hypothalamo-gonadal axis. Moreover, all such intoxicated stress induced ill- effects were improved under post- treatment with Curcumin which is not only a well-known anti-stress agent but also possess many other valuable properties (Abdel-Aziz and Ahmed, 2011; Elsayed *et al*., 2016; Badawy, 2018; Shashi and Tikka, 2021).

Previously, many researchers have found that there was significant increase in thelevels of adrenaline and nor-adrenaline in fluorotic individuals ($P<0.001$) as compared to controls (Michael *et al.* 1996; Barot, 1998; Bagmut *et al*. 2018). This might be due to the stress in adrenal gland or accumulation of fluoride in the body which enhanced the secretion of these hormones

Transmission electron microscopic examination of rat adrenal gland treated with fluoride during present experimental investigation revealed degenerated swollen and damaged mitochondria with disrupted cristae, vacuoles of variable sizes, numerous free ribosomes, microvilli, collagen fibres, widening of cellular junctions, shrunken nucleus with dilated perinuclear space fused lipid droplets, dilated SER, dilated capillaries and autophagic body.

These results are in accordance with Saker and Sabry (2016) who reported deformed mitochondria with abnormal cristae, dilated SER, vacuolated cytoplasm, numerous lysosomes and increased amount of lipid droplets. El-Drieny *et al*. (2009) also reported that Di (2-ethylexyl) Phthalate treatment to rat stimulated the

degenerative changes in the form of cytoplasmic vacuolation, swollen damaged mitochondria, increased lipid droplets and pyknotic nuclei. Mohamed and Hagag (2011) investigated that adrenal cortex of animal treated with lead acetate showed increase in the vacuolation of different zones. These vacuolization in the cortex might be due to the lipid droplets accumulation resulted from failure of their release because of reduction in the use of cholesterol for the synthesis of aldosterone or because of dilation of SER and swollen mitochondria. The impairment of steroid synthesis might be due serious lesions noticed in the mitochondria and endoplasmic reticulum. These organelles play a vital role in steroidogenesis within cortex. The steroid synthesis occurred by the coordinated actions of cytochrome P450 and enzyme 3ß-hydrosteroid dehydrogenase which are localized between the mitochondria and SER (Gurerrero *et al*., 2010).The damage caused in mitochondria and SER induced by fluoride might be due to oxidative stress which changes the structure and permeability of cell membranes (Blokhina *et al.,* 2003). The increase in the lipid droplets and widening of cellular junctions under fluoride toxicity was observed in the present finding. These results coincide with the study by Badawy (2018) who reported the swollen mitochondria, accumulation of lipid droplets, irregular nuclei and widening of cellular junctions between the cells and irregular nuclei in fetal adrenal gland treated with betamethasone.

Elshennawy and Aboelwafa (2011) observed that swelling and vacuolation of mitochondria in the ZF appeared from suppressing the conversion of cholesterol to pregnenolone. However, the mitochondria and SER plays a great role in the steroid synthesis; the damage noticed in them were sufficient to hinder the steroid production which showed further accumulation of cholesterol in the mitochondria. Similar finding were observed in the present study of fluoride induced toxicity in adrenal gland. The adrenal gland of fluoridated rats showed irregular and indented nucleus and increase in lysosomes. The finding are similar with Altayeb and Salem (2017) who demonstrated that stress caused changes in the nuclear envelops in the form of irregularities, indentations, these might be due to compression and condensed lipid droplets in adrenocortical cells. The ZF showed swelling of cells, vacuolation, pyknotic and shrunken nuclei, dilate SER and autophagic vacuoles containing degenerated mitochondria under nicotine induced toxicity (Khalaf *et al.,* 2017). Similar to present study results, many other researchers reported similar findings under the effect of various toxicants and demonstrated swollen and damaged mitochondria, pyknotic nuclei and increase in lipid droplets and numerous free ribosomes (Abdel Aziz; 2010; Abdelaleem *et al,* 2017; El-Tahawy and Abozaid, 2019).

After post-treatment with Curcumin all changes in adrenal gland by fluoride were restored. Other researcher observed similar findings and claimed that Curcumin attenuated the nicotine, betamethasone and streptozotocin toxicity in mice and rats (Abdel-Aziz, 2010; Abdel-Aziz and Ahmed, 2011; Badawy *et al.,* 2018; Baimai *et al*., 2021). Collectively, present study in accordance with previously published reports proved clinical efficacy of Curcumin as a valuable therapeutic agent and finally it was suggested that Curcumin administration may be effective in alleviating the adverse effects of fluoride on adrenal endocrine functions.

5. CONCLUSION

In conclusion, the results suggest that adrenal gland of rats exposed to NaF toxicity changes the hormonal profile of corticotropic and adrenal hormones. The treatment with Curcumin showed the curative effects against fluoride induced stress on adrenal gland. This is affirmed by the reduced levels of ACTH by Curcumin post-treatment. The Curcumin treatment also restored the levels of plasma aldosterone, cortisol and catecholamines. Curcumin administration also resulted in improving the ultrastructural changes caused by NaF toxicity to the normal status. Overall results revealed that, Curcumin helps in ameliorating the NaF induced injury to adrenal gland of rat.

Ethical approval

The experiments were performed under the approval of Institutional Animal Ethics Committee of Punjabi University, Patiala (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.

ACKNOWLEDGEMENT

The financial assistance from CSIR-UGC joint test JRF fellowship under University Grants Commission fellowship schemes, Govt. of India is greatly acknowledged. The authors are thankful to all faculties and staff associated with SAIF facility at All India Institute of Medical Sciences, New Delhi, India where transmission electron microscopy was performed.

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., 2016; 23(15): 15262-15274. 10.1007/s11356-016-6637-x.
2. Abdelaleem, S.A., Hassan, O.A., Ahmed, R.F., Zehom, N.M., Rifaai, R.A., El-Thawy, N.F., 2017. Tramadol induced adrenal insufficiency:histological, immunohistochemical, ultrastructural and biochemical genetic experimental study. J. Toxicol, 2017; 1-15. 10.1155/2017/9815853.
3. Abdel-Aziz, H.O. Morphological evaluation on the protective effect of curcumin on nicotine induced histological changes of the adrenal cortex in mice.

Egypt. J. Histol, 2010; 33(3): 552-559.
4. Abdel-Aziz, H.O., Ahmed, S.A., Curcumin protection against nicotine induced histological changes of the chromaffin cells of adrenal gland. J Am Sci., 2011; 7(9): 698- 703.
5. Abdo, F.K., Hassan, Z.A., Mohamed, D.A., Mousa, H.S. Monosodium glutamate induced histological change in the zona fasiculata of rats adrenal and the possible amelioration effect of vitamin C supplementation. J. Med. Health Sci. Res., 2018; 1(1): 1-7. https://doi.org/10.21839/jmhsr. 2018.v1i1.136.
6. Altayeb, Z.M., and Salem, M.M. Light and electron microscopic study on the effect of immobilization stress on adrenal cortex of adult rats and possible ameliorative role of vitamin E. J. Med. Histol, 2017; 1(1): 44-56.
7. Badawy GM. Curcumin ameliorates the hazard effect of prenatal betamethasone administration on the fetal adrenal gland of albino rats. EJPMR, 2018; 5(12): 133–148.
8. Bagmut, I., Kolisyk, I., Titkova, A., Petrenko, T. and Filipchenko, S. Content of catecholamines in blood serum of rats under fluoride intoxication. Georgian Med. News, 2018; 280-281: 125-129.
9. Baimai, S., Bhanichkul, P., Lanula, P., Niyomchan, A., Sricharoenvej, S. Modification of adrenal gland ultrastructure in streptozotocin-induced diabetic model rats. Int. J. Morphol, 2021; 39(1): 109-115.
10. Barot, V.V. Occurrence of endemic fluorosis in human population of North Gujrat, India: Human Health risk. Bull. Environ. Contam. Toxicol., 1998; 61(3): 303-310.
11. Basha, P.M., Sujitha N.S. Combined impact of exercise and temperature in learning and memory performance of fluoride toxicated rats. Biol. Trace Elem. Res., 2012; 150(1-3): 306-13. doi: 10.1007/s12011-012-9489-3.
12. Blokhina, O., Virolainen, E., Fagerstedt, K.V. Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Bot., 2003; 91(2): 179-94. 10.1093/aob/mcf118.
13. Brook, C., Marshall, N. Essential Endocrinology. Blackwell Science, 1996.
14. Burford, N.G., Webster, N.A., Cruz-Topete, D. Hypothalamic-pituitary-adrenal Axis modulation of glucocorticoids in the cardiovascular system. Int. J. Mol. Sci., 2017; 18(10): 2150-2166. 10.3390/ijms18102150.
15. Carbone, E., Borges, R., Eiden, L.E., Garcia, A.G., Hernandez-Cruz, A. Chromaffin cells of the adrenal medulla: physiology, pharmacology, and disease. Compr. Physiol, 2019; 9(4): 1443-1502. 10.1002/cphy.c190003.
16. Caride, A., Fernandez-Perez, B., Calbaleiro, T., Tarasco, M., Esquifino, A.I., Lafuente, A. Cadmium chronotoxicity at pituitary level: effects on plasma ACTH, GH, and TSH daily pattern. J. Physiol. Biochem, 2010; 66(3): 213-220. 10.1007/s13105-010-0027-5.
17. Costa, R., Carvalho, M.S.M., Brandao, J.D.P., Moreira, R.P., Cunha, T.S., Casarini, D.E., Marcondes, F.K. Modulatory action of environmental enrichment on hormonal and behavioral responses induced by chronic stress in rats: Hypothalamic renin- angiotensin system components. Behav. Brain Res., 2021; 397: 112928. 10.1016/j.bbr.2020.112928.
18. De Falco, M., Sellitti, A., Sciarrillo, R., Capaldo, A., Valiante, S., Iachetta, G., Forte, M., Laforgia, V. Nonylphenol effects on HPA axis of the bioindicator vertebrate, Podarcis sicula lizard. Chemosphere, 2014; 104: 190–196. https://doi.org/10.1016/j.chemosphere.2013.11.014.
19. Deuschle, M., Lecei, O., Stalla, K. G., Landgraf, R., Hamann, B., Lederbogen, F., Uhr, M., Luppa, P., Maras, A., Colla, M., Heuser, I. Steroid synthesis inhibition with ketoconazole and its effect upon the regulation of the hypothalamus-pituitary adrenal system in healthy humans. Neuropsychopharmacology, 2003; 28(2): 379–383. 10.1038/sj.npp.1300044.
20. El-Drieny, E. A., Soliman, G.M., Bayomy, N.A. Histological study of the effect of Di(2-ethylexyl) Phthalate (DEHP) on the adrenal cortex of adult male albino rats and possible protective role of Ginseng. Egypt. J. Histol., 2009; 32(1): 109-117.
21. Elsayed, A.S.I. Curcumin is antioxidant herb, with emphasize on its effects against some diseases. Int. J. Appl. Bio Pharm. Tech, 2016; 7(1): 26-40.
22. Elshennawy W.W., Aboelwafa R.H. Structural and ultrastructural alterations in mammalian adrenal cortex under influence of steroido-genesis inhibitors drug. J. Am. Sci., 2011; 7(8): 567-576.
23. El-Tahawy, N.F.G., Abozaid, S.M.M. The possible structural changes in the adrenal gland cortex after induction of hepatic ischemia-reperfusion injury in male albino rats: Light and electron microscopic study. J. Cell. Physiol, 2019; 234(1): 1-9. 10.1002/jcp.28196.
24. Enyeart, J.A., Liu, H., Enyeart, J.J. Curcumin inhibits bTREK-1 K+ channels and stimulates cortisol secretion from adrenocortical cells. Biochem Biophys Res Commun, 2008; 370(4): 623-628. 10.1016/j.bbrc.2008.04.001.
25. Giulivo, M., Lopez de Alda, M., Capri, E., Barcelo, D. Human exposure to endocrine disrupting compounds: their role in reproductive systems, metabolic syndrome and breast cancer. a review. Environ. Res., 2016; 151: 251–264. https://doi.org/10.1016/j.envres.2016.07.011.
26. Guerrero, B., Finol, H.J., Reyes-Lugo, M., Salazar, A.M., Sanchez, E.E., Estrella, A., Roschman-Gonzalez, A., Ibarra, C., Salvi, I., Rodriguez-Acosta, A. Activitiesagainst hemostatic proteins and adrenal gland ultrastructural changes caused by the brown widow spider Latrodectus geometricus (Araneae: Theridiidae) venom. Comp. Biochem. Physiol. C Toxicol Pharmacol, 2010; 151(1): 113-121. 10.1016/j.cbpc.2009.09.005.

27. Hart, K.A., Barton, M.H. Adrenocortical insufficiency in horses and foals. Vet. Clin. North Am. Equine Pract., 2011; 27(1); 19-34. 10.1016/j.cveq.2010.12.005.
28. Harvey, P.W. Adrenocortical endocrine disruption. J. Steroid Biochem. Mol. Biol., 2016; 155: 199–206. https://doi.org/10.1016/j.jsbmb.2014.10.009.
29. Harwood, J.E. The use of an ion selective electrode for routine fluoride analysis on water sample. Water Res., 1969; 3(4): 273-280. https://doi.org/10.1016/0043-1354(69)90024-4.
30. Hinson, J. P., Vinson, G. P., Kapas, S., Teja, R. The relationship between adrenal vascular events and steroid secretion: the role of mast cells and endothelin. J. Steroid Biochem. Mol. Biol., 1991; 40(1-3): 381-9. 10.1016/0960-0760(91)90205-j.
31. Hinson, J.P., Raven, P.W. Effects of endocrine-disrupting chemicals on adrenal function. Best Pract. Res. Clin. Endocrinol. Metab., 2006; 20; 111–120. 10.1016/j.beem.2005.09.006.
32. Holland, O.B., Carr, B. Modulation of aldosterone synthase messenger ribonucleic acid levels by dietary sodium and potassium and by adrenocorticotropin. Endocrinology, 1993; 132(6): 2666-73. 10.1210/endo.132.6.8389287.
33. Inkielewicz, I., Czarnowski, W. Determination of fluoride in soft tissues. Fluoride, 2003; 36(1): 16-20.
34. Karnovsky, M.J. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol., 1965; 27: 137-138.
35. 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(3): 123-131. 10.1016/j.jmau.2016.11.001.
36. Khalil, M.S. Vitamin D3 may ameliorate the ketoconazole induced adrenal injury:histological and immunohistochemical studies on albino rats. Acta Histochem. Cytochem, 2015; 48(4): 103-113. 10.1267/ahc.14062.
37. Kumar, N. K., Nageshwar, M., Reddy, K. P. Protective effect of curcumin on hippocampal and behavior changes in rats exposed to fluoride during pre- and post- natal period. Basic clin. Neurosci, 2020; 11(3): 289–299. https://doi.org/10.32598 /bcn.11.2.1189.1.
38. Lehoux, J.G., Fleury, A. and Ducharme, L. The acute and chronic effects of adrenocorticotropin on the levels of messenger ribonucleic acid and protein of steroidogenic enzymes in rat adrenal in vivo. Endocrinology, 1998; 139(9): 3913-3922. https://doi.org/10.1210/endo.139.9.6196.
39. Liyanarachchi, K., Ross. R., Debono, M. Human studies on hypothalamo-pituitary- adrenal (HPA) axis. Best Pract Res Clin Endocrinol Metab., 2017; 31(5); 459-473. 10.1016/j.beem.2017.10.011.
40. McQuade, R., Young, A.H. Future therapeutic targets in mood disorders: the glucocorticoid receptor. Brit. J. Psychiatry, 2000; 177: 390-395. https://doi.org/10.1192/bjp.177.5.390.
41. Michael, M., Barot, V.V., Chinoy, N.J. Investigation of soft tissue functions in fluorotic individuals of North Gujrat. Fluoride, 1996; 29(2): 63-71.
42. Minnetti, M., Caiulo, S., Ferrigno, R., Baldini-Ferroli, B., Bottaro, G., Gianfrilli, D., Sbardella, E., De Martino, M.C. and Savage, M.O. Abnormal linear growth in paediatric adrenal diseases: Pathogenesis, prevalence and management. Clin. Endocrinol, 2020; 92(2): 98-108. https://doi.org/10.1111/cen.14131.
43. Mohamed, D.S. and Hagag, K.E.A. Effect of lead acetate on the histological structure of the adrenal cortex of male albino rats and the possible protective role of vitamin E. Egypt. J. Histol., 2011; 34: 496-504.
44. Morgan, E. T., Ullrich, V., Daiber, A., Schmidt, P., Takaya, N., Shoun, H., McGiff, J. C., Oyekan, A., Hanke, C. J., Campbell, W. B. Cytochromes P450 and flavin monooxygenases: targets and sources of nitric oxide. Drug Metab Dispos., 2001; 29(11): 1366-1376.
45. Nabavi, S.F., Nabavi, S.M., Hellio, C., Alinezhad, H., Zare, M., Azimi, R., Bahafar, R.Antioxidant and antihemolytic activities of methanol extract of Hyssopus angustifolius. J. Appl. Bot. Food Qual, 2012; 85: 198-201.
46. Raber, J. Detrimental effects of chronic hypothalamic pituitary-adrenal axis activation. Mol. Neurobiol., 1998; 18: 1-22. 10.1007/BF02741457.
47. Rao, K., Susheela, A.K. Effect of sodium fluoride on adrenal gland of rabbit studies on ascorbic acid and delta 5-3beta hydroxysteroid dehydrogenase activity. Fluoride, 1979; 12: 65-71.
48. Rosol, T.J., Yarrington, J.T., Latendresse, J., Capen, C.C. Adrenal gland: structure, function, and mechanisms of toxicity. Toxicol. Pathol, 2001; 29(1): 41-48. 10.1080/019262301301418847.
49. Sakr, S.M., Sabry, S.A., Midazolum impact on the histological and ultrastructural chacteristics of mice adrenal cortex. Merit Res. J. Med. Med. Sci., 2016; 4(1): 59-67.
50. Schaaf, C., Shan, B., Buchfelder, M., Losa, M., Kreutzer, J., Rachinger, W., Stalla, G.K., Schilling, T., Arzt, E., Perone, M.J., Renner, U., Curcumin acts as anti- tumorigenic and hormone-suppressive agent in murine and human pituitary tumour cells in vitro and in vivo. Endocr. Relat. Cancer, 2009; 16(4): 1339-1350. 10.1677/ERC-09-0129.
51. Shashi, A, Bhardwaj, M., Prevalence of dental fluorosis in endemic fluoride areas of Punjab, India. Biosci. Biotech. Res. Comm, 2011; 4(2): 155-163.
52. Shashi, A., Fluoride and adrenal functions in rabbits. Fluoride, 2003; 36(4): 241-251.
53. Shashi, A., Kaushal, P., Curative effects of Curcumin on gonadotropin and steroid hormones in female rats exposedto fluoride toxicity. BJMHS, 2020; 2(8): 420-427.

54. Shashi, A., Khan, I., Efficacy of *Boerhaavia diffusa* L. on disruption of gonadotropins and testosterone in fluoride intoxicated male rats. Asian J. Pharm. Clin. Res., 2017; 10(12): 68-73. 10.22159/ajpcr.2017.v10i12.20604.
55. Shashi, A., Kumar, M., Bhardwaj, M. Incidence of skeletal deformities in endemic fluorosis. Trop. Doct., 2008; 38(4): 231-233. 10.1258/td.2008.070379.
56. Shashi, A., Tikka, M., Therapeutic effect of curcumin on scanning electron microscopy of rat adrenal gland in experimental fluorosis. J. Pharm. Res. Int., 2021; 33(41): 330-344. https://doi.org/10.9734/jpri/2021 /v33i41A32333.
57. Soliman, H.M., El-Haleem, M.R.A., Tarhouny, S.A.E., Histomorphometrical and electron microscopic study of adrenocorticocytes following surgically induced extrahepatic biliary obstruction in adult female albino rats. Folia Biol., 2015; 61(1): 14-25.
58. Soliman, M.A. and Noya, D.A., The possible ameliorating effect of barley grain on the histological structure of the adrenal cortex in streptozotocin (STZ)-induced diabetes in adult male albino rats. Egypt. J. Histol, 2019; 43(2): 555-568. 10.21608/EJH.2019.16258.1157.
59. 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(1-2): 71-8. 10.1016/s0960-0760(00)00049-2.
60. Wotus, C., Levay-Young, B.K., Rogers, L.M., Gomez-Sanchez, C.E., England, W.C., Development of adrenal zonation in fetal rats defined by expression of aldosterone synthase and 11B-hydroxylase. J. Endocrinol, 1998; 139(10): 4397-4403. https://doi.org/10.1210/endo.139.10.6230.