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HISTOPATHOLOGICAL CHANGES IN ADRENAL GLAND OF WISTAR RATS ON CURCUMIN THERAPY AND FLUORIDE TREATMENT

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.

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ABSTRACT
The present study focused on the efficacy of Curcumin on biochemical and pathological alterations in adrenal gland of rat exposed to sodium fluoride (NaF). Thirty six Wistar albino rats were used in this study. The rats were divided into six groups. Each group consisted of six rats. The group I served as control and given 1 mL of of deionized water/kg b.w./day orally by oral gavage for 40 days. The groups II and III were treated with 300 and 600 mg of NaF/kg b.w./day for the same period respectively. The group IV was administered with 200 mg/kg b.w./day of Curcumin only for 20 days. The groups V and VI were firstly given 300 and 600 mg of NaF/kg b.w./day respectively for 40 days followed by 200 mg/kg b.w./day of Curcumin for next 20 days. After the treatment period, the rats were sacrificed, adrenal tissue was taken out, weighed, and processed for biochemical and histopathological analysis. The activity of acetylcholinesterase and cholesterol was determined. The activity of acetylcholinesterase enzyme significantly ($P<0.0001$) declined while the amount of cholesterol significantly ($P<0.0001$) elevated in adrenal tissue of rat after 40 days of fluoride treatment. Pearson's bivariate correlation and simple linear regression analysis showed significant ($P<0.0001$) negative correlation was observed between the level of adrenal fluoride and activity of acetylcholinesterase (Pearson r =-0.955, $R^2 = 0.913$, Y = 11.237-3.108X) while positive correlation between the levels of tissue fluoride and cholesterol ((Pearson r = 0.954, $R^2 = 0.910$, Y = 10.497+7.851X). The pathological examination of adrenal gland demonstrated disorganization of cells in zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR). The cytoplasmic vacuolization, karyolytic nuclei, swollen cells, single cell necrosis, apoptosis, accumulation of lipid droplets and dilation of blood capillaries were prominent in adrenal cortex. The adrenal medulla showed hypertrophied cells, interstitial fibrosis, and damaged and shrunken chromaffin cells. However, Curcumin administration restored most of the changes caused by sodium fluoride.

KEYWORDS: Acetylcholinesterase, Adrenal gland, Cholesterol, Curcumin, Fluorosis, Histopathology.

INTRODUCTION
Fluorosis is a serious public health problem in many parts of world. Fluoride is apparently the first inorganic ion which brings attention to the scientific world for its harmful effects and now toxicity of fluoride through drinking water is well-known as a global problem. It is mainly classified into dental[1], skeletal[2] and non-skeletal fluorosis.[3,4] The pathological alteration in the structure and functions of some endocrine glands such as hypothalamus[5] parathyroid[6], thyroid[7], and pancreas[8] have been described adequately but less information is available on the adrenal gland in fluorosis.

The adrenal gland is an endocrine gland located above the kidney and also known as suprarenal gland. It is divided into two parts: outer adrenal cortex which produces steroid hormones and inner adrenal medulla which synthesizes epinephrine and norepinephrine are together known as catecholamines. The adrenal cortex is made up of three distinct layers. The zona glomerulosa is outermost zone and contained a thin region of columnar cells arranged in an arched pattern. The zona glomerulosa synthesize the steroid hormone aldosterone, which plays important role in increasing sodium reabsorption and stimulates the excretion of potassium by kidneys and thereby regulating extracellular fluid volume indirectly. The inability or loss of this zone to produce aldosterone might result in death due to excessive loss of sodium, chloride, water and retention of high levels of potassium. The zona fasciculata is the thickest and longest zone. It contained columns of secretory cells separated by capillaries. The cells are polyhedral, multinucleated having many intracellular lipid droplets. This zone synthesizes glucocorticoid hormones. The zona reticularis is also made up of polyhedral cells, which have less linear arrangement and more as round nests or clumps of cells. This zone secrets glucocorticoids, and small amounts of androgens. The

adrenal cortex is responsible for synthesis of both mineralocorticoids and glucocorticoids. The cholesterol is the precursor of steroid hormones. The cytochrome P-450 enzyme converts the cholesterol into steroid hormone in the mitochondria and smooth endoplasmic reticulum.

The cells of the adrenal medulla are derived from the neural crest cell. The secretory cells of adrenal medulla are called as chromaffin cells because after exposure to oxidizing agents such as chromate it forms colored polymers of catecholamines. In response to acetylcholine or calcium ion, they secrete epinephrine and norepinephrine into the blood.[9]

Researchers demonstrated that some toxicants such as fluoride, nicotine, betamethasone and streptozotocin toxicity in mice and rats altered the structure and functions of adrenal gland.[3,10,11,12,13]

Curcumin is diferuloylmethane, a main curcuminoid present in turmeric and give it a yellow color. Curcumin have significant wound healing properties and takes parts in various stages of the natural wound healing process to accelerate healing.[14] It protects the wound tissue from bacterial infection, lessens the inflammation, stimulates cell proliferation and promote the repair of damaged tissue.[15] It promotes cutaneous wound healing via remodeling of tissue, granulation, tissue formation, and collagen deposition.[16] Curcumin has been shown to possess various therapeutic properties. The current study was an attempt to investigate the biochemical and pathological changes in adrenal gland during experimental fluorosis and curative role of curcumin on it.

MATERIALS AND METHODS

Sodium fluoride and Curcumin were purchased from Loba Chemie Pvt. Ltd, Mumbai, India.

Experimental design

Thirty six 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 acclimatization, rats were randomly divided into six groups. Each group consisted of six rats. The group I was administered with 1 mL of deionized water orally for 40 days. The groups II and III were administered with 300 and 600 mg of NaF/kg b.w./day orally for same periods respectively. Group IV was given 200 mg/kg b.w./day of Curcumin only for 20 days. However, groups V and VI initially treated with 300 mg and 600 mg of NaF/kg b.w./day for 40 days, were post-treated with 200 mg/kg b.w./day of Curcumin for next 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 immediately weighed and processed further for biochemical, and histopathological analysis. Body weight was monitored daily.

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. The level of cholesterol in adrenal tissue of rat was measured by using diagnostic kit (Estrom Angstrom Biotech Pvt. Ltd.) following method given in respective datasheets on UV-VIS spectrophotometer (Labtronics model-LT). The activity of AChE enzyme in adrenal tissue was measured by the method of Ellman *et al.*[17]

Statistical analysis

Results were expressed as mean ±SD. All analysis was performed using SPSS 20.0 statistical software (IBM). Data was analyzed using complete randomized design (CRD) analysis followed by critical difference test for body weight and organ weight. The biochemical parameters were analyzed by 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.

Histopathological examination

Adrenal glands from control, fluoridated and fluoridated 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.[18] For Mallory stain, slide of serial section was put in 1% of aqueous solution of acid fuchsin for 2 to 5 minutes, wash quickly in water, then in 1% aqueous solution of phosphomolybdic acid for 1 to 2 minutes, wash in two changes of water and then put the slide in the aniline blue and methyl orange for 1 to 3 minutes.[19] Histopathological changes were studied under research binocular microscope (Leica microsystem) and subsequently microphotographed.

RESULTS

GROSS OBSERVATION

Body weight

There was significant ($P<0.0001$) reduction in the body weight of rats in groups II and III after 40 ($F=29.885$) days of fluoride administration, as compared to control group-I. The decline of -20.91 in 300 mg NaF/kg b.w./day and -28.18% in 600 mg NaF/kg b.w./day dose group was observed in test rats. The critical difference (CD) value was 14.787 for fluoridated groups (Table 1, Fig. 1A)

Table 1: Mean body weight (g) of control and experimental rats.

Treatment groups	Dose (mg NaF/kg b.w.)	Body weight (g) Mean ± SD	% Decline	CRD analysis
Control-1	1mL deionized Water	183.333 ± 10.327		F= 29.885 P<0.0001
II	300	145.00 ± 13.784[a]	-20.91	CD = 14.787
III	600	131.667 ± 11.690[ab]	-28.18	

Table 1 showed values expressed in mean ±SD. [a]P<0.0001 Group II and III compared with control-1. [ab]P<0.167 Group II compared with Group III. Complete randomized design followed by critical difference test.

The fluoridated groups IV and V post-treated with 200 mg Curcumin showed significant (P<0.01) increase in the body weight. The CD value for group V compared with group II was 3.56, while CD value for group VI compared with group III was 3.63 (Table 2, Fig. 1B).

Table 2: Mean body weight (g) of experimental rats post-treated with Curcumin.

Treatment Groups	Body Weight (g) Mean ± SD	CRD Analysis
300 mg NaF	145.000±13.784	F=5.196
300 mg NaF+ 200 mg Curcumin	175.000±10.488[#]	P<0.01 CD=3.56
600 mg NaF	131.667±11.690	F=4.715
600 mg NaF+ 200 mg Curcumin	160.000±12.649[#]	P<0.01 CD= 3.63

Table 2 showed values expressed as Mean±SD. [#]P< 0.01compared to respective NaF treated groups. Complete randomized design followed by critical difference test.

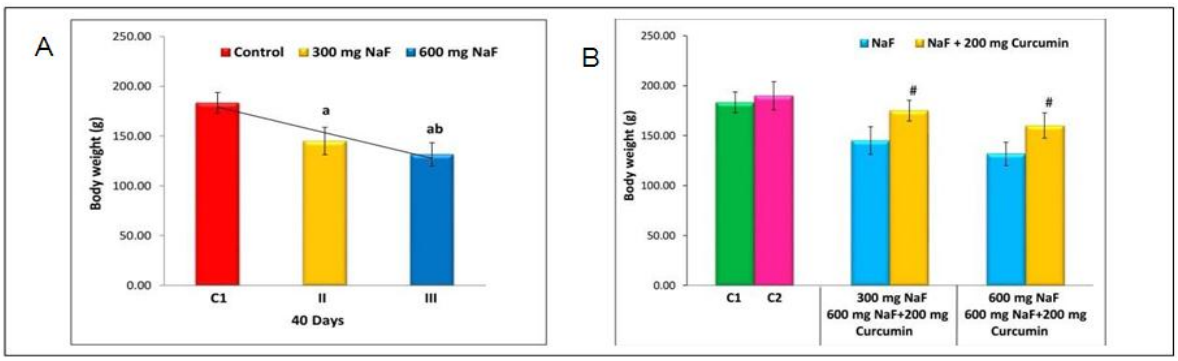

Fig.1 A. Mean body weight (g) of control and fluoridated rats. [a]P<0.0001 Group II and III compared with control-1. [ab]P<0.167 Group II compared with Group III. **B.** Mean body weight (g) of fluoridated rats post-treated with Curcumin. [#]P< 0.01 values were significantly different as compared to respective NaF treated groups. Complete randomized design followed by critical difference test.

Adrenal weight

There was significant increase (P<0.0001) in the organ weight of rats after 40 (F=18.673) days of fluoride administration, as compared to control-1. The elevation of 7.96% in 300 mg NaF/kg b.w./day dose group and 19.43% in 600 mg NaF/kg b.w./day dose group was observed in experimental rats. (Table 3, Fig.2A). In Table 3, the weight of adrenal was increased in group II in comparison to control-I, but the increase was not statistically significant. In addition, the weight of adrenal was significantly (P<0.0001) increased in group III as compared to control-1. The CD value was 1.969 for fluoridated groups.

Table 3: Mean adrenal weight (mg) of control and experimental rats.

Treatment groups	Dose (mg NaF/kg b.w.)	Adrenal weight (mg) Mean ± SD	% Elevation	CRD analysis
Control-1	1mL deionized Water	28.900±1.068		F= 18.673 P<0.0001
II	300	31.200 ±1.418[a]	+7.96	CD = 1.969
III	600	34.516 ±2.129[ab]	+19.43	

Table 3 showed values expressed as Mean±SD. [a]P<0.06, [b]P<0.0001 Group II and III compared with control-1. [ab]P<0.01 Group II compared with Group III. Complete randomized design followed by critical difference test There was statistically significant decrease in the weight of adrenal in group V (P<0.05, CD=1.52) and group VI (P<0.01), CD=1.62. (Table 4, Fig. 2B).

Table 4: Mean adrenal weight (mg) of fluoridated rats post treated with Curcumin.

Treatment Groups	Adrenal Weight (mg) Mean ± SD	CRD Analysis
300 mg NaF	31.200±1.418	F=3.910
300 ppm NaF+ 200 mg Curcumin	27.100±2.334$^{\#}$	P<0.05 CD=1.52
600 mg NaF	34.516±2.129	F=4.176
600 mg NaF+ 200 mg Curcumin	29.483±1.423$^{\#\#}$	P<0.01 CD= 1.62

Table 4 showed values expressed as Mean±SD. $^{\#}P< 0.05$, $^{\#\#}P< 0.01$compared to respective NaF treated groups. Complete randomized design followed by critical difference test.

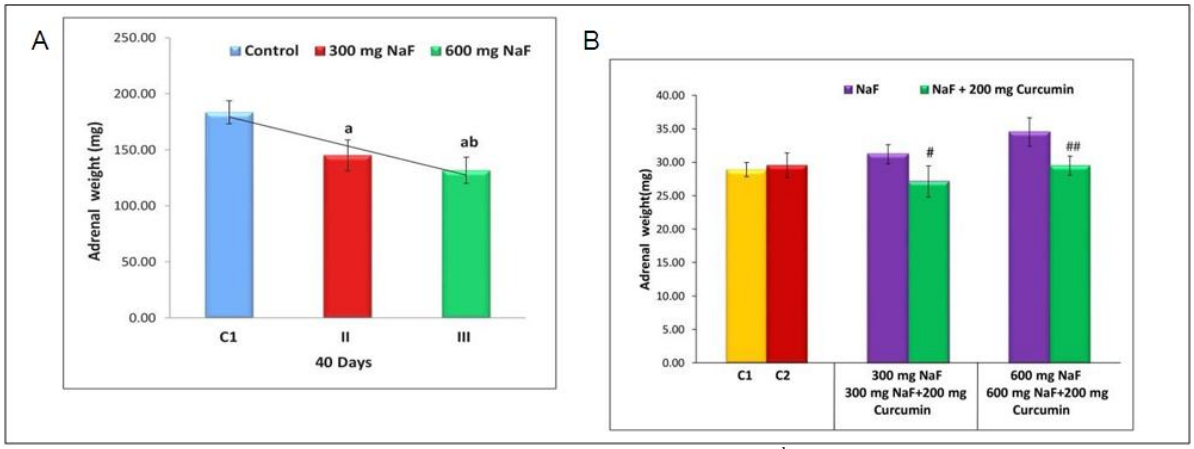

Fig. 2 A. Mean adrenal weight (mg) of control and fluoridated rats. $^{a}P<0.06$, $^{b}P<0.0001$ Group II and III compared with control-1. $^{ab}P<0.01$ Group II compared with Group III. **B.** Mean adrenal weight (mg) of fluoridated rats post-treated with Curcumin. $^{\#}P< 0.05$,$^{\#\#}P< 0.01$ values were significantly different as compared to respective NaF treated groups. Complete randomized design followed by critical difference test.

BIOCHEMICAL ANALYSIS

Acetylcholinesterase (EC 3.1.17.)

The activity of acetylcholinesterase (AChE) showed significant (P<0.0001) decrease in group II and III in comparison to control group-I in adrenal gland after 40 days (F= 106.708) fluoride treatment. The decrease of -32.988% in 300 mg NaF/kg b.w./day dose group and -59.425% 600 mg NaF/kg b.w./day dose group was reported in fluoridated rats (Table 5, Fig.3A).

Post hoc Tukey's HSD test after ANOVA showed significant (P<0.0001) decrease in activity of AChE in adrenal gland between and within groups (95% CI = 2.445 to 4.040; Mean difference = 3.600 to 2.885) after fluoride intoxication for 40 days.

Table 5: Mean activity of acetylcholinesterase (AChE) in control and experimental rats.

Treatment groups	Dose (mg NaF/kg b.w.)	AChE (µ moles/min/ mg protein) Mean±SD	% Decline	F-value
Control-1	1mL deionized Water	10.913±0.819		
II	300	7.313 ±0.667^{a}	-32.988	106.708
III	600	4.428 ±0.816^{aa}	-59.425	P<0.0001

Table 5 showed values expressed as Mean±SD. One way ANOVA followed by Post hoc Tukey's HSD. $^{a}P<0.0001$ Group II-III compared with control-1. $^{aa}P<0.0001$ Group II compared with groupIII.

Pearson's bivariate correlation and simple linear regression analysis demonstrated significant (P<0.0001) negative relationship between levels of fluoride (µg/g) and AChE (µ moles/min/mg protein) activity in adrenal gland (Pearson r =-0.955, R^2 = 0.913, Y = 11.237-3.108X; Fig. 3B) after 40 days of fluoride exposure.

Bonferroni multiple comparison after ANOVA showed significant (P<0.01-0.001) increase in the activity of AChE in adrenal gland of all NaF treated groups (95% CI = -2.880 to -2.524; Mean difference = -1.940 to -4.207) post-treated with 200 mg/kg b.w./day of Curcumin for 20 days (group V and group VI) (Table 6, Fig. 3C).

Table 6: Mean activity of AChE (µ moles/min/ mg protein) fluoridated rats post-treated with Curcumin.

Treatment Groups	AChE (µ moles/min/ mg protein) Mean±SD	Significance
300 mg NaF	7.313±0.667	P<0.01
300 ppm NaF+ 200 mg Curcumin	9.253±0.959[#]	
600 mg NaF	4.428±0.816	P<0.001
600 mg NaF+ 200 mg Curcumin	8.635±0.842[##]	

Table 6 showed values expressed as Mean±SD. The pairwise comparison was done by Bonferroni multiple comparison test. [#]P<0.01, [##]P<0.001 values were significantly different as compared to respective NaF treated groups II and III.

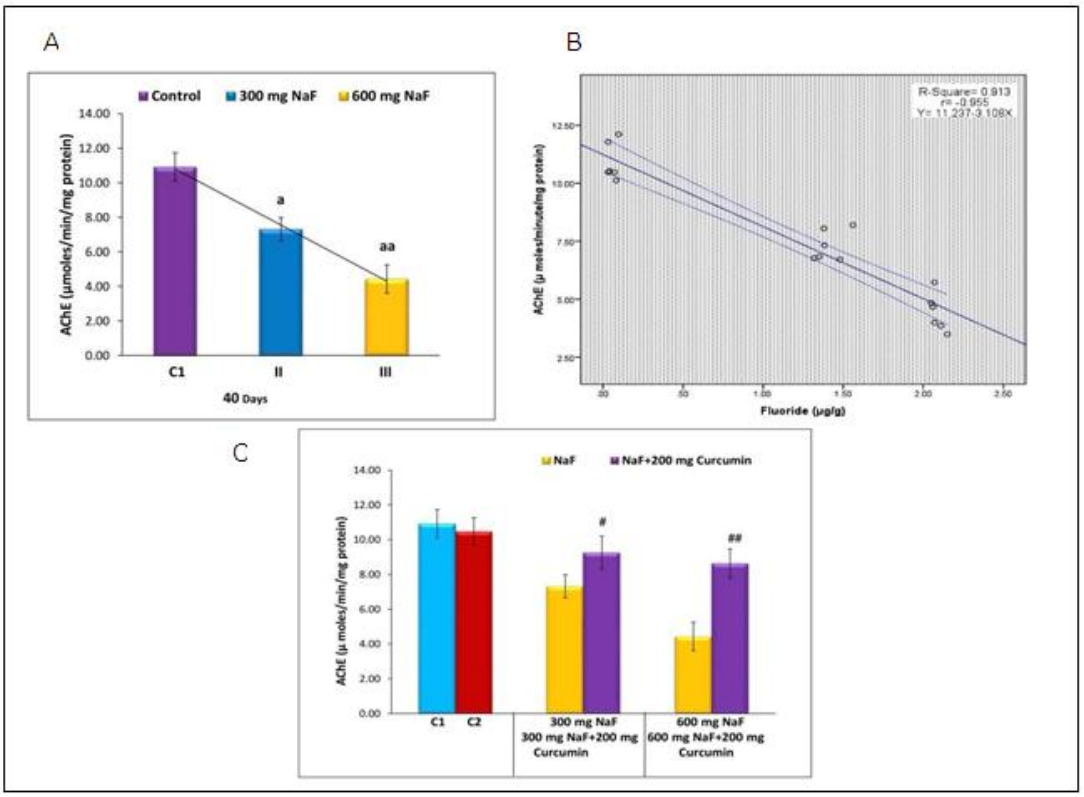

Fig. 3 Mean activity of AChE (µ moles/minute/mg protein) in adrenal gland of control and fluoride exposed rats. [a]P<0.0001 Group II-III compared with control-1. [aa]P<0.0001 Group II compared with group III. **B.** Scatterplot showing Pearson's bivariate correlation and simple linear regression between level of fluoride (µg/g) and AChE activity (µ moles/minute/mg protein) in adrenal gland of rats after 40 days of fluoride treatment. **C.** Mean activity of AChE (µ moles/minute/mg protein) in adrenal gland of fluoridated rats post-treated with Curcumin. [#]P<0.01, [##]P<0.001 values were significantly different as compared to respective NaF treated group.

Cholesterol

The levels of cholesterol in adrenal gland of fluoride treated rats showed significant (P<0.0001) increase in groups II and III as compared to control group-1 after 40 days (F= 137.737) of fluoride treatment. The increase of 72.977% in 300 mg NaF b.w./day dose group and 144.151% in 600 mg NaF/kg b.w./day dose group was observed in test rats (Table 7, Fig.4A)

Post Hoc Tukey's HSD test after ANOVA showed significant (P<0.0001) increase in levels of cholesterol in adrenal gland between and within groups (95% CI = -10.972 to -5.586; Mean difference = -8.385 to -8.178) after 40 days of fluoride treatment.

Table 7: Mean level of Cholesterol (mg/dl) in control and experimental.

Treatment groups	Dose (mg NaF/kg b.w.)	Cholesterol (mg/dl)	% Elevation	F-value
Control-1	1mL deionized Water	11.490±1.159		
II	300	19.875 ±1.926[a]	+72.977	137.737
III	600	28.053 ±1.977[aa]	+144.151	P<0.0001

Table 7 showed values expressed as Mean±SD. One way ANOVA followed by Post hoc Tukey's HSD. [a]P<0.0001 Group II-III compared with control-1. [aa]P<0.0001 Group II compared with group III.

Pearson's bivariate correlation and simple linear regression analysis demonstrated significant (P<0.0001) positive relationship between levels of fluoride and cholesterol in adrenal gland (Pearson r = 0.954, R^2 = 0.910, Y = 10.497+7.851X) after fluoride exposure for 40 days (Fig. 4B).

Bonferroni multiple comparison test after ANOVA showed significant (P<0.05-0.001) decrease in levels of cholesterol in adrenal gland of all NaF treated groups (95% CI = 0.276 to 8.261; Mean difference = 3.933 to 6.107) post-treated with 200 mg/kg b.w./day of Curcumin for 20 days (group V and group VI) (Table 8, Fig 4C).

Table 8: Mean level of Cholesterol (mg/dl) fluoridated rats post-treated with Curcumin.

Treatment Groups	Cholesterol (mg/dl)	Significance
300 mg NaF	19.875±1.926	P<0.05
300 ppm NaF+ 200 mg Curcumin	15.941±1.950#	
600 mg NaF	28.053±1.977	P<0.001
600 mg NaF+ 200 mg Curcumin	21.947±1.600##	

Table 8 showed values expressed as Mean±SD. The pairwise comparison was done by Bonferroni multiple comparison test. #P<0.05, ##P<0.001 values were significantly different as compared to respective NaF treated groups II and III.

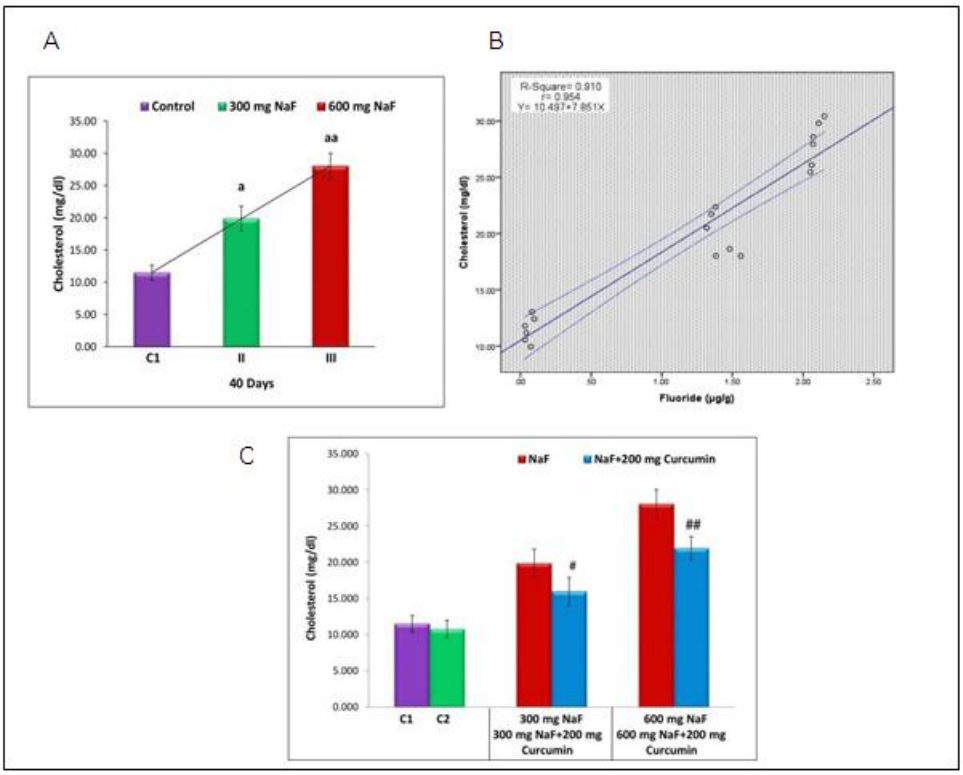

Fig. 4 Mean level of cholesterol (mg/dl) in adrenal gland of control and fluoride treated rats for 40 days. [a]P<0.0001 Group II-III compared with control-1 .[aa]P<0.0001 Group II compared with group III. **B.** Scatterplot showing Pearson's bivariate correlation and simple linear regression between level of fluoride (µg/g) and cholesterol (mg/dl) in adrenal gland of test rats after 40 days of fluoride exposure. C. Mean level of cholesterol in adrenal gland of fluoride treated rats post- treated with Curcumin. #P<0.05, ##P<0.001 values were significantly different as compared to respective NaF treated group.

HISTOPATHOLGICAL EXAMINATION

Adrenal cortex

The adrenal cortex of control rat consists of zona glomerulosa (ZR), zona fasciculata (ZF), and zona reticularis (ZR). The zona glomerulosa layer had oval and rounded clusters with deeply stained nuclei (Fig. 5A). The zona fasciculata was the widest cortical zone and has multinucleated cells with acidophilic cytoplasm. The cytoplasm was foamy in appearance due to presence of small and large oval lipid droplets (Fig. 5B). The cells of zona reticularis were small and deeply stained. Cells were irregularly arranged and formed a network (Fig. 5C).

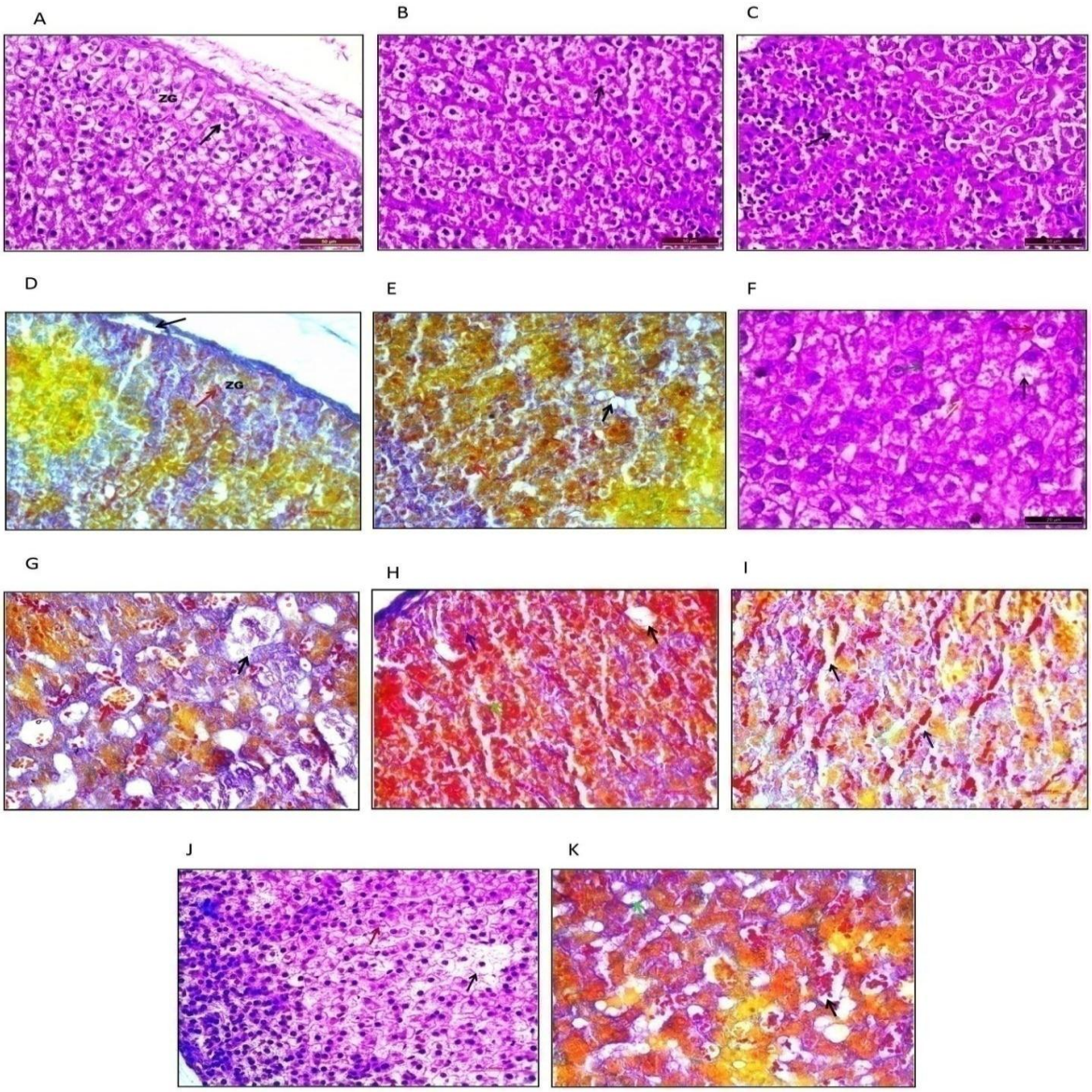

Fig. 5. T.S. of adrenal cortex of control rat showing A. The cells of zona glomerulosa(ZG) arranged in oval and rounded (↑) clusters with deeply stained nuclei. B. Zona fasciculate having multinucleated cells (↑) with eosinophilic cytoplasm. C. Zona reticularis irregularly arranged and form a network (↑) Haematoxylin and Eosin X400. The adrenal cortex of rat treated with 300 mg/kg b.w./day of NaF showing D. Disintegration of capsule (↑) and distorted (↑) zona glomerulosa cells (ZG). E. Cell vacuolation (↑) and disrupted cell membrane (↑) in zona fasciculata. Mallory triple stain X400. F. Zona fasciculata cells having swollen nuclei (↑) karyolytic (↑) or lost nuclei (↑) and single cell necrosis (↑). Haematoxylin and Eosin stain X1000. G. Zona reticularis showed irregular anastomosing cords separated by wide blood capillaries (↑) and necrotic area (↑) Mallory triple stain X400. The adrenal cortex of rat treated with 600 mg/kg b.w./day of NaF showing H. Irregular orientation of zona glomerulosa (↑), swelling (↑) and vacuolation of some of the cells of zona fasciculata (↑). I. Zona fasciculata cells, dilation (↑) and congestion of blood sinusoid with stagnant blood (↑) Mallory triple stainX400. J. Zona fasciculata cells with excessive lipid droplets (↑), hypertrophied (↑) and radially arranged in cords with foamy cytoplasm. Haematoxylin and Eosin X400. K. Zona reticularis contained trabeculae (↑) and loss their arrangement with enlarged blood sinusoid loaded with blood (↑). Mallory triple stain X400.

The rats treated with 300 mg/kg b.w./day NaF for 40 days showed disintegration of capsule and distorted zona glomerulosa (Fig. 5D). The cells of zona fasciculata appeared ballooned with disruption in cell membranes and cytoplasmic vacuolation (Fig. 5E). The cells appeared swollen and distended with highly vacuolated cytoplasm. Some cells were karyolytic and others had lost nuclei (Fig. 5F). The zona reticularis exhibited irregular anastomosing cords separated by wide blood capillaries filled with blood, cytoplasmic vacuolation, and necrotic area (Fig. 5G).

The rats treated with 600 mg/kg b.w./day NaF for 40 days revealed zona glomerulosa cells having irregular orientation. Most of zona glomerulosa cells showed swelling and marked cytoplasmic vacuolation (Fig 5H). Blood sinusoid of zona fasciculata became dilated and filled with stagnant blood in their lumina which was lined by pyknotic endothelial cells (Fig. 5I). The adrenal cortex was disorganized with highly vacuolated zona glomerulosa and zona fasciculata cells. The ballooned zona fasciculata cells were radially arranged in cords along with accumulation of lipid droplets, and formed foamy cytoplasm (Fig. 5J). The zona reticularis showed unclear distributed network of its own cells. The cells were enlarged and contained well demarcated vacuoles. Vascular network showed engorged blood vessels (Fig. 5K).

The rat treated with 200 mg/kg bw/day of Curcumin showed ZG, ZF, and ZR similar to control (Fig. 6A, 6B).

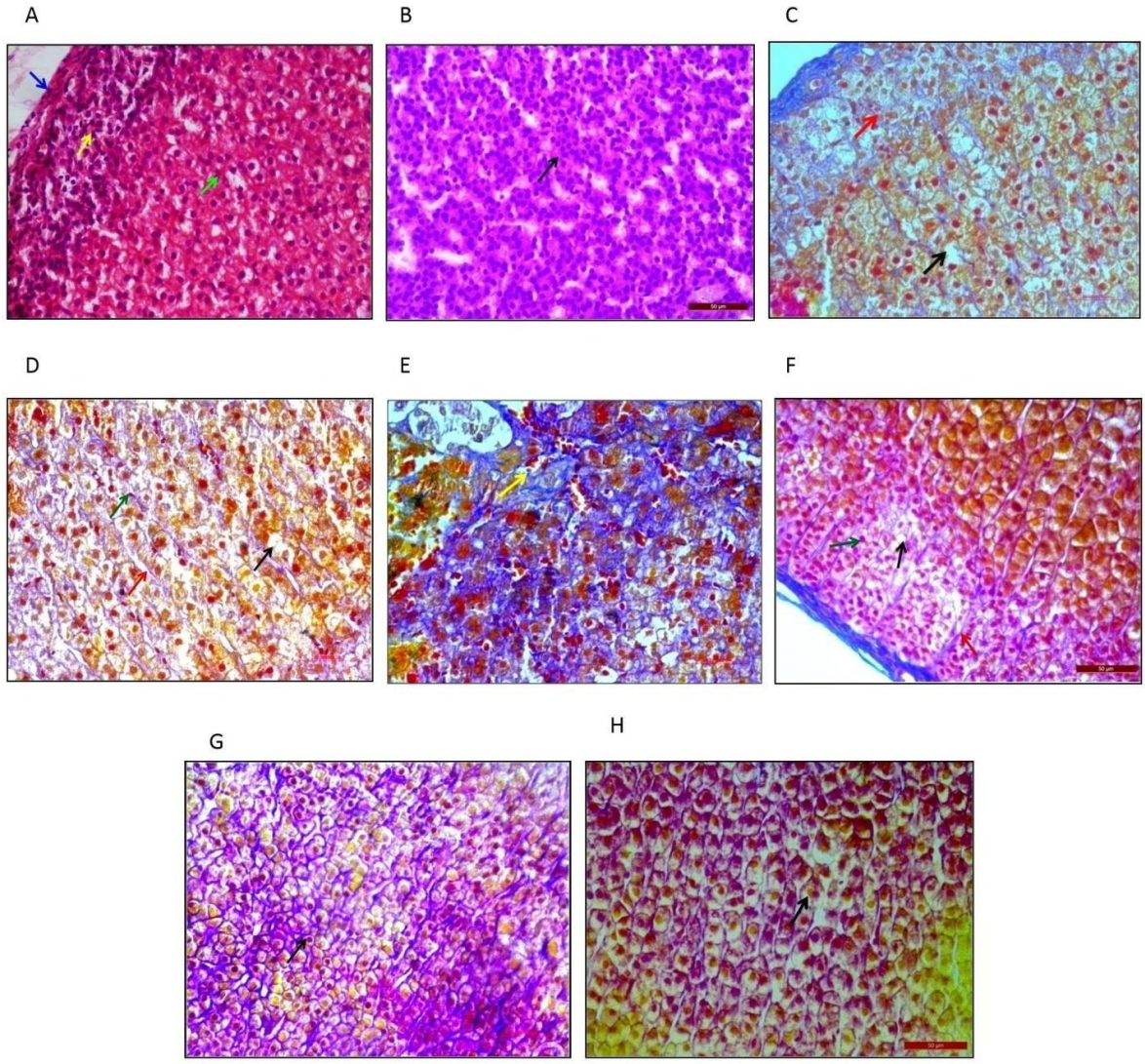

Fig. 6 T.S of adrenal gland of treated with 200 mg/kg b.w./day of Curcumin showing A. capsule (↑) normal cells of zona glomerulosa (↑) and zona fasciculata (↑). A. Zona reticularis (↑). Haematoxylin and Eosin stain X 400. The adrenal cortex of rat treated with 300 mg/kg b.w./day of NaF post-treated with 200 mg/kg b.w./day of Curcumin showing B. Reduction in lipid droplets (↑) and few vacuolated cytoplasm (↑). D. Reapperance of cords (↑) in zona fasciculata, some cells had normal, large nuclei, some cells had shrunken nuclei (↑) and

cytoplasmic vacoulations (↑). E. Zona reticularis cells appeared with multiple congested blood sinusoids (↑) with apparent normal cells. Mallory triple stain X400. The adrenal cortex of rat treated with 600 mg/kg b.w./day of NaF post-treated with 200 mg/kg b.w./day of Curcumin showing F. Zona glomerulosa with few vacuolated cells (↑) and few darkly stained nuclei (↑) and fine trabeculae (↑). G. Zona fasciculata cells arranged in parallel columns, some of nuclei are darkly stained (↑) and their cytoplasm showed minimal vacuolation. H. Zona fasciculata with its polyhydral cells having rounded nuclei (↑) and few vacuoles in cytoplasm. Mallory triple stain X400.

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. There were reduction in lipid droplets in zona glomerulosa, zona fasciculata cells and few cytoplasmic vacuolation in zona fasciculata (Fig. 6C). Some cells had normal large rounded nuclei. However, some of the cells had small shrunken nuclei and cytoplasmic vacuolation. The reappearance of the cord like arrangement was also seen in zona fasciculata. In zona fasciculata, most of the adverse changes that were observed in group II, were restored in curcumin post-treated group (Fig. 6D). The zona reticularis showed multiple congested blood sinusoids. Some of the cells were normal (Fig. 6E).

In the rats post-treated with 200 mg/kg b.w./day of curcumin after 600 mg/kg b.w./day of NaF demonstrated zona glomerulosa with few vacuolated cells, few darkly stained nuclei and fine trabeculae (Fig. 6F). The zona fasciculata cells were arranged in parallel columns. The nuclei of cells were darkly stained and their cytoplasm showed minimal vacuolation (Fig. 6G). Most of the adverse changes observed in group III were reduced in post-treated group VI. The cells were polyhedral in shape having rounded nuclei (Fig. 6H).

Adrenal medulla

The adrenal medulla is central part of adrenal gland. In control rats, it has chromaffin cells having deeply stained granular cytoplasm (Fig. 7A). The rats treated with 300 mg/kg b.w./day NaF for 40 days showed that in adrenal medulla most of chromaffin cells appeared hypertrophied with ill defined boundaries. The nuclei and cytoplasm was pale in staining. Interstitial fibrosis and small clusters of macrophages were distributed among chromaffin cells (Fig. 7B). The rats treated with 600 mg/kg b.w./day NaF for 40 days, adrenal medulla had edema of cytoplasm, vacuolar degeneration and depletion of secretory granules. Existence of small amount of connective tissues was also observed (Fig. 7C).

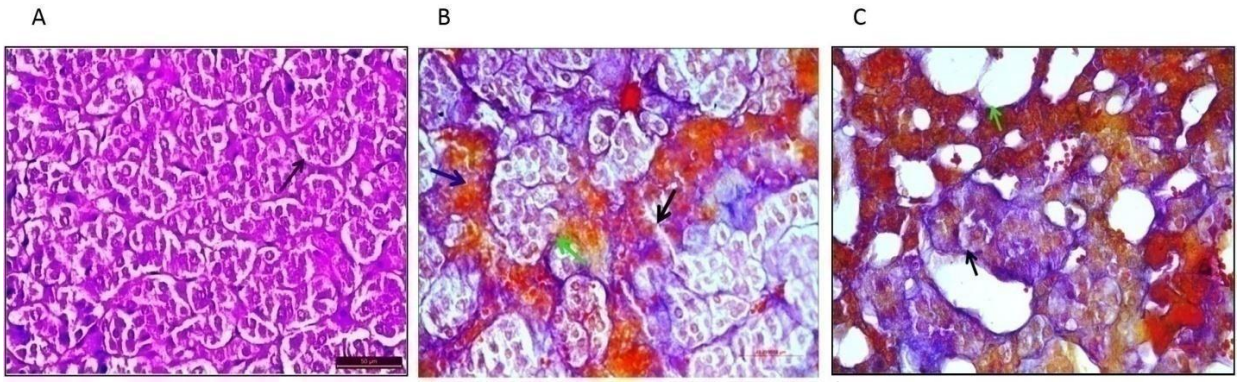

Fig. 7 A. T.S. of adrenal medulla of control rat showing chromaffin cells (↑). Haematoxylin and Eosin X400. B. The adrenal medulla of rat treated with 300 mg/kg b.w./day of NaF showing The hypertrophied chromaffin cells (↑) with ill defined boundaries, fibrosis (↑) and macrophages infiltration (↑). C. The adrenal medulla of rat treated with 600 mg/kg b.w./day of NaF showed edema, cytoplasm Edema, cytoplasm vacuolar degeneration (↑), cells are irregular and damaged (↑), and small amount of connective tissue. Mallory triple stain X400.

In the rat treated with 200 mg/kg b.w./day of Curcumin the adrenal medulla contained chromaffin cells with blood sinusoids (Fig. 8A). 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 that normal chromaffin cells reappeared with few damaged cells in adrenal medulla (Fig. 8B). The cells had large nuclei with rounded, prominent nucleolus (Fig. 8C). In the rats post-treated with 200 mg/kg b.w./day of curcumin after 600 mg/kg b.w./day of NaF, the adrenal medulla exhibited most of chromaffin cells having granular cytoplasm and few cells with vacuolated cytoplasm (Fig. 8D, 8E).

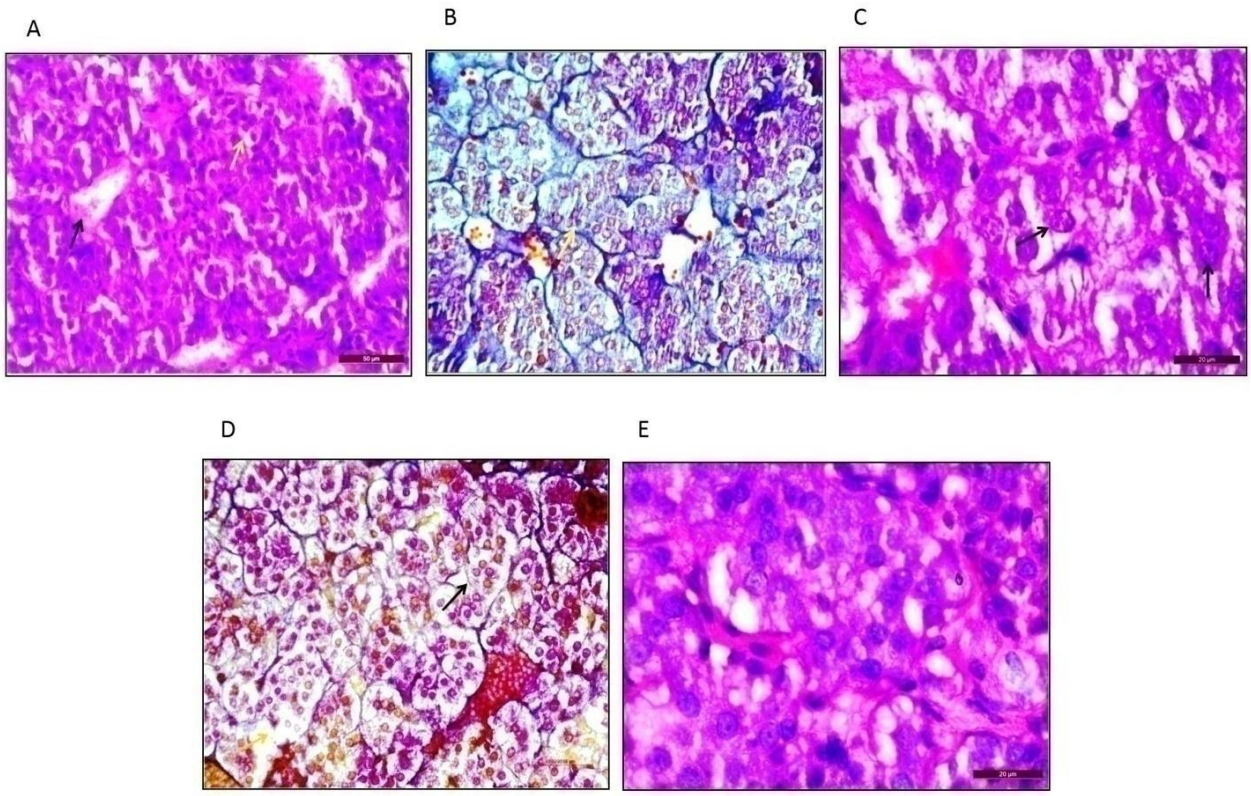

Fig. 8 T.S of adrenal medulla of rat treated with 200 mg/kg b.w./day of Curcumin showing A. chromaffin cells (↑) and blood sinusoid (↑). Haematoxylin and Eosin X400. The adrenal medulla of rat treated with 300 mg/kg b.w./day of NaF post-treated with 200 mg/kg b.w./day of Curcumin showing B. Clusters of chromaffin cells (↑) with few vacuolar degeneration. Mallory triple stain X400. C. The cells of the adrenal medulla with nuclei appeared large rounded with prominent nucleolus (↑). Haematoxylin and Eosin X1000. The adrenal medulla treated with 600 mg/kg b.w./day of NaF for 40 days and post-treated with 200 mg/kg b.w./day of Curcumin showing D. Most of the chromaffin cells appeared with granular cytoplasm (↑) and few cells with vacuolated cytoplasm (↑). Mallory Triple stain X400. E. Restoration of chromaffin granules (↑ X Haematoxylin and EosinX100.

DISCUSSION

The results of present study demonstrated that fluoride induced significant (P<0.0001) reduction in the body weight and increase in adrenal weight of experimental rats. Furthermore, Curcumin administration confirmed its curative role in alleviating the toxicity by significantly increasing the body weight and reducing adrenal weight in fluoridated rats. The results are in accordance with Zabulyte *et al.*[20] who demonstrated that rats treated with NaF and NaF plus nitrate showed reduction in the body weight and enlarged adrenal gland in comparison to control.

Similar observation was recorded by other researchers who investigated that diabetes caused reduction in the body weight and increase in adrenal weight.[21,22] The reduction in the body weight in case of diabetes occurs due to the excessive loss of tissue protein or lacking of carbohydrates required for the energy metabolism which leads to the degradation of structural proteins and muscle wasting.[23] Mustuga *et al.*[24] reported that aminoglutethimide caused decrease in the body weight and increase in the adrenal weight. Abass *et al.*[25] observed that atrazine toxicity caused the significant (P<0.05) decrease in the body weight as compared to control. Similar finding was reported by Zaya *et al.*[26] who studied difference mechanism responsible for the reduced body weight. The increase in the energy demand for the atrazine detoxification caused beta oxidation of lipid and mobilization of fat.

Same observation was noticed in case of present study which suggested that body weight might be reduced by the loss of tissue proteins and detoxification of fluoride toxicity and beta oxidation of lipids. Ulrich-Lai *et al.*[27] investigated that chronic variable stress increased the adrenal weight. The increase in adrenal weight might be related with increase in ACTH hormone level which may have occurred due to feedback mechanism as a result of reduction in the production of aldosterone and corticosterone. The hypersecretion of ACTH stimulated the hyperplasia of the adrenal gland, leading to extracellular signal regulated kinases (ERKs) activation and hence displayed a necessary role in cellular proliferation and stimulation.[28,29] The present study also demonstrated that increase in the adrenal weight of

fluoridated rats might be due to hypersecretion of ACTH and reduction of aldosterone and cortisol and accumulation of lipid droplets which stored cholesterol. These changes were reversed by Curcumin administration.

During present study, the level of cholesterol was elevated in adrenal gland of fluorotic rats in comparison with control. Previously from our laboratory reported that cholesterol content in adrenal gland showed significant (P<0.001) rise in male animals followed by rapid decline (P<0.001).[3] The accumulation of cholesterol in the organs is a sign of alteration in steroid metabolism. Similar finding was recorded by Sharma *et al.*[30] who revealed that concentration of cholesterol in ovary and adrenal gland was increased significantly (P<0.001) which might have caused changes in the steroid synthesis and affected the function of organs. The cholesterol acts as a precursor molecule in ovary and adrenal gland for the synthesis of steroidal hormone.[31,32] Mehta and Singh (2012)[33] observed a significant (P<0.001) increase in the levels of cholesterol in liver and testis under fluoride toxicity. Results suggested that fluoride increased the cholesterol in adrenal gland which might have inhibited the steroid hormone synthesis.

During present study, activity of AChE decreased in all NaF treated groups while AChE activity was restored when fluoridated rats were post-treated with Curcumin. These results are in agreement with Zabulyte *et al.*[20] who reported 24% decrease in the activity of AChE enzyme in NaF and NaF plus nitrate treated group in comparison to control.

The AChE is the main enzyme which governs the cholinergic transmission in both central and peripheral nervous system. Fluoride caused toxic effect in the brain region by inhibiting the AChE causing abruption in the cholinergic transmission which in turn alters the learning and memory ability.[34] Fluoride also showed adverse effect on AchE activity which caused the hydrolysis of esters of choline. This toxic effect might cause alteration in the utilization of acetylcholine, thus affecting the transmission of the nerve impulse in brain tissue.[35,36] Basha and Sujitha[37] demonstrated that rats exposed to fluoride showed inhibition in AChE activity which is in accordance with the previous reports.[38,39]

The present finding revealed the microscopic examination of adrenal gland of NaF treated rats showed increase in cytoplasmic vacuolation in the cells of zona glomerulosa, zona fasciculata and accumulation of lipid droplets, which might be due to the impairment the synthesis of glucocorticoids. As the zona fasciculata is responsible for the synthesis and secretion of glucocorticoids so the disrupted steroidogenesis had a vital role in the toxicity of adrenal cortex. This may be occurring due to disruption of enzyme cytochrome 450 and therefore, cholesterol biosynthesis is inhibited, and lead to accumulation of lipid droplets and cytoplasmic vacuolation in the cells of zona fasciculate.[40]

Khalaf *et al.*[41] reported accumulation of lipid droplets and appearance of cytoplasmic vacuolation in the zona fasciculata cells after administration of nicotine This also was in accordance with the data of previous researcher who noticed accumulation of lipid droplets in the zona fasciculata and zona reticularis after suppression of steroidogenesis via dexamethasone administration.[42]

The present study demonstrated that NaF induced pathological changes in zona glomerulosa and zona fasciculata in the form of disorganization of cells. The cells became ballooned and vacuolated with pyknotic nuclei also showed karyolyis, single cell necrosis, and dilated and hyperemic blood capillaries.

The effects of different treatments on adrenal gland has been studied by different researchers, as Lorentle *et al.*[43] reported the change in the rat adrenal zona glomerulosa under the effect of chronic hypoxia. Hinsel *et al.*[44] recorded ketonazole induced dilation and engorged blood sinusoid mainly in zona fasciculata and zona reticularis. Saker and sabry[45] revealed that midazolum treatment in mice showed hypertrophy in the cells of zona glomerulaosa, zona fasciculata and zona reticularis with large vacuole in their cytoplasm. Blood sinusoids of zona fasciculata became dilated and loaded with stagnant blood. The results are in agreement with the study of Cekic *et al.*[46] and Bojanovic *et al.*[47] who reported that the monosodium glutamate treated rat showed hypertrophy of the adrenal gland. They documented that the cortex was widened and zona fasciculata showed large cells and abundant intracytoplasmic lipid droplets. Interstitial fibrosis was also observed in the all zones of adrenal cortex and medulla in rats with acute heat stress.[48]

El-Helbawy *et al.*[49] investigated that monosodium glutamate caused histopathological changes in the cells of zona fasciculata. The cells were swollen with karyolytic nuclei or other lost their nuclei. Cytoplasmic syncetium was observed. Mustuga *et al.*[24] reported that aminoglutethimide treated mice showed cytoplasmic vacuolation and single cell necrosis was observed in zona fasciculata cells. Abdo *et al.*[50] found that monosodium glutamate administration caused loss of parallel arrangement of zona fasciculata cells. Dilated capillaries were observed in between the cells of zona fasciculata. Many macrophages were seen in the lining of sinusoids.

The present finding showed the depletion of chromaffin granules and apoptotic changes in the chromaffin cells of medulla treated with NaF. Most of chromaffin cells appeared hypertrophied. Similar finding was observed by Abdel-Aziz[11] who reported that nicotine toxicity caused hypertrophied cells and vacuolated cytoplasm of most of chromaffin cells in adrenal medulla. Raees *et al.*[51] found that diazinon exposure group showed the depletion of

cytoplasmic granules in chromaffin cells of the adrenal medulla. Also previous study has demonstrated the ameliorative role of curcumin against betamethasone on the fetal adrenal gland of albino rats.[12] In addition, Abdel-Aziz[10] investigated that curcumin administration has protective effect on the cells of the adrenal cortex against the toxicity of nicotine. All studies suggested that Curcumin is effective to cure fluoride-induced toxicity in adrenal gland of rat.

CONCLUSION

In conclusion, the present study demonstrated reduction in the relative body weight and increase in the adrenal weight of rats under fluoride induction. The level of cholesterol was increased whereas inhibited acetylcholinesterase activity was seen in fluoridated rats. Furthermore, histopathological changes characterized by single cell necrosis, cytoplasmic vacuolation, increase in intracellular lipid droplets, swelling, dilation of blood vessels, hypertrophied cells and necrotic area were also observed in the adrenal gland. Expectedly, all of the above mentioned adversities caused due to fluoride induction were normalized when Curcumin was administered. It is worth noticing that pathological changes in the different zones of adrenal gland of fluoridated rats were improved but not completely reversed in Curcumin post-treatment.

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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