

Protective Effect of Curcumin against Toxic Effects of Sodium Fluoride on Lungs of Adult Male Albino Rats

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ABSTRACT

Sodium fluoride (Na-F) is added to drinking water as a decay preventive ingredient. Lungs are susceptible to oxidative injury because of their constant exposure to air containing toxic fluoride particles. Curcumin exhibits anti-inflammatory, anti-tumor and antioxidant properties. To study the toxic effects of Na-F on the lungs and to evaluate the protective role of curcumin, 3 albino rat groups; control, Na-F treated & curcumin protected, were used. Lung tissues were visualized by light and electron microscope. Plasma malondialdehyde (MDA) levels and erythrocyte superoxide dismutase (SOD) & catalase (CAT) activities were determined. Na-F treated group showed collapse of the alveoli with marked thickening of inter-alveolar septa with dilated congested blood vessels, increased collagen deposition. MDA level showed significant increase while erythrocyte SOD and CAT activities showed significant decrease. Curcumin protected group revealed normal shaped alveoli separated by thin inter-alveolar septa with minimal localized cellular infiltration. Moreover, MDA level, SOD and CAT activities drew closer to values of control group.

Keywords: Fluoride, lung, curcumin

Introduction:

Fluoride is an essential trace element from the halogen group which is widely distributed in the environment [1]. The wide spread distribution of fluoride in nature and chronic exposure of millions of people worldwide is an endemic problem in a number of countries [2]. Sea food, bony meals, kale, barley, rice [1] and dark green vegetables as the tea plant [3] are very rich in fluoride. Moreover, food additives, insecticides, anti-carcinogenic drugs and some inhalational anesthetics such as methoxiflurane are other sources of fluoride exposure [4]. Also, cooking in Teflon lining cookware may increase the concentration of fluoride in food prepared inside them [5]. Sodium fluoride is the most commonly used compound in oral caries prevention in the form of fluorinated drinking water, salts or milk, tooth pastes, mouth washes and fluoride tablets [6].

Barot, 1998 and Ortiz et al., 2003[7&8] stated that Egypt among about 21 countries that had problems with endemic fluorosis where the main pathway of fluoride exposure is the ingestion of tap water from contaminated ground water sources. The source of drinking water in the two governorates of Marsa Matrouh

and Arish however, is groundwater coming from artesian wells. This water contained higher levels of fluoride with an average of 0.761 and 0.926 mg/L for Marsa Matrouh and Arish respectively [9].

Fluoride is known to cross the cell membranes and to enter soft tissues [10]. Fluorosis is a slow and progressive process causing metabolic, functional and structural damages affecting many tissues particularly musculoskeletal, dental systems [11], kidney [4], liver [12] and brain [13]. The existence of correlations between fluorosis and oxidative stress in some organs had been proven [14 & 15].

It is known that fluoride-induced increase in the generation of free oxygen radicals and decrease in antioxidant enzyme capacity play an important role in fluorosis [16 & 17]. Increase in tissue levels of lipid peroxidants, which are generated as a result of oxidative stress, is prevented by antioxidant defense systems, which are either endogenous and enzymatic or non-enzymatic [18]. In circumstances where these systems are insufficient, antioxidants are used to prevent oxidative stress.

Curcumin is the active polyphenolic compound in the *Curcuma longa* Linn. It is used for the treatment of dental diseases, digestive disorders such as dyspepsia and acidity, indigestion, flatulence, ulcers, and to alleviate the hallucinatory effects of marijuana and other psychotropic drugs [19]. In food industries, curcumin is used in perfumes and as a natural yellow food additive to flavor various types of curries and mustards [19&20]. Curcumin exhibits anti-inflammatory, anti-tumour, and antioxidant properties [21].

Curcumin in the spice turmeric, have tumour-suppressing properties in rodent models of carcinogenesis, and it interferes with cellular processes involved in tumour promotion and progression. Kinases, telomerase, cyclooxygenase-2, triggers of apoptosis, and transcription factors AP1 and nuclear factor kappa B are among the cellular targets [22].

Studies have found that polyphenolic compounds like quercetin and curcumin can reduce or prevent oxidative damage caused by toxicants and oxidative materials [23].

The aim of this work was to study the toxic effects of sodium fluoride on the lungs of adult male albino rats and evaluate the possible protective role of curcumin supplementation against oxidative stress induced by sodium fluoride.

Materials and methods

Animals

Thirty healthy adult male albino rats, (3- 5 months aged) weighting about 200-250 grams, were used in this study. They were obtained from the breeding animal house, Faculty of Medicine, Zagazig University. The animals were housed in stainless steel cages and were maintained at a room temperature with normal light-dark cycle. They were allowed standard balanced diet and water ad- libitum. They were divided into three groups.

Group I (control group- 10 rats): was divided into two equal subgroups:

Group IA: The animals were given distilled water only.

Group IB: The animals were given daily oral dose of curcumin (80 mg/kg of body weight).

Group II (Na-F treated group - 10 rats):

The animals were treated with daily oral dose of sodium fluoride 20 mg/kg/ body weight dissolved in distilled water for 4 weeks [24].

Group III (protected group – 10 rats):

The animals were given a daily oral dose of sodium fluoride in the same dose of the previous group concomitantly with curcumin 80 mg/kg body weight for 4 weeks [25].

Chemicals

Sodium fluoride was obtained in the form of powder from El Gomhoria Company for Chemical and Medical Trading, Zagazig, Egypt. Sodium fluoride solution was prepared by dissolving 1 gm sodium fluoride

in 250 ml distilled water so each 1 ml would have 4 mg sodium fluoride. Each rat was given daily 1-1.25 ml of this solution according to its weight by oral intubation for 4 weeks.

Curcumin, 5,5-dithiobis (2- nitrobenzoic acid) [DTNB, (Ellman's reagent)], glacial acetic acid, heparin, nitro blue tetrazolium chloride (NBT), potassium dihydrogen phosphate (KH₂PO₄), reduced glutathione (GSH), sodium dihydrogen phosphate (NaH₂PO₄), sodium fluoride (NaF), trichloroacetic acid (TCA), thiobarbituric acid (TBA), and hydrogen peroxide were obtained from Sigma-Aldrich Chemical Company, (St. Louis, MO), USA [23].

Biochemical study

Collection of blood samples

At the beginning of the trial and on days 14 and 28, blood was drawn from the heart of each animal into tubes with heparin under light ether anesthesia. The blood samples were centrifuged at 3000 rpm, and erythrocytes were separated from the plasma.

Washing of erythrocytes and haemolysis

Erythrocytes were washed in phosphate buffered saline and diluted with an equal volume of this solution. A 400 µl portion of the washed erythrocytes was subjected to haemolysis by mixing with 1600 µl of ice-cold water [26].

Determination of plasma malondialdehyde levels, erythrocyte superoxide dismutase and catalase activities

Plasma MDA levels, and erythrocyte SOD and CAT activities were determined as described by [27, 28 & 29], respectively. Erythrocyte hemoglobin levels were measured in compliance with the method described by [30].

After four weeks, the animals were sacrificed by ether anesthesia; the lungs were dissected out, rinsed in phosphate buffer saline, prepared and examined by the following techniques:

Histological study

For light microscopic study, specimens were fixed in 10% buffered formaldehyde and 5 µm thick paraffin sections were prepared and stained with hematoxylin and eosin [31], Mallory's trichrome stains and Orcein stains [32].

For electron microscopic study, small pieces of 1 mm³ of the lung were excised, fixed in 2% gluteraldehyde buffered with 0.1 M phosphate buffer at pH 7.4 for 2h at 4°C, and post-fixed in 1% osmium tetroxide. They were then dehydrated with ascending grades of ethanol and placed in propylene oxide for 30 min at room temperature, followed by impregnation in a mixture of propylene oxide and resin (1 : 1) for 1h and then in a mixture of the previous reagents at 48°C for 1h. The specimens were embedded in an EM bed-812 resin in BEEM capsules at 60°C for 24h. Ultrathin sections were cut and double stained with uranyl acetate and lead citrate [33] and were examined with a JEOL transmission electron microscope, Department of Histology and Cell Biology, Faculty of Medicine, Zagazig University, Egypt.

Morphometric study

The image analyzer computer system Leica Qwin 500, UK in the pathology Department, Faculty of dentistry, Cairo University, was used to obtain the alveolar diameter and also thickness of inter-alveolar structures. Ten non-overlapping high-power fields from each slide of all animals of each group were used.

Statistical analysis

The data obtained from the image analyzer were expressed as means ± standard deviations. The morphometric results were analyzed using an analysis of variance one-way test. The results were considered statistically significant when the p value < 0.05 [34].

Results:

Biochemical results

At the beginning of the trial, there were no significant differences between the three groups for MDA, CAT or SOD ($p > 0.05$). This demonstrated that the animals included in the different groups were physiologically equivalent for the three parameters. On day 14 and until day 28, plasma MDA levels were observed to be significantly increased, while SOD and CAT activities were determined to have significantly decreased in group II which was given sodium fluoride alone when compared to the controls ($p < 0.05$). In group III, which received curcumin and fluoride, samples on day 14 until day 28, plasma MDA levels were observed to have decreased and SOD and CAT activities were ascertained to have increased in comparison to group II, which was administered fluoride alone. In other words, it was determined that, the indicated parameters of the trial group III had drawn closer to the values of the control group (table 1, 2 &3).

Table (1): Plasma MDA levels (nmol/ml) in control and experimental groups

Group	Mean± SD (range)		P- value
I (control)	Beginning	6.7 ± 0.7	
	Day 14	6.5 ± 0.9	
	Day 28	6.8 ± 1.1	
II (Na-F treated)	Beginning	6.9 ± 0.4	<0.001*
	Day 14	7.9 ± 0.9	
	Day 28	9.9 ± 0.8	
III (protected)	Beginning	6.8 ± 0.6	
	Day 14	6.9 ± 0.9	
	Day 28	7.2 ± 1.2	

*: statistically significant

Table (2): Erythrocyte CAT activity (U/mgHb) in control and experimental groups

Group	Mean± SD (range)		P- value
I (control)	Beginning	1.40 ± 0.2	
	Day 14	1.36 ± 0.3	
	Day 28	1.32 ± 0.2	
II (Na-F treated)	Beginning	1.49 ± 0.5	<0.001*
	Day 14	1.44 ± 0.2	
	Day 28	0.82 ± 0.4	
III (protected)	Beginning	1.46 ± 0.1	
	Day 14	1.43 ± 0.1	
	Day 28	1.23 ± 0.2	

*: statistically significant

Table (3): Erythrocyte SOD activity (U/mgHb) in control and experimental groups

Group	Mean± SD (range)		P- value
I (control)	Beginning	0.6 ± 0.1	
	Day 14	0.58 ± 0.2	
	Day 28	0.61 ± 0.1	
II (Na-F treated)	Beginning	0.58 ± 0.2	<0.001*
	Day 14	0.61 ± 0.3	
	Day 28	0.31 ± 0.1	
III (protected)	Beginning	0.59 ± 0.1	
	Day 14	0.57 ± 0.1	
	Day 28	0.54 ± 0.1	

*: statistically significant

Histological results

Control Group (I)

Light microscopic examination of H & E stained sections revealed that the lung showed the normal adult architecture with rounded or polygonal alveoli, alveolar sacs and the interstitial tissue contained blood vessels (Figure 1.a). Mallory's trichrome stained sections showed few collagen fibers in the inter-alveolar septa and around the blood vessels (Figure 1.b). Orcein stained sections showed many elastic fibers in the inter-alveolar septa, wall of bronchioles and also wall of the blood vessels (Figure 1.c). Toluidine Blue stained sections showed the alveolar cells, some are polygonal cells and others are flat cells. These polygonal alveolar cells had large rounded pale nuclei with prominent nuclei while the flat cells contain thin dark flat nuclei. Moreover the intra-alveolar septa are also formed by the same cells (Figure 1.d). Ultra-thin sections showed that the alveolar wall and the inter-alveolar septum contain different types of cells. These cells included small sized interstitial cells having small euochromatic nuclei and pale cytoplasm with minimal cell organelles. The wall of alveoli contained pneumocyte type II with indented euochromatic nuclei, multi-lamellar bodies and also apical intact microvilli were seen lining the alveolar walls. The blood air barrier was formed of attenuated type I pneumocytes process, fused basal lamina and the cytoplasm of the capillary endothelial cell (data not shown).

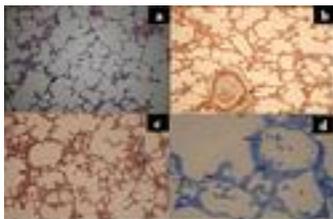


Figure (1): (a) A photomicrograph of a section of adult rat lung from the control group (group I) showing alveoli (a) separated by relatively thin inter-alveolar septa (arrow). Blood vessels (bc) is also seen (H&E X 400). (b) A photomicrograph of a section of adult rat lung (group A) showing few or faint collagen fibers in the inter-alveolar septa and around blood vessel (arrow) (Mallory's trichrome stain X 400). (c) A photomicrograph of a section of adult rat lung (group A) showing many elastic fibers in the inter-alveolar septa (thin arrow), in the walls of bronchiole (thick arrow) and also in walls of blood vessel (red arrow). (Orcein X 400).

(d) A photomicrograph of a section of adult rat lung (group I) showing cells with large rounded pale nuclei (**thick arrow**) and others with dark flat nuclei (thin arrow), both are lining walls of the alveoli (a). Blood capillaries (**bc**) are seen on one sides of primary septa (**Toluidine blue x1000**).

Na-F Treated Group (II)

In this group, most of the alveoli were collapsed with marked thickening of the inter-alveolar septa that showed marked cellular infiltration. The septa contained dilated blood vessels that appeared congested with extravasated erythrocytes into the alveolar lumen and in the inter-alveolar septa (Figs. 2.a). Mallory's trichrome stained sections showed increased collagen deposition in the inter-alveolar septa and around the blood vessels (Fig. 2.b). Orcein stained sections showed few elastic fibers in the inter-alveolar septa (Fig. 2.c). Toluidine Blue stained sections showed that the inter-alveolar septa contained interstitial cells, some of them contained large rounded nuclei and others have irregular nuclei. Moreover, some of these cells showed multi-lobed nuclei with darkly stained nucleoli and other cells with large nuclei and vacuolated cytoplasm. Extensive hemorrhage in the inter-alveolar septa was seen with extravasation of erythrocytes in the alveolar lumens (Fig. 2.d).

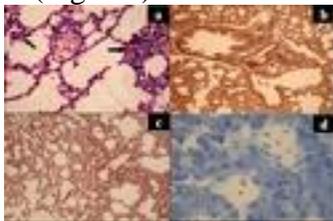


Figure (2): (a) A photomicrograph of a section of adult rat lung (group II) showing relatively thick inter-alveolar septa (thick arrow) and large congested blood vessel (bc) with extensive or marked cellular infiltration (IF). Also numerous blood capillaries (bc1) are observed in the walls of alveoli (**H&E X 100**). (b) A photomicrograph of a section of adult rat lung (group II) showing abundant collagen fibers around the blood vessels (arrow) (**Mallory's trichrome stain X 400**). (c) A photomicrograph of a section of adult rat lung (group II) showing few or scanty elastic fibers in the inter-alveolar septa (arrow) (**Orcein X 400**). (d) A photomicrograph of a section of adult rat lung (group II) showing interstitial cells with large rounded and also irregular (**I**) nuclei. Some cells with multi-lobed nuclei (**N**) and others with darkly stained nuclei (**n**) are observed in thick inter-alveolar septa. Numerous cells with large nuclei and also vacuolated cytoplasm (**P2**) are seen. Extensive hemorrhage (**H**) and also extravasated erythrocytes (**e**) in alveolar lumens (**a**) are seen (**Toluidine blue x1000**).

Ultra-thin sections showed thickened inter-alveolar septa containing congested blood capillaries with extravasation of RBCs into the alveolar lumen (Fig. 3.a). The alveoli were lined by pneumocytes type II with irregular heterochromatic nuclei and lamellar bodies in their cytoplasm. The cell membrane of the cells showed numerous folding of variable sizes and shapes with their cytoplasm containing many vacuoles. The interstitial cells contained long flat heterochromatic nuclei and many cytoplasmic vacuoles within the interstitium (Fig. 3.b). The inter-alveolar septa contained abundant bundles of collagen fibers and blood capillaries (Fig. 3.c). The blood air barriers were formed of the cytoplasm of pneumocyte type I, thick basal lamina and cytoplasm of endothelial cells (Fig. 3.d).

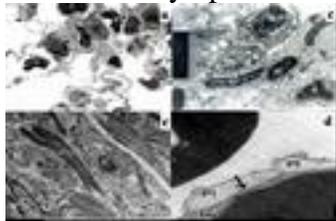


Figure (3): (a) An electron micrograph of a section of adult rat lung from sodium fluoride treated group (group II) showing interstitium with many congested blood capillaries (bc) containing red blood cells (R). Also numerous interstitial cells (IC) with variable shaped nuclei are observed (**TEM X 5000**). (b) An electron micrograph of a section of adult rat lung (group II) showing many pneumocyte type II (P2) with heterochromatic nuclei (N), numerous lamellar bodies (arrows) and many vacuoles (V). The cell

membrane showed numerous enfoldings (arrow heads) are observed on its surface. Interstitial cells (IC) with flat heterochromatic nuclei and many vacuoles (V) within the interstitium are also seen (**TEM X 4000**). (c) An electron micrograph of a section of adult rat lung (group II) showing blood capillaries (bc) containing red blood cells (R), numerous interstitial cells (IC) with variable shaped nuclei (N) and bundles of collagen fibers (CO) in interstitium (**TEM X 5000**). (d) An electron micrograph of a section of adult rat lung (group II) showing the blood air barrier formed of cytoplasm of pneumocyte type I (P1), thick basal lamina (arrows) and cytoplasm of the capillary endothelial cells (bc) (**TEM X 30.000**).

Curcumin Protected Group (III)

Examination of H&E stained sections from the sodium fluoride and curcumin treated lung revealed normal shaped alveoli separated by thin inter-alveolar septa. The septa showed minimal localized cellular infiltration (Fig 4.a). Mallory's trichrome stained sections showed few collagen fibers in the inter-alveolar septa and around the blood vessels (Fig. 4.b) while Orcein stained sections showed few elastic fibers in the septa and around bronchioles (Fig. 4.c). The wall of the alveoli and the inter-alveolar septa showed cells with large pale nuclei and vacuolated cytoplasm and others with flat nuclei. The septa are composed of interstitial cells; some of them have large pale nuclei with and vacuolated cytoplasm whereas others have dark nuclei. Also blood capillaries were seen on both sides of these septa with extravasated erythrocytes observed in alveolar lumen (Fig. 4.d)

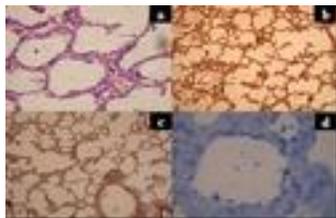


Figure (4): (a) A photomicrograph of a section from protected group (group III) showing normal alveoli (a) and relatively thin inter-alveolar septa (arrows). Small blood vessels (bv) and little cellular infiltration (IF) are also observed (**H&E X 400**). (b) A photomicrograph of a section of adult rat lung (group III) showing very few collagen fibers in the inter-alveolar septa and around the blood vessel (arrow) (**Mallory's trichrome stain X 400**). (c) A photomicrograph of a section of adult rat lung (group III) showing some elastic fibers in the inter-alveolar septa and around the bronchiole (arrows) (**Orcein X 400**). (d) A photomicrograph of a section of adult rat lung, subgroup III (protected group), showing cells with large pale nuclei (N) and a vacuolated cytoplasm (*) and others with flat nuclei (n), both lining walls of the alveoli (A). Inter-alveolar septa are composed of interstitial cells (IC); some of these cells have large pale nuclei (N1) with cytoplasmic vacuoles whereas others have dark nuclei (n1). Blood capillaries (bc) can be seen on both sides of this septa. Some extravasated erythrocytes (e) also are observed in alveolar lumen (**Toluidine blue, x 1000**).

Ultra-thin sections showed many pneumocyte type II with large rounded euchromatic nuclei and many lamellar bodies. The interstitial cells between the alveoli showed irregular euchromatic nuclei (Fig. 5.a). The blood air barrier was formed of the cytoplasm of pneumocytes type I, thick basal lamina and cytoplasm of endothelial cells (Fig. 5.b).

Statistical results.

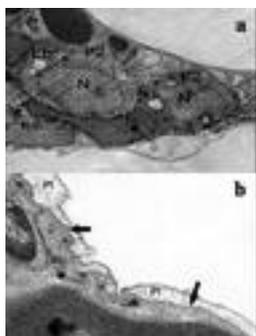


Figure (5): (a) An electron micrograph of a section of adult rat lung (group III) showing the blood air barrier formed of irregular swollen cytoplasm of pneumocyte type I (P1), thick basal lamina (arrows) and cytoplasm of the capillary endothelial cells (bc) (TEM X 30.000). (b) An electron micrograph of a section of adult rat lung (group III) showing the blood air barrier formed of cytoplasm of pneumocyte type I (P1), thick basal lamina (arrows) and cytoplasm of the capillary endothelial cells (bc) (TEM X 30.000).

Statistical analysis of the mean alveolar diameter and inter-alveolar septal thickness revealed a highly significant statistical decrease and increase consequently ($P < 0.001$) in sodium fluoride treated when compared with the control group. Also, there was a non significant statistical difference between control and curcumin supplemented group (Table 4 & 5).

Table (4): The thickness of the inter-alveolar septa of the adult rat lung in control and experimental groups

Group	Mean± SD (range)	P- value
I (control)	70.77± 24.7	
II (Na-F treated)	469.3± 133.5	<0.001*
III (protected)	75.57± 23.4	

*: statistically significant

Table (5): The alveolar diameter of the adult rat lung in control and experimental groups

Group	Mean± SD (range)	P- value
I (control)	190.9 ±8.6	
II (Na-F treated)	98.57± 9.8	<0.001*
III (protected)	179.3± 8.5	

*: statistically significant

Discussion :

Increased MDA level in Na-F treated group is attributed to the fact that fluoride generates free radicals, which result in lipid peroxidation. Contrariwise, erythrocyte CAT and SOD activities were decreased because free radicals are converted to less harmful compounds through these enzymes and this leads to their depletion.

In curcumin protected group, MDA levels and SOD and CAT activities had drawn closer to values of control group as curcumin raises the antioxidant status through increasing the activities of antioxidant enzymes and induction of HO-1 that increases heme oxygenase activity and donates H atoms from phenolic group and inhibits iNOS induction leading to the antioxidant effect [18, 35 & 36].

In the present study, examination of lung sections in second group revealed collapsed alveoli with marked cellular infiltration and thickened inter-alveolar septa. These results might be attributed to the fact that fluoride causes an increase in various mediators of inflammation and chemokines such as cytokine-induced neutrophil chemo-attractant that plays an important role in neutrophilic lung inflammation [10] or due to generation of mediators such as IL-8 which attract the inflammatory cells to the respiratory system [37].

The collapsed alveoli of the treated group were thought to be due to pressure from the expanded interstitium making less surface area available for gas exchange. This collapse may be also as a result of large decrease in surfactant synthesis due to lipid peroxidation of the phospholipids present in pneumocyte type II or due to oxidative stress causing degenerative changes in type II pneumocytes [38].

Increased collagen fibers in second group in the inter-alveolar septa and around the blood vessels could be attributed to injury to the epithelium and basement membranes representing an initial step of pulmonary

fibrosis, after which several inflammatory and immune cells migrate to areas of injury and release numerous cytokines that lead to further cell recruitment, inflammation, and eventual matrix remodeling [39]. There were also decreased amount of elastic fibers which might be attributed to the fact that neutrophils secreted elastase enzyme that causes fragmentation of elastic fibers [40].

EM examination of fluoride treated group shows similar results to others who reported that fluoride causes failure of sodium/ potassium pump at plasma membrane which leads to cloudy swelling and cytoplasmic vacuolation as well as disarrangement of the cells [41]. Fluoride also had been found to cause an increase in intracellular calcium concentration that is proposed to induce apoptosis [42]. In this study, the irregular enfolding of the cell membrane of the surface of type II cells was attributed to increased lipid peroxidation caused by fluoride may lead to functional damage of the apical membrane enfolding of type-II pneumocytes, thereby eliciting an impairment of the extrusion of lamellar bodies by these cells [43,44].

In this study, the observed structural changes in the treated groups were much less abundant. The alveoli appeared normal with thin inter-alveolar septa. Few collagen and abundant elastic fibers were seen. Also, electron microscope examination revealed apparently normal pneumocytes type II with lamellar bodies. These results were in accordance with other investigators who attributed the preserved normal structures in the protected group by curcumin to the maintenance of a high intracellular level of antioxidants that protect the cells from oxidative stress. Curcumin, can also act to overcome the oxidative stress, being part of the antioxidant system [45].

Conclusion

It was determined that fluoride led to oxidative stress at the indicated dose and period. Moreover, concomitant treatment with curcumin at the doses of 80 mg/kg body weight with sodium fluoride administration restored plasma malondialdehyde (MDA) levels and erythrocyte superoxide dismutase (SOD) & catalase (CAT) activities in adult male rat.

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