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Research Article

MELATONIN AMELIORATES FLUORIDE INDUCED NEUROTOXICITY IN YOUNG RATS: AN *IN VIVO* EVIDENCE

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ABSTRACT

Objective: Developing brain is highly vulnerable to environmental toxins. Recently, fluoride was declared as a developmental neurotoxin and heralded search for natural neuroprotectant. In this study, we have evaluated the neuroprotective and anti-inflammatory efficacy of melatonin in fluoride-induced neurotoxicity.

Methods: Animals were divided into following groups; the first group was used as a control. Groups 2, 3, and 4 were treated with melatonin (10 mg/kg body weight [BW]), sodium fluoride (NaF 4 mg/kg BW) and NaF (4 mg/kg BW) plus melatonin (10 mg/kg BW), respectively. Young rats were orally administered their respective doses daily for 60 days. Biochemical and behavioral analysis were performed. The level of proinflammatory cytokine, tumor necrosis factor alpha (TNF-α) was also determined.

Results: Data obtained showed that NaF significantly ($p<0.001$) increased thiobarbituric acid reactive substances (TBARS), reactive oxygen species (ROS) concentration and decreased the activities of glutathione (GSH) and GSH peroxidase. On the other hand, melatonin plus NaF treated group showed a significant decrease in the levels of TBARS and ROS while it increased the activities of antioxidant enzymes and GSH content. In addition, melatonin significantly attenuated the fluoride-induced increase in the TNF-α level of the brain. Melatonin also prevented the cognitive deficit as shown by the increased retention latency in the passive avoidance task ($p<0.001$).

Conclusion: This study suggests that melatonin has therapeutic potential since it suppresses fluoride-induced inflammation, cognitive impairment, and oxidative stress in the brain.

Keywords: Oxidative stress, Inflammation, Melatonin, Tumor necrosis factor alpha, Fluoride.

INTRODUCTION

Developmental neurotoxins can cause multiple effects on the brain and learning-memory disabilities. Recently, fluoride was classified as a developmental neurotoxin by medical authorities [1]. Fluoride availability in drinking water beyond the safe limits has been a global problem [2]. Enhanced oxidative stress, decreased antioxidant pool, and neurodegeneration have been established by a number of studies in fluorosis [3-5]. Varied neurological manifestations are also observed in advance stages of fluorosis [6].

Fluoride exerts powerful effects on various enzymes, oxidant/antioxidant systems and cellular functions [4]. The underlying mechanism is however not clear. In spite of the severe health hazards associated with the slow toxin fluoride, there are only limited reports on the development of suitable neuroprotectant against it. Thus, our aim was to search for a potent neuroprotectant against fluoride neurotoxicity for a mitigating effect. In this regard, the pineal hormone melatonin is an important biomolecule, which is a potent neuroprotectant. Melatonin (nacetyl-5-methoxytryptamine) is synthesized mainly in the pineal gland and has powerful antioxidant properties as proven by several *in vivo* and *in vitro* studies [7-10].

Studies have reported relationship between fluoride and oxidative stress, but none of these reports illustrate blended interactions of fluoride, oxidative stress, inflammation and behavioral alterations which could be an added advantage in understanding the mechanism of cognitive alterations in fluoride neurotoxicity. Hence, this study is aimed to quantify the biochemical, behavioral, and inflammatory parameters and to correlate their interactions with supplementation of a biomolecule, which may help further in understanding the mechanism of fluoride perturbation in central nervous system.

METHODS

Animals

Young Wistar rats, weighing 50-60 g (18 days old) were used in this study. The rats were housed in a temperature-controlled room 22-24°C with a 12:12 light: Dark cycle. Water and food were given ad libitum. All protocols described were reviewed and approved by the Local Institutional Committee for the Ethical Use of Animals and the laboratory was approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. (Approval no. #973/ac/06/CPCSEA).

The animals were randomly divided into the following groups, Group I - Control, Group II - melatonin, Group III - NaF, Group IV - NaF + melatonin. The rats in the NaF treated, Group III and Group IV, had access to drinking water with a 3 mg L^{-1} NaF solution for 60 days. Melatonin was administered between 6 and 7 p.m. administered by gastric gavages (0.1 ml 10 g^{-1} body weight [BW]) at a dose of 10 mg kg^{-1} bw^{-1}.

Biochemical tests

At the end of the treatment period, the animals were sacrificed by decapitation. Brain were dissected out, weighed, rinsed in ice-cold saline and used immediately or stored frozen at −70°C until analysis. Brain tissue samples were thawed and homogenized in 10% (w v^{-1}) ice-cold 0.1 M phosphate buffer (pH 7.4) and centrifuged for 10 minutes at the temperature of 4°C at 10,000 g. The supernatants were collected, and aliquots (stored at −20°C) were prepared to determine various parameters. Total protein content was estimated by the method of Lowry *et al.*, 1951 [11]. Then the hemolysate was used for the estimation of oxidative stress parameters.

Lipid peroxidation assay
Lipid peroxidation was measured as thiobarbituric acid reactive substances (TBARS) production in the thiobarbituric acid reaction in brain homogenates as described by Mihara and Uchiyama, 1978 [12].

Glutathione (GSH) assay
GSH level was estimated in the deproteinized supernatant fraction of brain homogenate using 5,5-dithiobis (2-nitrobenzoic acid) and recording absorption at 412 nm Sedlak and Lindsay, 1968 [13].

GSH peroxidase (GPx) assay
GPx activity was assayed in brain homogenates by a coupled test system (Günzler *et al.*, 1974) [14].

Reactive oxygen species (ROS) estimation
ROS were estimated as described by Socci *et al.*, 1999 [15]. DCFH-detectable ROS intensity was expressed as fluorescent units (FIU at 530 nm).

Tumor necrosis factor alpha (TNF-α) assay
The brain was homogenized in 1 ml of ice-cold lysis buffer radio-immunoprecipitation assay, containing 50 mM Tris–HCl (pH 8.0), 150 mM sodium chloride, 1.0% iepal CA-630 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% phosphatase inhibitor cocktail and a protease inhibitor cocktail. The lysate was centrifuged (15000×g–4°C) for 15 minutes, and the supernatant was added to 96-well enzyme-linked immunosorbent assay (ELISA) plates. The TNF-α concentration was then determined by reading the ELISA plate.

Behavioral test
Passive avoidance test: Memory retention deficit was evaluated by step-through passive avoidance apparatus. The apparatus consist of an equal sized light and dark compartments (30 cm × 20 cm × 30 cm). A 40-W lamp was fixed 30 cm above its floor in the center of the light compartment. The floor consisted of metal grid connected to a shock scrambler. The two compartments were separated by a trap door that could be raised to 10 cm. To improve the reliability and validity of the footshock avoidance test, the grid as well as the rat paw was moistened with water before delivering the foot shock as this is known to reduce the wide inter animal variability in paw skin resistance of the rats [16].

Statistical analysis
Results are expressed as the mean±standard deviation. Data comparisons were carried out using one-way Analysis of Variance followed by Bonferroni post-test to compare all pairs of groups. Data were analyzed with the Prism Software program (Graphpad Software Inc. USA).

RESULTS

All rats were observed once daily with detailed evaluation on general appearance and physical condition including moving, activities, appetite, appearance of hair, eyes and limbs, and no obvious change was noticed. Compared with control group, rats in NaF treated group and NaF+melatonin treatment group, gained less BW from week 3 to the end of the experimental period. After the period of exposure, a difference of the BW of rats among four groups was more obvious, but did not show a significant difference in BW compared with control rats during the whole experimental period.

The results of TBARS, GPx, GSH and ROS levels in brain tissues are shown in Figs. 1-4 respectively. The levels of TBARS and ROS in brain tissues were significantly higher ($p<0.001$) in rats receiving NaF alone compared to that of Group I animals used as a control. On the other hand, compared with the NaF group, the levels of TBARS and ROS were significantly lower in rats receiving NaF and melatonin co-administration. GSH and GPx level were significantly ($p<0.001$) lower in brain tissue, in NaF treated rats, when compared with the control animals. Melatonin treatment in NaF received rats, increased GSH and GPx levels significantly ($p<0.001$) as compared to NaF treated rats. It

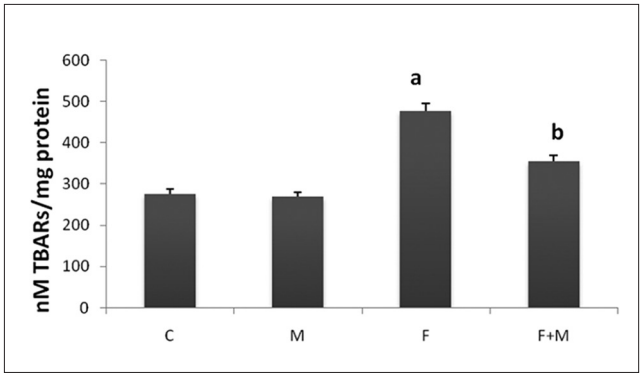

Fig. 1: Effect of 60 days treatment with melatonin on levels of thiobarbituric acid reactive substances in fluoride induced neurotoxicity in rats. Each value represents the mean±standard error of mean. [a]p<0.001 compared to control, [b]p<0.001, compared to group treated with fluoride. 'M' represents melatonin, 'F' represents fluoride, 'C' represents control groups

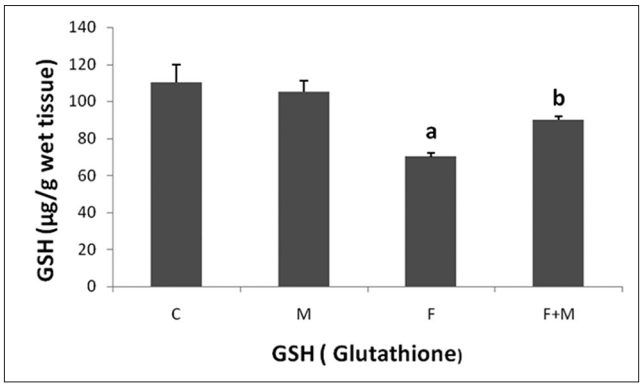

Fig. 2: Effect of 60 days treatment with melatonin on levels of glutathione in fluoride induced neurotoxicity in rats. Each value represents the mean±standard error of mean. [a]p< 0.001 compared to control, [b]p< 0.001, compared to group treated with fluoride

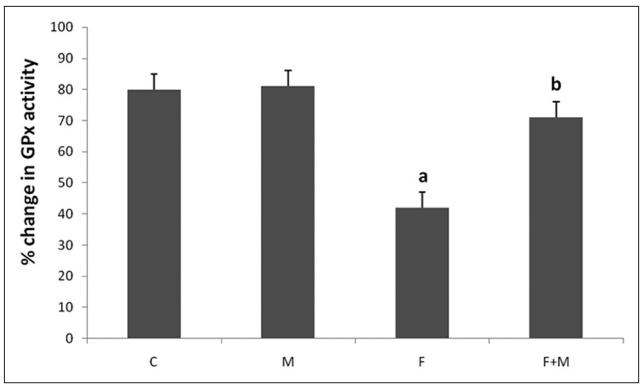

Fig. 3: Effect of 60 days treatment with melatonin on levels of glutathione peroxidase activity in fluoride induced neurotoxicity in rats. Each value represents the mean±standard error of mean. [a]p<0.001 compared to control, [b]p<0.001, compared to group treated with fluoride

was observed that 60 days treatment had no significant effect on the TBARS, GPx, GSH and ROS levels in the melatonin *per se* Group II.

A marked increase in TNF-α concentration was seen after NaF-administration (631.11±09.28 pg/ml) as compared to the control group rats (269.40±11.15 pg/ml) ($p<0.001$). In the melatonin+NaF

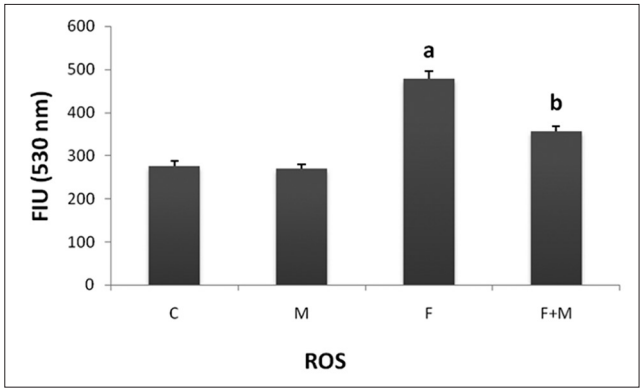

Fig. 4: Effect of 60 days treatment with melatonin on levels of reactive oxygen species levels in Fluoride induced neurotoxicity in rats. Each value represents the mean±standard error of mean. [a]p<0.001 compared to control, [b]p<0.001, compared to group treated with fluoride

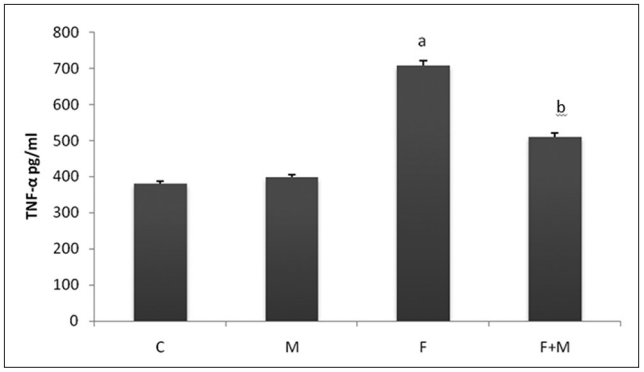

Fig. 5: Effect of 60 days treatment with melatonin on levels of tumor necrosis factor alpha in Fluoride induced neurotoxicity in rats. Each value represents the mean±standard error of mean. [a]p<0.001 compared to control, [b]p<0.001, compared to group treated with fluoride

group significant (p<0.001) attenuation of NaF induced rise in brain levels of TNF-α, was observed (402.10±20.23 pg/ml) (Fig. 5). Further, in the melatonin, *per se* group, the 60 days treatment had no significant effect on the brain TNF-α level.

Behavioral profiles have been presented in Fig. 6. Altered passive avoidance task was observed with a significance of (p<0.001) in NaF treated rats. These changes were found to be markedly (p<0.001) reversed following the co-administration of melatonin in Group IV animals. The retention latency in the NaF treated, Group III was significantly (p<0.001) less when compared with the control group rats. This indicates significant cognitive impairment due to NaF treatment. The mean retention latencies in the melatonin+NaF improved when compared with Group III animals. Melatonin treatment produced a significant reversal of F-induced cognitive deficit Melatonin treatment alone in Group II did not have any significant changes in the retention latencies when compared to control group.

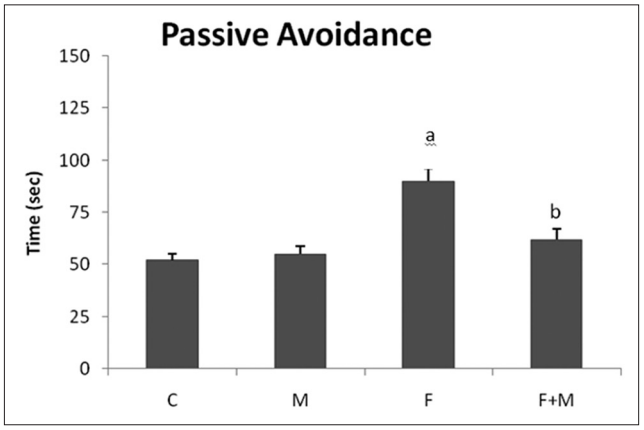

Fig. 6: Effect of 60 days treatment with melatonin on passive avoidance in fluoride induced neurotoxicity in rats. Each value represents the mean±standard error of mean. [a]p<0.001 compared to control, [b]p<0.001, compared to group treated with fluoride

DISCUSSION

Different studies have revealed that fluoride interferes with the cellular functions and dental formation [2,5,17,18] but there is a paucity of literature on the mechanism of fluoride in developmental neurotoxicity. Thus, the present study was planned to investigate the possible neuroprotective role of melatonin in fluoride-induced toxicity in young rats and to illustrate further the mechanism of F toxicity.

In our experiments, we observed a significant increase in the levels of TBARS and ROS in fluoride exposed animals, suggesting that free radicals were involved in oxidative stress. Wang *et al.*, (1997) have also reported that production of OH• and •O2− radicals is dependent on fluoride concentration [19]. Also, we observed GSH and GPx level decreased significantly, which was consistent with the report of Shivarajashankara *et al.*, [20] and Chouhan and Flora [21]. Our results are suggestive of fluoride-induced oxidative stress which is an imbalance between the production of free radicals and the body's antioxidant defense system. The brain is the main organ for F toxicity, as it crosses the blood-brain barrier and its exposure further induces both biochemical and behavioral changes [22]. Recent studies from our lab have also shown that fluoride generates reactive radicals, which in turn cause cellular damages by depleting important enzyme activities and damaging membrane lipid bilayer [23,24].

GSH is known to be an important mediator in detoxifying lipid peroxides. In the process of F toxicity, it can bind to thiol-containing proteins, further inhibiting their activity, and ultimately interrupting metabolic processes such as glycolysis, synthesis of proteins and antioxidative pathways [25]. The mechanism of action of F is not yet established but certain hypotheses have been proposed suggesting the role of ROS and lipid peroxidation. Being a prooxidant, F may cause GSH depletion, protein enzyme oxidation and lipid per-oxidation [26]. Low levels of GSH, GPx with high TBARS and ROS concentrations as found in our experiments, could be used as an indicator of oxidative stress. It is also suggested from our data that depletion of GSH and GPx might be the mechanism leading to increases in levels of ROS and TBARS molecules and thereby increasing to the fluoride induced toxicity.

TNF-α has an important role in cell signaling, regulation of immune cells and homeostasis [27]. Elevated TNF-α level may have a mechanism leading to a marked rise in inflammation and cellular disturbances. We also observed elevated levels of TNF-α in F treated rats and significant attenuation was found in treatment groups with melatonin supplementation. Our results are consistent with earlier reports of increased expression of TNF-α in F toxicity [28]. Therefore, it is reasonable to assume for the current study that the peroxidative effect of F might be related to thiol depletion, enhanced TNF-α concentration and lowered pool of antioxidant enzymes (GSH, GPx) in brain.

Under physiological conditions, the balance between the oxidant/antioxidant statues of the tissue has remarkable effects on cellular functioning. In our study melatonin administration in F treated animals was seen to stabilize GSH and GPx concentration and also decline ROS production. Significant reduction in the level of TNF-alpha and TBARS was also detected in brain tissue, with the use of melatonin. This can be correlated with melatonin strong antioxidative properties and

capability of quenching oxygen free radicals, which are important for the initiation of LPO, in a cascade reaction. According to Reiter *et al.*, melatonin may cause stabilization of membrane structure and decrease ROS generation [29]. Decreased levels of tissue ROS, TBARS and TNF-α level as a result of melatonin treatment clearly show the neuroprotective effect of melatonin in our study.

TNF-α has also been implicated as potential modulators of apoptosis and ROS also plays a significant role in this process. The ability of TNF-α and ROS to initiate apoptosis has also been associated with enhanced oxidative stress [30]. This interpretation is further supported by a recent report on chronic exposure of chicken broilers to F that caused reduced cellular and humoral immunity as exhibited by reduced cytokine interleukin-6, TNF-α, and interferon-γ content in the cecal tonsil [31]. Our findings suggests that melatonin treatment have potent free radical scavenger activity to prevent free radical production and it also safeguard cellular structure and functions, through its receptor mediated and independent functions [32]. In this context, this was the first experiment in which we tried to find out some possible mode of action of melatonin and fluoride at the cellular level. In this study, we observed that the administration of F was associated with cognitive impairment as evidenced by reduction of retention latency in passive avoidance behavior. These results are in conformity with findings of other workers who also demonstrated cognitive impairment after administration of fluoride [33]. The administration of melatonin prevented cognitive deficit associated with fluoride toxicity as indicated by increased retention latency in passive avoidance behavior. The present study has demonstrated the beneficial effect of melatonin in attenuating the fluoride induced neurotoxicity as well as cognitive impairment.

CONCLUSION

The present study reveals that fluoride exerts its toxic effects possibly ascribed to the enhanced oxidative stress and inflammation which further causes cognitive dysfunctions. One of interesting finding of the study is that melatonin exerts its neuroprotective effects on deleterious effects of fluoride and also on inflammation and cognitive dysfunctions. The study also provides important data about the possible mode of action of fluoride. However, the underlying molecular mechanisms are warranted for further studies.

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REFERENCES

1. Grandjean P, Landrigan PJ. Neurobehavioural effects of developmental toxicity. Lancet 2014;13:330-8.
2. Amanlou M, Hosseinpour M, Azizian H, Khoshayand MR, Navabpoor M, Souri E. Determination of fluoride in the bottled drinking waters in Iran. Iran J Pharm Res 2010;9(1):37-42.
3. Agency for Toxic Substances and Disease Registry. Toxicological Profile for Fluorides, Hydrogen-Fluoride, and Fluorine (Update). Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2003.
4. Bhatnagar M, Rao P, Saxena A, Bhatnagar R, Meena P, Barbar S, *et al.* Biochemical changes in brain and other tissues of young adult female mice from fluoride in their drinking water. Fluoride 2006;39(4):280-4.
5. Bhatnagar M, Bhatnagar C, Regar BC. Fluoride-induced histopathological changes in gill, kidney, and intestine of fresh water teleost, *Labeo rohita*. Fluoride 2007;40:55-61.
6. Choi AL, Sun G, Zhang Y, Grandjean P. Developmental fluoride neurotoxicity: A systematic review and meta-analysis. Environ Health Perspect 2012;120(10):1362-8.
7. Jain A, Bhatnagar M. Melatonin- A magic biomolecule. Ann Neurosci 2007;14:108-14.
8. Ghosh D, Mitra E, Dey M, Firdaus SB, Ghosh AK, Mukherjee D, *et al.* Melatonin protects against lead-induced oxidative stress in rat liver and kidney. Asian J Pharm Clin Res 2013;6(2):137-45.
9. Giusti P, Franceschini D, Petrone M, Manev H, Floreani M. *In vitro* and *in vivo* protection against kainate-induced excitotoxicity by melatonin. J Pineal Res 1996;20(4):226-31.
10. Jain A, Sharma D, Suhalka P, Sukhwal P, Bhatnagar M. Changes in the density of nitrergic neurons in the hippocampus of rats following kainic acid and melatonin administration. Physiol Res 2013;62(2):197-203.
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193(1):265-75.
12. Mihara M, Uchiyama M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 1978;86(1):271-8.
13. Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman‘s reagent. Anal Biochem 1968;25(1):192-205.
14. Günzler WA, Kremers H, Flohé L. An improved coupled test procedure for glutathione peroxidase (EC 1-11-1-9-) in blood. Z Klin Chem Klin Biochem 1974;12(10):444-8.
15. Socci DJ, Bjugstad KB, Jones HC, Pattisapu JV, Arendash GW. Evidence that oxidative stress is associated with the pathophysiology of inherited hydrocephalus in the H-Tx rat model. Exp Neurol 1999;155(1):109-17.
16. Stubley-Weatherly L, Harding JW, Wright JW. Effects of discrete kainic acid-induced hippocampal lesions on spatial and contextual learning and memory in rats. Brain Res 1996;716(1-2):29-38.
17. Choubisa SL. Osteo-dental fluorosis in relation to chemical constituents of drinking waters. J Environ Sci Eng 2012;54(1):153-8.
18. Amanlou M, Jafari S, Afzalianmand N, Bahrampour Omrany Z, Farsam H, Nabati F, *et al*. Association of saliva fluoride level and socioeconomic factors with dental caries in 3-6 years old children in tehran-iran. Iran J Pharm Res 2011;10(1):159-66.
19. Wang YY, Zhao BL, Li XJ. Spin trapping technique studies on active oxygen radicals from human polymorphonuclear leukocytes during fluoride-stimulated respiratory burst. Fluoride 1997;30:5-15.
20. Shivarajashankara YM, Shivashankara AR, Gopalakrishna Bhat P, Hanumanth Roa S. Effect fluoride intoxication on lipid peroxidation and antioxidant systems in rats. Fluoride 2001;34(2):108-13.
21. Chouhan S, Flora SJ. Effects of fluoride on the tissue oxidative stress and apoptosis in rats: Biochemical assays supported by IR spectroscopy data. Toxicology 2008;254(1-2):61-7.
22. Blaylock RL. Excitotoxicity: A possible central mechanism in fluoride neurotoxicity. Fluoride 2004;37:301-14.
23. Bhatnagar M, Sukhwal P, Suhalka P, Jain A, Joshi C, Sharma D. Effects of fluoride in drinking water on NADPH-diaphorase neurons in brain: A possible mechanism of fluoride neurotoxicity. Fluoride 2011;44:195-209.
24. Sharma C, Suhalka P, Sukhwal P, Jaiswal N, Bhatnagar M. Curcumin attenuates neurotoxicity induced by fluoride: An *in vivo* evidence. Pharmacogn Mag 2014;10(37):61-5.
25. Forman HJ, Zhang H, Rinna A. Glutathione: Overview of its protective roles, measurement, and biosynthesis. Mol Aspects Med 2009;30(1-2):1-12.
26. Varner JA, Jensen KF, Horvath W, Isaacson RL. Chronic administration of aluminum-fluoride or sodium-fluoride to rats in drinking water: Alterations in neuronal and cerebrovascular integrity. Brain Res 1998;784(1-2):284-98.
27. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: Integrating mammalian biology. Cell 2001;104:487-501.
28. Yan L, Liu S, Wang C, Wang F, Song Y, Yan N, *et al*. JNK and NADPH oxidase involved in fluoride-induced oxidative stress in BV-2 microglia cells. Mediators Inflamm 2013;2013:895975.
29. Reiter RJ, Tan DX, Qi W, Manchester LC, Karbownik M, Calvo JR. Pharmacology and physiology of melatonin in the reduction of oxidative stress *in vivo*. Biol Signals Recept 2000;9(3-4):160-71.
30. Kim JJ, Lee SB, Park JK, Yoo YD. TNF-alpha-induced ROS production triggering apoptosis is directly linked to Romo1 and Bcl-X(L). Cell Death Differ 2010;17(9):1420-34.
31. Liu J, Cui HM, Peng X, Fang J, Zuo ZC, Wang H, *et al*. Changes induced by high dietary fluorine in the cecal tonsil cytokine content of broilers. Fluoride 2012;45:94-9.
32. Thalhammer T. Melatonin: A therapeutic potential for the neurohormone in gallbladder disorders. Iran J Pharm Res 2004;2(2):12.
33. Liu F, Ma J, Zhang H, Liu P, Liu YP, Xing B, *et al*. Fluoride exposure during development affects both cognition and emotion in mice. Physiol Behav 2014;124:1-7.