FLUORIDE-INDUCED NEURONAL OXIDATIVE STRESS AND ITS AMELIORATION BY ANTIOXIDANTS IN DEVELOPING RATS

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SUMMARY: Premated 3-month-old albino rats received 200-ppm fluoride ion (F) in their drinking water; the pups born to them were separately administered, in groups of six, daily doses of clinoptilolite, zinc, selenium, vitamin C, vitamin D, and propolis. On post-partum day 45, the pups were sacrificed, brain regions separated, and oxidative stress markers were analyzed. Prenatal (maternal) and postnatal F exposure in the developing rats caused a significant increase in the activity of lipid peroxidation and a decrease in catalase, superoxide dismutase, and glutathione peroxidase activity, thus indicating vulnerability of the developing brain to oxidative stress. Alterations were region specific, and oral supplementation of the listed antioxidants not only inhibited oxidative stress but also enhanced the activity of antioxidant enzymes. Administration of antioxidants during F exposure significantly overcame neuronal F toxicity (mostly with $p<0.05$ or <0.01) and therefore may be a therapeutic strategy for fluorotic victims.

Keywords: Antioxidants; Clinoptilolite; Fluoride neurotoxicity; Lipid peroxidation; Oxidative stress; Propolis; Rat brain; Selenium; Vitamin C; Vitamin E; Zinc.

INTRODUCTION

Fluoride (F) is highly electronegative anion with cumulative toxic effects, from prolonged ingestion that can lead to the pathogenesis known as fluorosis, a condition especially persistent in third world countries, where populations have little choice as to the main source of their oftentimes F-contaminated drinking water. Even in developed nations, where governmental agencies regulate the F content of public water systems, other sources include private water supplies, dietary ingredients, dental products, industrial emissions, and/or occupational exposure, which can cause an individual's total F intake to exceed safe doses.[1] High-level exposure can allow excessive amounts of F to penetrate the blood brain barrier and cause neuronal degeneration by a combination of events that may impair normal functioning.[2-4] During fetal and neonatal development, F has been shown to affect growth, cell differentiation, and subcellular organization in brain cells of rats by processes involving production of free radicals and lipid peroxidation (LPO).[3,5-7] The exact mechanisms of these effects on various parts of the brain are still under investigation.

Previous studies have shown promise for the use of antioxidants and antioxidant rich foods as antidotes for F intoxication and management of fluorosis.[8-13] However, findings in the literature on developing stages of fluorosis, especially in soft tissues, remain unclear. In view of what has been reported and the tentative nature of current information concerning the neuroprotective efficacy of antioxidants, the present study aimed to evaluate the oxidative damage caused by F in developing rats and the ameliorative role of the natural zeolite clinoptilolite,

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propolis (honey bee glue), zinc, selenium, vitamin C, and vitamin E against soft tissue fluorosis.

MATERIALS AND METHODS

Twenty-four premated Wistar-strain albino rats, *Rattus norvegicus,* 3-months old, weighing 160–170 g, were obtained from Sri Raghavendra Enterprises, Bangalore, and were acclimatized to laboratory conditions (12-hr dark/light, 25±2ºC). The animals were fed a standard rodent pellet diet (< 1-ppm water-extractable F) *ad libitum* and were maintained in accordance with the guidelines of National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India, and approved by the Institutional animal ethical committee, Bangalore University. The animals were divided into 8 groups of 3 rats each: a control group (I) was given tap water (< 1-ppm F), and all experimental groups (II, III, IV, V, VI, VII, and VIII) received 200-ppm F (from 442 NaF) in their drinking water during gestation and post-gestational periods. The higher dosage of 200-ppm F ion was used to induce significant toxic effects through mother rat to pups, since this mode of exposure is maternal. The NOAEL (no observable adverse effect level) of F for rats is between 150–200 ppm F (≅18 mg/kg bw/day) in drinking water,[14,15] and no mortality was observed in the present study with 200-ppm F. The live-born litter size was restricted to six pups in each of the groups III, IV, V, VI, VII, and VIII that were orally administered (gavage technique) the respective antioxidants daily in water (w/v) except vitamin E (in olive oil) with a dosage/kg bw/day of: clinoptilolite (10 mg), zinc (200 µg), selenium (40 µg), vitamin E (400 µg), vitamin C (20 mg), and propolis (2 mg). After 45 days, the pups (n=6) from each group were randomly pooled, sacrificed, required brain tissues, viz., cerebral cortex, medulla oblongata and cerebellum, were removed and homogenates of them were made for biochemical assays.

Epinephrine and DTNB (Ellman's reagent) were procured from Sigma Chemicals, USA, clinoptilolite from Zeo Inc, Augusta/McKinney, TX 75070, US, and other AR grade chemicals from Merck Ltd. Propolis was extracted in water at 50ºC from natural honeybee hives.

The biochemical estimations were done spectrophotometrically according to the following methods: LPO by Niehius and Samuelsson,[16] catalase (CAT) activity by Aebi,[17] superoxide dismutase (SOD) activity by Misra and Fridovich,[18] glutathione peroxidase (GSH-Px) activity by Rotruck et al.,[19] and protein by Lowry et al.[20]

The values in the table are expressed as mean ± SEM (standard error of the mean) with the percent change shown in parenthesis. Figures 1–4 represent the % recovery made by the antioxidants against the toxicity level or activity of F without addition of any anti-oxidant. The % recovery was calculated using the formula:

$$\% \text{ Recovery} = \left[\frac{\% \text{ change from control by F} - (\% \text{ change from the control by F} + \text{antioxidant supplement})}{\% \text{ change from control by F}} \right] \times 100$$

The statistical analysis was carried out by using SPSS 15.0 software by adapting one-way analysis of variance (ANOVA) with the Bonferroni post hoc-test at 0.05 level of significance.

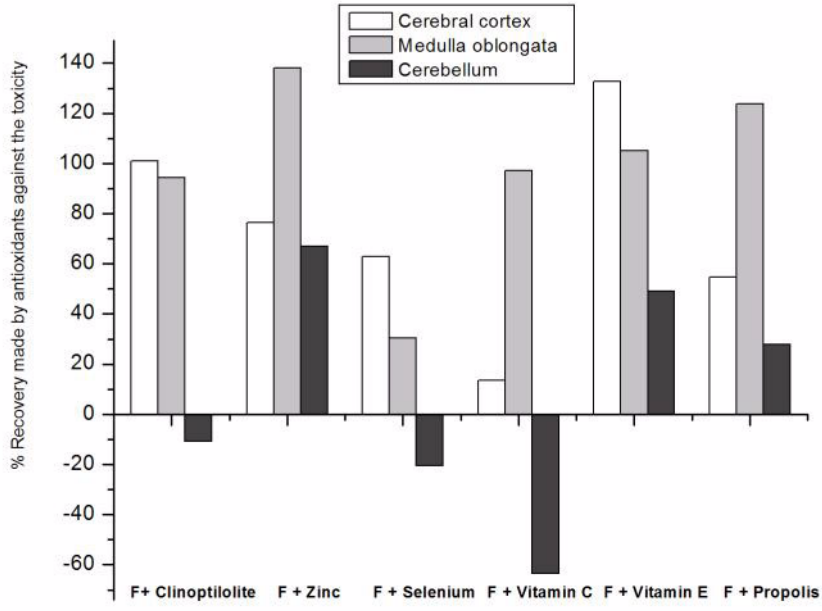

Figure 1. Percent recovery in experimental groups treated with antioxidants against the F-induced level of LPO.

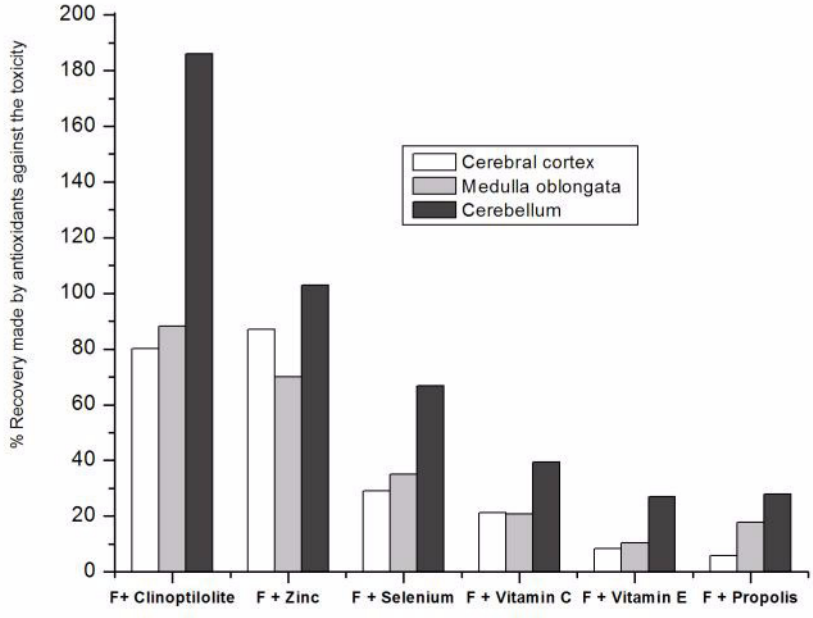

Figure 2. Percent recovery in experimental groups treated with antioxidants against the F-induced activity of CAT.

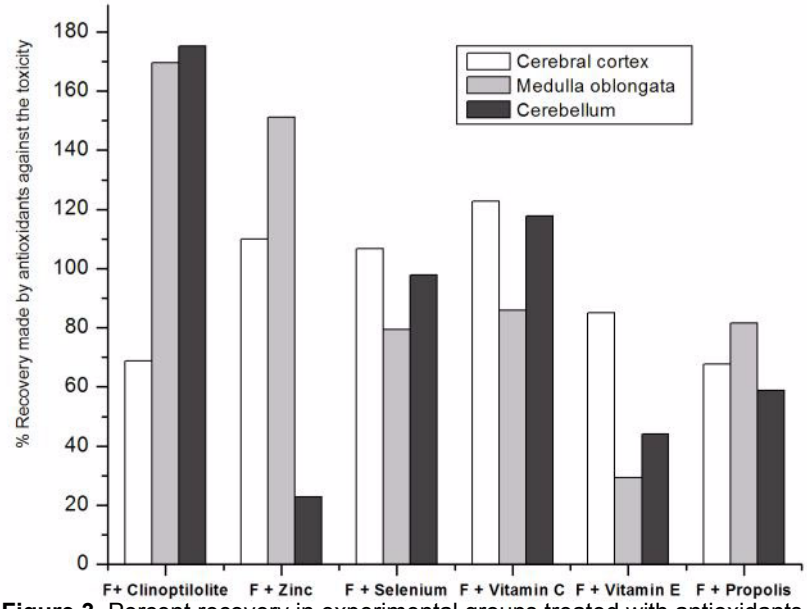

Figure 3. Percent recovery in experimental groups treated with antioxidants against the F-induced activity of SOD.

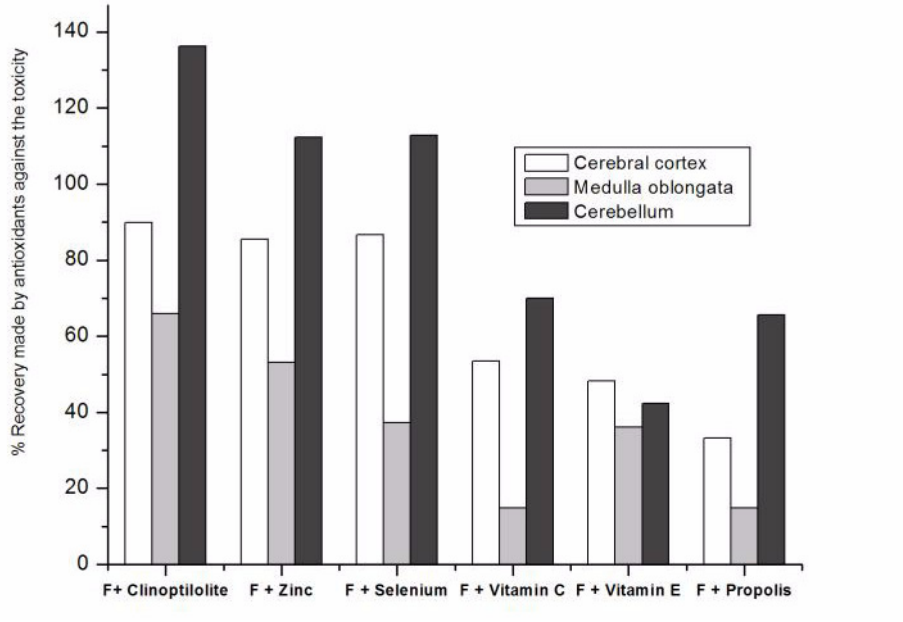

Figure 4. Percent recovery in experimental groups treated with antioxidants against the F-induced activity of GSH-Px.

RESULTS

The findings of this study confirm the deleterious effect of 200-ppm F in drinking water during maternal exposure on the antioxidant systems of the brain in developing rats (Table). In comparison to the control group, the F study group showed a marked increase in the concentration of malondialdehyde (MDA) in all

the regions of the brain, indicating intensified LPO. The F study group also exhibited decreased activity of CAT, SOD, and GSH-Px in discrete regions, validating the suppressed antioxidant efficiency in combating the F arbitrated free radical damage.

Table. Changes in the level of LPO and the activities of CAT, SOD, and GSH-Px in discrete regions of the brain in developing rats with maternal exposure to 200-ppm F drinking water and amelioration by selected antioxidants (Values (n=6) are mean activity±SEM, Values in parenthesis are % change: '–' sign indicates decrease, '+' sign indicates increase over controls)

Group	Cerebral cortex	Medulla oblongata	Cerebellum
LPO (nmoles of MDA/mg tissue)			
Control	4.81±0.22	5.10±0.09	3.47±0.09
Fluoride (F)	6.89±0.10 (+43.24)*	8.30±0.05 (+62.75)*	7.09±0.10 (+104.32)*
F + Clinoptilolite	4.79±0.07 (–0.42)†	5.28±0.07 (+3.53)†	7.48±0.08 (+115.56)*
F + Zinc	5.30±0.07 (+10.19)†	3.88±0.08 (–23.92)*†	4.67±0.06 (+34.58)*†
F + Selenium	5.58±0.12 (+16.01)*†	7.33±0.10 (+43.73)*†	7.83±0.11 (+125.65)*†
F + Vitamin C	6.61±0.09 (+37.42)*	5.19±0.06 (+1.76)†	9.39±0.10 (+170.61)*†
F + Vitamin E	4.13±0.08 (–14.14)*†	4.94±0.10 (–3.14)†	5.31±0.05 (+53.03)*†
F + Propolis	5.75±0.09 (+19.54)*†	4.34±0.05 (–14.90)*†	6.08±0.11 (+75.22)*†
CAT (µmols/min/mg protein)			
Control	109.49±2.51	140.35±3.78	89.37±3.04
Fluoride (F)	46.50±4.29 (–57.53)*	35.93±2.40 (–74.40)*	34.79±2.84 (–61.07)*
F + Clinoptilolite	96.92±4.33 (–11.48)†	127.98±3.43 (–8.81)†	136.27±3.91 (+52.48)*†
F + Zinc	101.30±6.34 (–7.48)†	109.13±4.05 (–22.24)*†	90.93±3.55 (+1.75)†
F + Selenium	64.71±2.10 (–40.90)*†	72.62±2.28 (–48.26)*†	71.23±1.65 (–20.30)*†
F + Vitamin C	59.86±2.18 (–45.33)*	57.46±1.43 (–59.06)*†	56.31±2.84 (–36.99)*†
F + Vitamin E	51.78±2.33 (–52.71)*	46.70±2.15 (–66.73)*	49.51±2.27 (–44.60)*†
F + Propolis	50.18±1.51 (–54.17)*	54.44±1.94 (–61.21)*†	50.04±2.42 (–44.01)*†
SOD (µmols/min/mg protein)			
Control	7.22±0.11	7.50±0.12	7.64±0.12
Fluoride (F)	5.42±0.12 (–24.93)*	6.58±0.09 (–12.27)*	6.23±0.05 (–18.46)*
F + Clinoptilolite	6.66±0.09 (–7.76)†	8.14±0.19 (+8.53)*†	8.70±0.42 (+13.87)*†
F + Zinc	7.40±0.08 (+2.49)†	7.97±0.07 (+6.27)†	6.55±0.09 (–14.27)*
F + Selenium	7.34±0.15 (+1.66)†	7.31±0.09 (–2.53)†	7.61±0.07 (–0.39)†
F + Vitamin C	7.63±0.13 (+5.68)†	7.37±0.07 (–1.73)†	7.89±0.15 (+3.27)†
F + Vitamin E	6.95±0.12 (–3.74)†	6.85±0.11 (–8.67)*	6.85±0.13 (–10.34)†
F + Propolis	6.64±0.13 (–8.03)*†	7.33±0.12 (–2.27)†	7.06±0.17 (–7.59)*†
GSH-Px (µg of GSH consumed/min/mg protein)			
Control	5.11±0.08	2.23±0.017	4.10±0.08
Fluoride (F)	2.64±0.08 (–48.34)*	1.29±0.03 (–42.15)*	2.47±0.05 (–39.76)*
F + Clinoptilolite	4.86±0.03 (–4.89)†	1.91±0.04 (–14.35)†	4.69±0.06 (+14.39)*†
F + Zinc	4.75±0.11 (–7.05)†	1.79±0.09 (–19.73)*†	4.30±0.06 (+4.88)†
F + Selenium	4.78±0.06 (–6.46)†	1.64±0.02 (–26.46)*	4.31±0.06 (+5.12)†
F + Vitamin C	3.96±0.07 (–22.50)*†	1.43±0.04 (–35.87)*	3.61±0.08 (–11.95)*†
F + Vitamin E	3.83±0.11 (–25.05)*†	1.63±0.05 (–26.91)*	3.16±0.05 (–22.93)*†
F + Propolis	3.46±0.08 (–32.29)*†	1.43±0.06 (–35.87)*	3.54±0.11 (–13.66)*†

*$p < 0.05$ compared to control group; †$p<0.05$ compared to F group.

As seen in the Table, regional brain changes in oxidative stress markers in the F group were specific and heterogeneous (variable). Among the three regions studied in the developing rats, the cerebral cortex showed the greatest percentage decreases in the activity of CAT (–57.53), SOD (–24.99), and GSH-Px (–48.40), and smallest increase in LPO (+43.14). In the medulla oblongata, decreased activities were greatest in CAT (–74.40), moderate in LPO (+62.57) and GSH-Px (–42.15), and least in SOD (–12.16). The cerebellum showed the highest activity increase in LPO (+104.74), moderate decreases in CAT (–61.07) and SOD (–18.46), and smallest in GSH-Px (–39.89).

Dietary antioxidant supplementation proved to be effective in restoring oxidative damage evidenced by diminishing elevated MDA levels and enhancing the inhibited activities of CAT, SOD, and GSH-Px expressed in terms of % recovery against the MDA level and enzyme activities induced by F exposure (Figures 1–4). In the cerebral cortex, the maximum ameliorative effect of supplementation was observed with vitamin E on the level of LPO (132.83%) and on enzyme activities with zinc on CAT (86.99%), vitamin C on SOD (122.44%), and clinoptilolite on GSH-Px (89.90%). In the medulla oblongata, the maximum amelioration occurred with the supplementation of zinc on the LPO level (138.36%), and on the activities with clinoptilolite on CAT (88.16%), SOD (170.75%), and GSH-Px (65.78%). In the cerebellum, maximum amelioration occurred with zinc on the LPO level (66.88%), and on the activities with clinoptilolite on CAT (185.92%), SOD (174.94%), and GSH-Px (135.54%).

DISCUSSION

Reactive oxygen species (ROS) are implicated as important pathologic mediators in many neuronal disorders. Generation of free radicals, LPO, and altered antioxidant systems are considered to play a vital role in posing toxic effects of F. Increased oxidative stress has also been directly linked to oxidation of cellular macromolecules that may cause injury to the brain or induce a variety of adverse cellular responses.[3,5,11,21] A high rate of oxygen consumption coupled with low potential of brain to obviate oxidative stress may be the main triggering factor for their enhanced release of ROS during F exposure.[3,5,22] In the presence of free radicals, F induces neurotoxicity by biphasic action where, it behaves as a pro-oxidant and the other as an inhibitor of antioxidant enzyme systems.

In the present study, the elevated levels of MDA in the cerebral cortex, medulla oblongata, and cerebellum appear to be due F-induced generation of free radical oxidative stress that may cause extensive cellular damage unless it is arrested by certain protective agents. The studies of Vani and Reddy[22] and by Chirumari and Reddy[23] corroborate our findings. In addition, the significant reduction ($p<0.05$–<0.01) in the activities of CAT and SOD may be in response to increased production of H_2O_2 and O_2. Similarly, a reduction in GSH-Px activity ($p<0.05$) observed in all the regions of brain indicates the widespread extent of cellular damage caused by oxidative stress and the inability of GSH-Px to check it. The regional changes observed in developing brain on oxidative stress markers in the F

group were specific and heterogeneous. These regional alterations caused could be due to their difference in cell types, composition, function, sensitivity, etc.

Among zeolites, clinoptilolite is reported to have a free radical scavenging ability and an antioxidant capacity to treat medical ailments including cancer in different pathological states.[24] In the present study, the antioxidant responsiveness against F intoxication mediated by clinoptilolite supplementation in discrete regions of brain may have significance in eliminating F-induced ROS radicals. This effect could be due to the enriched mineral content of clinoptilolite having composition SiO_2, Al_2O_3, Fe_2O_3, Na_2O, K_2O, MgO, CaO, MnO, TiO_2, and water.[25] The protection offered might be associated with the ion-exchange and cation binding properties of clinoptilolite, and its supplementation could help to prevent alterations in redox homeostasis of cells as it traps and encounters the free radical buildup, thereby helping the body to defend itself against a surge of ROS radicals.

Zinc, as a membrane stabilizer, prevents disruptive effects of LPO and protein oxidation and offers protection to membrane protein thiols, thereby promoting membrane skeletal and cytoskeletal protein integrity. Supplementation with zinc evidently counteracted LPO by reversing F toxicity in all the regions of developing nervous system. Moreover, zinc competes with pro-oxidants like iron and copper and diminishes their oxidation potential as a component of Cu-Zn SOD that potentially converts destructive $O_2^{\cdot -}$ to H_2O_2.[26,27] Similarly, selenium supplementation reduced the extent of LPO. The selenium dependent enzyme GSH-Px acts as an essential cofactor for selenoprotein-P and other selenoproteins.[28] Strikingly, a deficiency in the level or changes in the utilization of selenium during F intoxication may markedly decrease tissue GSH-Px activity that results in peroxidative damage and mitochondrial dysfunction.

The beneficial role of vitamin C and vitamin E in combating F toxicosis in relation to LPO observed in all the regions of the brain studied indicates their scavenging, detoxifying, and therapeutic properties. It is hoped that supplementation of these vitamins to F victims might have a beneficial role, i.e., to alleviate F-induced vitamin metabolism disturbances. [9] Vitamin C and vitamin E are key synergistic antioxidants; when vitamin E quenches free radicals, it becomes a vitamin E radical, which then uses vitamin C to return it to its antioxidant state and acts as chelating agent.[29] Antioxidants such as vitamin E, coenzyme Q, vitamin C, glutathione (GSH), and selenium may act synergistically, preventing LPO and cell destruction.

Green propolis contains valuable bioflavanoids and polyphenolic acids, which has been widely studied and used in view of its strong antioxidative, antibacterial, anti-inflammatory, and tumoricidal properties.[30] Studies by Hara et al.[31] indicate propolis offered reduction in neuronal damage caused by transient ischemia to forebrain and spinal cord. The protective action of propolis against F-induced oxidative stress in different areas of the brain is evidenced in the present study, and such protection by propolis in mouse brain homogenates is also indicated by the studies of Krol et al.[32] The present study further suggests that the food substances

having bioflavanoids and polyphenolic acid compounds might be effective antioxidants for human health and in prevention of neuronal oxidative stress and degeneration.

In conclusion, F toxicity in the developing brain may result in disruption of the pro-oxidant/antioxidant balance, which provides a strong coupling of altered equilibrium processes and loss of energy capacity to meet an oxidative challenge. Moreover, exposure to F can increase the effects of nutritional deficiencies. Exogenous supplementation of antioxidants has been found to counter nutritional deficiency and to facilitate reduction of the toxic effects induced by F, thereby bolstering the cellular antioxidant defense.

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