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ANTIOXIDANTS IN THE MANAGEMENT OF FLUORIDE INDUCED NEURAL OXIDATIVE STRESS IN DEVELOPING RATS

Mahaboob P. Basha*[1], Shabana Begum [2] and N. Madhusudhan [1]

Department of Zoology, Bangalore University [1], Bangalore 560056, Karnataka, India
Maharanis Science College for Women [2], Bangalore, 560001, Karnataka, India

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Correspondence to Author:

Dr. P. Mahaboob Basha

Associate Professor in Zoology, Bangalore University, Bangalore-560056, Karnataka, India

E-mail:pmbashabub@rediffmail.com

ABSTRACT: 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 F-contaminated drinking. In recent times many neurological problems among children are being addressed in endemic areas. Thereby reasons for the neurotoxicity have to be explicated thoroughly. In this study premated Wistar albino rats were exposed to 50 and 150 ppm fluoride in drinking water during gestation and pups born to them were used to analyze the extent of neurotoxicity imposed in discrete brain areas. Dose dependent toxicity was evident in different brain regions and fluoride exposure has significantly enhanced the levels of malondialdehyde ($P>0.05$), glutathione ($P>0.05$) and decreased the activities of superoxide dismutase ($P>0.05$), catalase ($P>0.05$), glutathione peroxidase ($P>0.05$) and glutathione- S-transferase ($P>0.05$). Alterations were region specific and oral supplementation of dietary antioxidants viz., vitamin-C (20mg), vitamin-E (400µg), zinc (200µg) and selenium (40µg) not only inhibited oxidative stress but also enhanced the activity of antioxidant enzymes. Thereby supplementation of antioxidants to toxicated animals significantly overcame toxic burden imposed by fluoride and therefore may be a therapeutic strategy for fluorotic victims.

INTRODUCTION: Fluoride is an essential trace element, plays a role in preventing dental carries and reducing tooth decay [1]. However, excessive fluoride intake for a long is shown to cause serious public health problem [2]. The guideline value of 1.5ppm (1.5 mg/L) set by WHO in 1984 and reaffirmed in 1993 needs to be revised. Concentrations above this value carry an increasing risk of dental fluorosis, and much higher concentrations lead to skeletal fluorosis and soft tissue fluorosis.

It is well known that the developing nervous system is more vulnerable to environmental contaminants than the adult nervous system [3] and the critical period of neuronal development where the essential process of regional brain structure and function are established [4, 5]. Perturbations of these processes (e.g. exposure to chemical agents), result in long-term irreversible consequences that affect their structure and function, and could account for qualitative differences in age-related susceptibility

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of the developing nervous system as compared with the adult nervous system [4, 5].

Dysfunction of the central nervous system (CNS) has been considered as a result of chronic fluorosis [6]. As in the case of many chronic degenerative diseases, increased production of reactive oxygen species (ROS) and lipid peroxidation have even been considered to play vital role in the pathogenesis of chronic fluoride toxicity. Interactions between fluoride and free-radical reactions have been studied in various biological systems including fluorosis [7]. It is known that fluoride can penetrate through the placental sac and be stored in certain organs of the fetus [8].

Recently, it was reported in children aged 3 to 10 years with endemic fluorosis and there was an increased oxidative stress [8]. However, the relationship between fluorosis/fluoride toxicity and oxidative stress is still not clear.

In the absence of conclusive evidence for an increased oxidative stress in fluorosis, some studies suggest the use of antioxidants and antioxidant-rich foods for the management of fluorosis [9, 10] and also for the beneficial effects of antioxidants as antidotes for fluoride toxicity.

Keeping in view of the significance of developing nervous system and lacuna in the literature on aspect connected to neurotoxic effects of fluoride, this study was initiated, and in addition, antioxidant efficacy of selected antioxidants was measured.

MATERIAL AND METHODS:

Animals: Laboratory bred premated albino rats *Rattus norvegicus*, Wistar strain obtained from Sri Raghavendra enterprises, Bangalore and made to acclimatize to laboratory conditions (12 hr dark/light, 25±2^{o}C). Standard rodent pellet diet was given *ad libitum*. The animals were maintained in accordance with the guidelines of National Institute of Nutrition, ICMR Hyderabad, India and approved by the ethical committee, Bangalore University.

Design: The animals were assorted into 3 groups. The control (I) group was given tap water (Less than 1ppm fluoride) and experimental group II & III received 50 ppm and 150 ppm fluoride in water during gestation and post gestation period respectively (pups were placentally and lactationally exposed to fluoride toxicity). The pups born to experimental animals were sorted further into various groups and were given orally respective antioxidants in water (w/v) except vitamin E (in olive oil w/v) during postnatal period till the day of autopsy with a daily dose/ kg body weight shown- vitamin - C (20mg), vitamin -E (400µg), zinc (200µg) and selenium (40µg).

At the end of 21st day of antioxidant treatment the pups were sacrificed and required brain tissues viz, cerebral cortex, medulla, cerebellum and spinal cord were separated and used for biochemical assays.

Chemicals: Epinephrine and DTNB (Ellman's reagent) procured from Sigma chemicals USA, and other AR grade chemicals from Merck Ltd were used for the assay.

Biochemical Assays: Tissue samples were homogenized in requisite buffer for the assessment of oxidative stress indices, such as CAT, SOD, GST, GPx and GSH activity/ levels and the adopted procedures are shown below:

1. **Lipid peroxidation** (LPO): Lipid peroxidation (LPO) product was estimated by measurement of thiobarbituric acid reactive substances using the method of Nehius and Samuelson [11]. The pink chromogen produced by the reaction of thiobarbituric acid with malondialdehyde (MDA), a secondary product of lipid peroxidation, was estimated at 535 nm.

2. **Superoxide Dismutase** (SOD, EC 1.15.1.1): SOD activity was assayed by measuring the inhibition of epinephrine auto-oxidation as described by Misra and Fridovich[12].The absorbance was recorded at 480 nm for 60 s. Results are expressed as units/mg protein.

3. **Catalase** (CAT, EC 1.11.1.6): CAT activity was measured as described by Aebi[13]. The rate constant of hydrogen peroxide (H_2O_2) decomposition was monitored by measuring the decrease in absorbance at 240 nm for 60s. Results are expressed as nmol of H_2O_2 consumed/min/mg protein.

4. **Glutathione Peroxidase Activity** (GPx, EC 1.11.1.9): GPx activity was estimated by measuring the oxidation of DTNB as described by Rotruck *et al*, (1973) [14] and change in absorbance was measured at 420 nm. An enzyme unit represents a decrease in GSH concentration of 0.01 log unit/min, after subtraction of Non-enzyme rate. Results are presented as µmol of GSH consumed /min/mg protein.

5. **Glutathione-S-transferase** (GST, EC 2.5.1.18): GST activity was estimated by the method of Habig *et al* [15] by following the increase in absorbance at 340 nm using 1-chloro-2, 4-dinitrobenze (CDNB) as substrate. The assay was conducted by monitoring the appearance of the conjugated complex of CDNB and GSH, viz., 2, 4-dinitro phenyl glutathione at 340 nm. Results are presented as nmol of 2, 4-dinitro phenyl glutathione formed/min/mg protein.

6. **Reduced Glutathione** (GSH): Reduced glutathione content was determined using 5, 51-dithio-bis (2-nitrobenzoic acid) (DTNB) as fluorescent reagent according to the method of Ellman[16]. GSH levels were monitored at 412 nm. Results are expressed as mg of GSH/g wet weight of tissue.

7. **Protein Assay**: Protein content was estimated by the method of Lowry *et al* [17], using bovine serum albumen (BSA) as a standard.

Statistical Analysis: The results are expressed as Mean ± S.E. Values in parenthesis indicate percentage change, '+' sign indicate increase, '-' sign indicate decrease over control. Data were analyzed by employing Student's-t test and values of $p<0.05$ were considered statistically significant.

The percentage recovery was calculated by using the formula –

$$\% \text{ Recovery} = \left(\frac{A - B}{A}\right) \times 100$$

Where A - % change noticed in fluoride exposed group and B - % change noticed in fluoride and antioxidants exposed group

RESULTS: The results of the present experiment are depicted in **figures 1-6**. It is evident from results that fluoride intoxication has augmented MDA levels indicating increased peroxidation followed by a marked decrease in the activity of catalase, SOD, GSH-Px and GST levels in discrete regions of CNS studied. The alterations were more apparent in 150 ppm exposure dose than the 50 ppm indicating dose dependent toxicity and brought perturbations in the antioxidant homeostasis. The toxicated rats supplemented with dietary antioxidants showed considerable protection in elevating the antioxidant levels to safeguarding the developing nervous system. The ameliorative efficacy offered by different antioxidants in terms of percentage (%) recovery is shown in figure 1-6. Comparatively, Zinc (200µg) and Vitamin E (400µg), supplementation offered higher percent recovery, than vitamin C (20mg) and selenium (40µg).

DISCUSSION: Brain consumes greatest amount of oxygen and has high polyunsaturated fatty acids with low concentrations endogenous antioxidants; thereby oxygen free radicals exert their cytotoxic effect by peroxidation of membrane phospholipids leading to change in permeability and loss of membrane integrity [3]. The source of oxygen toxicity arises from the mitochondria, where oxygen is the final acceptor of electrons in oxidative metabolism. Oxygen is reduced to water, as well as a number of ROS, including superoxide radical, hydrogen peroxide and hydroxyl radical. These ROS leak out of the mitochondria into the cytoplasm where they cause oxidative damage to a wide variety of biologically important molecules, including deoxyribonucleic acid, proteins and polyunsaturated fatty acids (PUFAs) [3, 4]. Oxidative stress is a pivotal factor in many chronic neurodegenerative disorders [3, 4].

Increased TBARS levels observed in discrete regions of CNS indicate increased lipid peroxidation in the immature rat brain as peroxidation of cell membrane lipid disrupts their fluidity and permeability. According to Shivarajashan kara *et al* [18] long-term high-fluoride exposure during the early developing stages in rats induce oxidative stress and disturb the endogenous antioxidants.

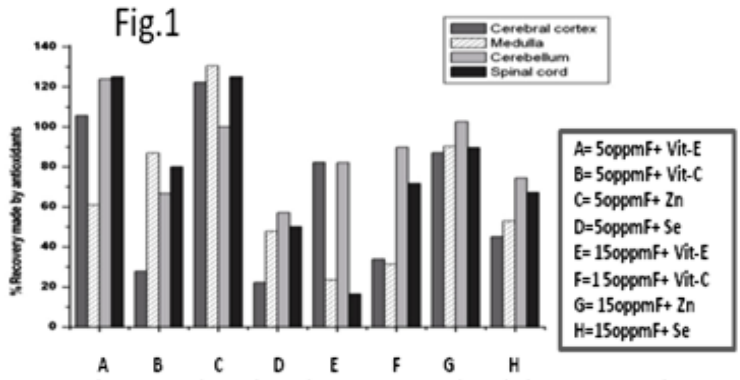

Fig 1: Recovery (%) in MDA levels in fluoride toxicated rats treated with antioxidants

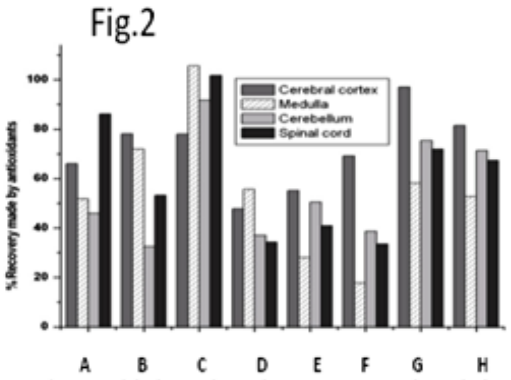

Fig 2: Recovery (%) in CAT activity in fluoride toxicated rats treated with antioxidants

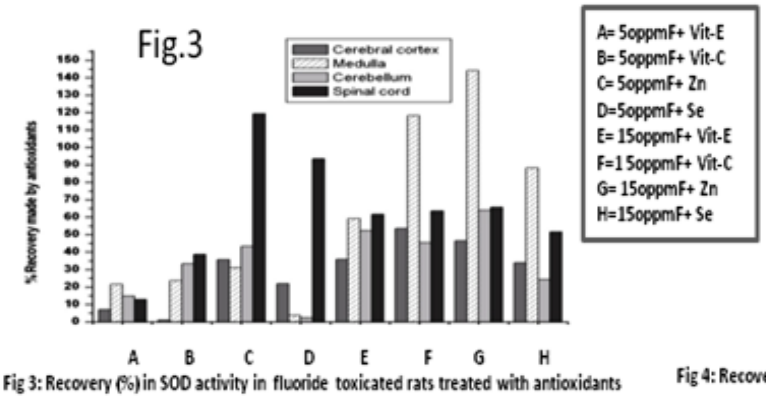

Fig 3: Recovery (%) in SOD activity in fluoride toxicated rats treated with antioxidants

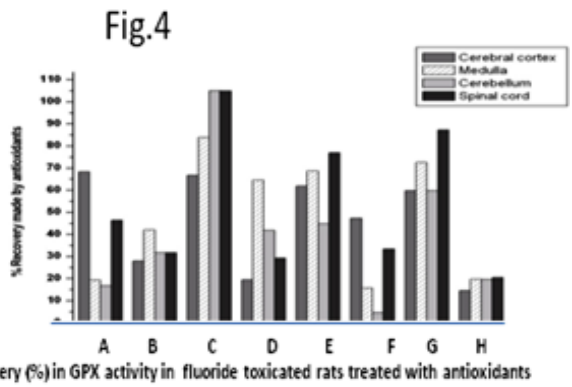

Fig 4: Recovery (%) in GPX activity in fluoride toxicated rats treated with antioxidants

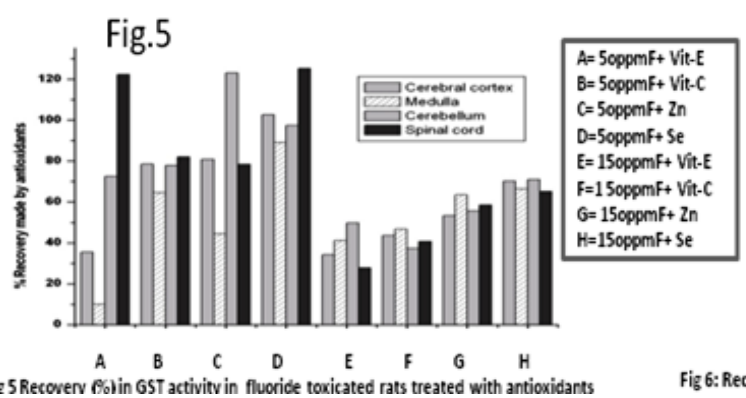

Fig 5 Recovery (%) in GST activity in fluoride toxicated rats treated with antioxidants

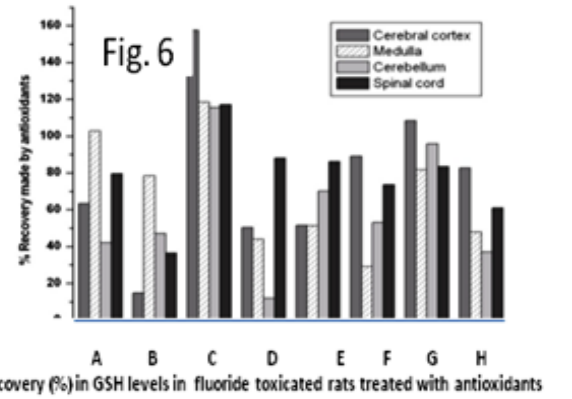

Fig 6: Recovery (%) in GSH levels in fluoride toxicated rats treated with antioxidants

The increased oxidative stress observed in fluoride toxicosis indicates the sensitivity of the developing nervous system and prone to lipid peroxidation and oxidative damage. Since the oxidative stress observed in the present study is the key factor in altering the antioxidant homeostasis, regardless of etiology and is associated with decreased SOD concentrations in nervous tissue.

The findings of Vani and Reddy (2000) [19], Shivarajashankara *et al* (2001) [18], Kasperczyk and Kasperczyk (2006) [20] showed similar results and corroborate the present findings. The activity levels of GST, GSH-Px found decreased in all the regions of CNS upon fluoride intoxication in 50 and 150ppm doses. This could be due to increased utilization of glutathione in curbing the free radical mediated toxic actions. When the tissues are exposed to oxidative stress they increase the activity and expression of antioxidant enzymes as a compensatory mechanism against free radical-mediated damage. Nevertheless, the increased activity of the antioxidant enzymes may be inadequate to counteract the potential damage in many conditions of oxidative stress [21]. And also the decreased SOD activity may lead to decreased production of hydrogen peroxide may in turn lead to reduced oxidation of glutathione by glutathione peroxidase [22].

Oxidants and antioxidants play an important role in maintaining a balance between free radical produced by metabolism or derived from environmental sources and antioxidant system of

the body. Deficiency of dietary antioxidants or precursors of antioxidants enhance oxidative damage [3, 4]. Restoring or maintaining oxidative buffering capacity therefore represents one useful therapeutic approach to minimize neuro-degeneration.

In the present experiment, supplementation of anti-oxidants to the fluoride intoxicated animals were carried out to assess the neuroprotective capability of vitamin C, vitamin E, selenium and Zinc.

Vitamin C is the most versatile antioxidant and a powerful scavenger of hydroxyl radical and regenerator of vitamin E, the key antioxidant glutathione, which reacts enzymatically (through GSH-Px) and non-enzymatically with a broad range of oxidants. Vitamin E, a known free radical scavenger and offer protection against lipid peroxidation [23].

When vitamin E quenches free radicals, it becomes a vitamin E radical, which then uses vitamin C to return it to its antioxidant state [23]. Thus supplementation of vitamin C (20mg/kg bw/day) and vitamin E(400µg/kg bw/day) considerably protected toxicated neuronal tissues suggesting a protective act by their synergistic action in minimizing the toxic effect of fluoride mediated oxidative stress.

An essential role of selenium is the activation of GSH-Px and its activity is shown to be proportional to the log of the dietary selenium concentration[3]. Therefore, selenium supplementation enhances the availability of glutathione, which is one of the most intrinsic antioxidant that prevents cell damage. Zinc is essential for brain maturation, function and has shown to have a relationship with many enzymes that can prevent cell damage through activation of the antioxidant system [24].

Thereby, zinc acts as an antioxidant, play key role in blood brain barrier (BBB) and offer protection to protein thiol groups.

In this study, the supplementation of selenium and zinc have considerably offered protection to brain compartments confirming the antioxidant ability to minimize the toxic burden imposed by fluoride.

In conclusion, fluoride in high dose duly proves to cause oxidative stress, and perturb the antioxidant homeostasis. The supplementation of dietary antioxidants offers a promising approach in minimizing toxicity indicating ameliorative role. Of all the antioxidants zinc, vitamin-E supplementation found to pronounce high percent recovery than vitamin-C and selenium.

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