Age Associated Fluoride Induced Ultra Structural and Biochemical Changes in Rat Brain

Abstract

Fluoride (F) ingestion is well known to be associated with oxidative stress in experimental animals, but the precise mechanism of its toxicity associated with advancing age has not yet been delineate. The present study attempts to assess the learning and memory capacity of rats using water maze test for cognitive functioning. The markers of oxidative stress i.e., Reactive oxygen species (ROS), protein carbonyl content (PC), lipid peroxide levels (LPx), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (Gpx) and reduced glutathione (GSH) were measured in the brain hippocampus of young and aged rats fed with fluoride (100 ppm) with drinking for 90 days. We observed significant changes of fluoride treated young and aged rats as compared with their respective controls. Lipofuscin content was significantly increased in fluoride treated aged rats. Ultra structural studies of the CA1 region in hippocampus of fluoride exposed rats revealed that the changes were more pronounced in the aged treated rats in terms of presence of clustered lipofuscin, vacuolization, lysosomal degradation and nucleolar fragmentation. On the basis of the results it may conclude that fluoride may be linked with neuro lipofuscinogenesis and alteration in neurobehavioral activity and these changes may be responsible for the development of age related disorders.

Keywords: Fluoride; Aging; Brain; Oxidative stress

Abbreviations: ANOVA: Analysis of Variance; CAT: Catalase; GSH: Glutathione; GSSG: Oxidized Glutathione; GPx: Glutathione Peroxidase; GR: Glutathione Reductase; LPO: Lipid Peroxidation; Lipofuscin (LIF); ROS: Reactive Oxygen Species; SOD: Superoxide Dismutase; PMS: Phenazinemethosulphate; NBT: Nitoblu Tetrazo lium; YC: Young Control; YEx: Young Experimental; OC: Old Control; OEx: Old Experimental

Introduction

Fluorine is abundantly found in the ground drinking water in worldwide and very little amount is required to humans. Fluorosis is one of the manifestations of chronic poisoning from long-term exposure to high levels of fluoride, and is a serious health problem in many parts of the world where drinking water contains more than 1-1.5-ppm of fluoride [1]. There have been numerous researches on the harmful effects of fluoride intake in excess on bones and teeth of human as well as animal models [2]. It is characterized by clinical manifestations in bones and teeth. Fluoride accumulation in brain was observed in chronic high-fluoride exposure through drinking water. It is reported that high fluoride is known to cause structural and functional changes in the brain of experimental animals [3]. Evidence from clinical and animal model studies demonstrates that fluoride content may increases with age. It may be either due to increased exposure with age or decreased ability to remove fluoride from the body. Therefore, knowledge of age related effects of fluoride in tissues and relative risk as well as extent of its retention in tissue is important for the understanding of its effects and associated disorders [4]. The possible mechanism of fluoride induced neurotoxicity has been related to cell damage via free radical production [5]. Increased lipid peroxidation (LPO) is one of the major consequences of oxidative stress [6]. Lipofuscin, a morphological biomarker of lipid peroxidation, represents an end - product of oxidative degradation of lipids by free radical mechanism [7]. Studies on age associated and fluoride mediated toxic effects are very important as there are several reports that, on one hand the efficiency of the blood brain barrier (BBB) may decline with advancing age [8], and on the other hand, cognitive decline may account for functional and morphological changes in the central nervous system (CNS). There is, however, a paucity of studies on the effect of fluoride intoxication with advancing age on oxidative stress.

In view of the aforementioned considerations, the present study was designed to assess fluoride induced neurochemical and ultra structural changes in the hippocampus of young and old rat brain. To achieve these objectives we administered sodium fluoride (100 ppm) through drinking water to young and aged rats for 90 days. Theretofore, the animals were sacrificed and neurochemical and ultra structural studies were carried out. In the present study, we attempt to assess the effect of intake of high-fluoride levels in drinking water during fetal, weaning, and post-weaning stages of life on oxidative stress in the brain of rats.

Materials and Methods

Animals

Forty male Rattus norigious, Wistar strain rats of two age groups, young (6 month, weight 182 ± 2.2g, n = 24) and old (24
Age Associated Fluoride Induced Ultra Structural and Biochemical Changes in Rat Brain

Data were

- M

2+

+ 2+

Tripathi S, Chauhan DS, Singh VP, Joshi DK, Choudhary M (2015) Age Associated Fluoride Induced Ultra Structural and Biochemical Changes in Rat Brain. MOJ Toxicol 1(5): 00024. DOI:

Citation:

adducts at 375nm. Carbonyl contents were calculated by using a

et al. [10] using bovine serum albumin (BSA) as standard. The

description

Measurement of Protein Oxidation and Lipid Peroxidation

The protein content was measured by the method of Lowry et al. [10] using bovine serum albumin (BSA) as standard. The protein oxidation was measured by estimating the protein carbonyl levels by the method of Liu et al. [11]. Protein carbonyl content was determined in the samples by measuring the DNP adducts at 375nm. Carbonyl contents were calculated by using a molar extinction coefficient (ε) of 22,000 M⁻¹·cm⁻¹. Data were expressed as nmol carbonyl/mg.

Lipid Oxidation and Lipofuscin

The lipid peroxide (LPx) levels were measured by the method of Ohkawa et al. [12]. The thiobarbituric acid reacting substances (TBARS) of the sample were estimated spectrophotometrically at 532 nm and expressed as nmole of MDA /g tissue. Lipid hydroperoxide (LOOH) levels were measured by the method of Halderbrandt [13]. In this method, ferrous-thiocyanate was measured on peroxidation of Fe²⁺ to Fe³⁺ by H₂O₂ and resulted in intense pale colour, which was read at 480 nm. Standard cumene hydroperoxide was used as control and results were expressed as nmole of cumene hydroperoxide per gram tissue. Conjugated dienes (CD) was measured by the method of Rakhnegel [14]. The cytosolic fraction was extracted from long chain fatty acids by chloroform: methanol. The absorbance of lipid extract was read at 220nm on spectrophotometer. The level was expressed as pmol dienes/100g lipid using the molar extinction coefficient of 2.1x10³. The lipofuscin (LF) was measured according to the method of Sohal [15]. Brain tissue was homogenized with 2:1 chloroform-methanol mixture. The mixture was centrifuged at 3000 rpm and the pellet discarded. The concentration was measured with a fluoro-spectrophotometer at an excitation maximum of 360nm and emission maximum of 420nm. The content of the fluorescence was determined using quinine fluorescence as a standard. Data were presented as U/g tissue. One unit (U) of lipofuscin is defined as fluorescence of 0.01 g/ml quinine sulphate.

Measurement of Endogenous Enzymes

An aliquot of cerebellum homogenate was used for the assay of enzymatic antioxidants. The superoxide dismutase (SOD EC 1: 15.1.1.1) activity was determined from its ability to inhibit the reduction of NBT in presence of PMS [16]. The reaction was monitored spectrophotometrically at 560nm. The SOD activity was expressed as U/mg protein (1 unit is the amount of enzyme that inhibit the reduction of NBT by one half in above reaction mixture). Catalase (CAT, EC 1.11.1.6) activity was assayed using hydrogen peroxide as substrate; the decomposition of H₂O₂ was followed at 240nm on spectrophotometer [17]. The CAT activity was expressed as U/mg protein. The glutathione peroxidase (GSHPx, EC 1.11.1.10) was assayed using GSH, NADPH and H₂O₂ as reactants. The oxidation of GSH into GSSG was measured in terms of oxidation of NADPH to NADP⁺ and assayed as decrease in the absorbance of reaction mixture at 340 nm on spectrophotometer [18]. The activity of GSHPx was expressed as nmol of NADPH oxidized/ min / mg protein. Glutathione reductase (EC 1.6.4.2, GR) activity was assayed by the method of Hazelon [19]. Activity of GR was expressed as nmole of NADPH oxidised/min/mg protein of cell extract.

Measurement of Reduced and Oxidized Glutathione

Reduced glutathione was measured in deproteinized supernatant of the cerebellum. Tissue homogenate was deproteinated with tetrachloroacetic acid, centrifuged and supernatant was used for the estimation of reduced glutathione (GSH) with the help of Ellman reagent (5, 5’ dithiobis (2-nitro benzoic acid)). The optical density of the pale colour was measured on the spectrophotometer at 412 nm. An appropriate
standard (pure GSH) was run simultaneously. The level of GSH was expressed as μg/g tissue [20]. The oxidized glutathione (GSSG) was estimated by the decrement of GSSG in the presence of NADPH and glutathione reductase and determined the decrement of NADPH absorbance at 340 nm. The result was expressed as μg/g tissue [21].

Electron Microscopic study

Ultra structural changes in frontal cortex region of the central nervous system were assessed using standard electron microscopic technique. The rats were anaesthetized with Nembutol (sodium pentobarbital) solution, 50 mg/kg b.w. and perfused through heart with Karnovsky’s fixative (0.1 M paraformaldehyde and glutaraldehyde solution in cacodylate buffer, pH 7.3). The brains were quickly removed from the cranium, placed on ice and frontal cortex was dissected out. Small pieces of 2-3 mm size were immersed in the same fixative for four hours, and thereafter washed with 0.1 M cacodylate buffer (pH 7.3). The samples were post fixed for three hours at 4°C in 1% osmium tetroxide prepared in 0.1 M cacodylate buffer. The specimen were washed with distilled water and left in 1% aqueous uranyl acetate overnight. Subsequently, dehydration was carried out in ascending grades of alcohol, acetone and in pure acetone. Following dehydration, the specimens were embedded in Epon 812 at room temperature. Sections were cut on an LKB-Ultramicrotome with a glass knife. Thereafter, sections were mounted on 300 mesh copper grids, stained with 1% uranyl acetate and lead citrate and examined with a Phillips (FEI Tecnai 12 twin) Transmission Electron Microscope.

Statistical Analysis

Experimental data were summarized as Mean ± SE (n=6). Groups were compared together by one way analysis of variance followed by Student Newman-Keuls post hoc test. The acceptance level of significance was p< 0.05. InStat (version 3) was used for analysis of data.

Results and Discussion

In the present study we report that exposure with fluoride caused significant oxidative degradation of lipids and proteins due to increased rate of ROS following structural changes in the hippocampus and these effects were more pronounced in aged rats. The fluoride exposed rat revealed reduced body and brain weight (Table 1). These changes seem to be linked with chronic fluoride neurotoxicity. Moreover, as these changes were more apparent in the aged rats, it is likely that they may be due to the perturbation in cellular and molecular metabolism of aged rats.

Although toxic effects of fluoride are well established but its mechanism of action is poorly understood. Following oral administration, retention of fluoride is reported in the brain, bone, kidney, muscle and heart [22]. Figure 1 embodies ROS, protein and lipid peroxidation products. The level of ROS was found to be significantly increased by 29% in fluoride treated young rats and 31% in old rats when compared with their respective controls. The ROS was 35% was found to increase in fluoride treated aged rats as comparison with fluoride treated young rats. The PC content was found to be increased by 44% in YEx and 70% OEx as compared with YC and OC respectively. Moreover, PC increased by 62% in OEx as compared to YEx. LOOH were found to be significantly elevated by 64% and 99% in fluoride treated YEx and OEx as compared to their age matched control rats. The OEx exhibited 86% elevation of LOOH as compared with the YEx. CD was also markedly increased by 63% in YEx and 60% in OEx as compared to their respective controls. While, it was elevated by 75% in OEx when compared with YEx. The concentration of MDA was found to be significantly increased by 67% in YEx and 62% in OEx when compared with their age matched control rats. The OEx showed 44% greater lipid peroxidation as compared with the YEx. The concentration of LIF was found to be markedly increased by 180% in YEx and 166% in OEx than that of YC and OC respectively. In addition, lipofuscinogenesis was significantly increased by 201% in OEx as compared with YEx.

**Table 1: Body and brain weight of control and experimental rats.**

| Groups | Terminal Body Wt (g) | Brain (g/kg) |
|--------|----------------------|--------------|
| YC     | 27.88 ± 6.3          | 3.9 ± 0.07   |
| YEx    | 247.5 ± 5.2          | 3.34 ± 0.05  |
| OC     | 534.2 ± 8.2          | 3.62 ± 0.07  |
| OEx    | 422.5 ±9.1           | 2.89 ± 0.08  |

The body and brain weight of control and experimental rats of young and old rats are presented as Mean ± SEM in six rat of each group. Superscripts relate significant (p< 0.05) comparison with YC (a), YEx (b) and OC (c).

Our results demonstrated oxidative damage of lipids, as measured by the increased levels of lipid peroxidation products (MDA, LOOH and CD) in the hippocampus of young and aged rats following fluoride exposure (Figure 1). This may be due to increased rate of ROS production in the brain. Moreover, as the brain contains large amount of lipids that are rich in polyunsaturated fatty acids, they can readily react with free radicals and undergo peroxidation [23]. These free radicals attack lipids and proteins to produce lipofuscin pigment deposition of which was revealed by our electron microscopic studies of fluoride treated rats. We also observed that the deposition of lipofuscin was accompanied by increased levels of lipid peroxide and protein carbonyl content in the hippocampal neuron of the young and aged rats following fluoride exposure.

The activities of antioxidant enzymes namely SOD, CAT, GPx and GR presented in Figure 2. The activity of SOD was found to be decreased 33 % and 37% in fluoride treated YEx and OEx treated rats when compared with their age matched controls YC and OC respectively. While, 35% decrease due to aging in OEx as compared with YEx. The activity of CAT was also markedly reduced by 34% and 65% in YEx and OEx as comparison with YC and OC respectively. The CAT reduced by 57% in OEx when compared with YEx. The glutathione containing enzyme GPx and GR were found to be significantly reduced by 22% and 17% in YEx, while 39% and 21% reduction in OEx as compared to their respective controls. Due to aging, GPx and GR were reduced by 34% and 29% in OEx as comparison with YEx.

Citation: Tripathi S, Chauhan DS, Singh VP, Josh DK, Choudary M (2015) Age Associated Fluoride Induced Ultra Structural and Biochemical Changes in Rat Brain. MOJ Toxicol 1(5): 00024. DOI: 10.15406/mojt.2015.01.00024
Figure 1: Levels of reacting oxygen species (ROS), Protein, Protein carbonyl content (PC), lipid peroxide levels (LPO) and lipofuscin (LIF) in control and fluoride treated group. The results are expressed as Mean ± SEM in six rats of each group. Superscripts relate significant (p< 0.05) comparison with YC (a), YEx (b) and OC (c).
Figure 2: Activity of superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GSHPx) and Glutathione reductase (GR) in control and fluoride treated group. The results are expressed as Mean ± SEM in six rat of each group. Superscripts relate significant (p< 0.05) comparison with YC (a), YEx (b) and OC (c).

Figure 3: Levels of reduced glutathione (GSH), oxidized glutathione (GSSG) and their ratio (GSH/GSSG) in control and fluoride treated group. The results are expressed as Mean ± SEM in six rat of each group. Superscripts relate significant (p< 0.05) comparison with YC (a), YEx (b) and OC (c).
The concentration of GSH, GSSG and their ratio are presented in Figure 3. The concentration of GSH was significantly reduced by 28% and 43% as compared to YC and OC respectively. The GSSG was reduced by 32% in OEx in comparison to YEx. On the other hand, the concentration of GSSG was found to markedly elevate by 63% and 34% in YEx and OEx when compared with YC and OC respectively. OEx exhibited 30% increment of GSSG as compared with the YEx. The GSH/GSSG ratio were markedly decreased by 56% in YEx and 58% in OEx when compared with their age matched controls respectively, while ratio was reduced by 48% in OEx as comparison with the YEx.

In the present study, we also found reduced a GSH/GSSG ratio in fluoride young and old rats (Figure 3). GSH/GSSG ratio determines the relative amount of reduced or working glutathione (GSH) compared to the oxidized glutathione (GSSG). A larger ratio reflects a more efficient glutathione redox system, because GSH is normally maintained in a highly reduced state of via NADPH dependent enzymes, specifically GR, while, GPx has protects from the H2O2 which produced during the metabolism of superoxide radicals [7].

Our electron microscopic examination of the hippocampal neuron showed significant deposition of the lipofuscin granules with advancing age. Electron microscopy of both the young and aged rat (Figure 4A-D) brains revealed that neurons of the hippocampal region exhibited occasional small, round to irregular lipofuscin granules mingled with mitochondrial profiles in the immediate vicinity of the nuclei. The aged control rats had mostly irregular lipofuscin and in the fluoride treated aged rats (Figure 4C & 4D). Moreover, the number and size of granules remarkably increased in fluoride exposed aged rats. Increasing intra-neuronal accumulation of lipofuscin is the most consistent cytological change, which has been correlated with fluoride neurotoxicity and ageing. It may be noted here that clustered

Citation: Tripathi S, Chauhan DS, Singh VP, Joshi DK, Choudhary M (2015) Age Associated Fluoride Induced Ultra Structural and Biochemical Changes in Rat Brain. MOJ Toxicol 1(5): 00024. DOI: 10.15406/moj.t.2015.01.00024
lipofuscin granules were also found in the fluoride treated aged rats. Although, the increased content of lipofuscin in fluoride exposed young rats may be the hallmark of natural aging (Figure 4). Furthermore, changes observed in fluoride exposed young rats resembled those of old control animals which indicate that fluoride is responsible for premature aging of animals. Lipofuscin in itself is a cytotoxic agent which may induce a variety of ultrastructural changes, like cell vacuolization, mitochondrial swelling and demyelination in brain tissue [28]. Hence elevated fluoride content along with high neuro lipofuscin levels may possibly play a role in the development of neurodegenerative disorders.

Conclusion

In the present study, aged rats were more prone to fluoride induced lipid peroxidation, increased lipofuscinogenesis and reduced antioxidant level. The biochemical and ultrastructural changes observed in fluoride treated young rats were comparable to those of untreated old control rats. Therefore, fluoride may play a role in the premature aging of animals. However, further in depth studies in other models are required to unravel the role of fluoride in the pathophysiology of aging.

Acknowledgement

Author wish to thank NIMS University for providing infrastructure for the research.

References

1. Reddy GR, Khandare AL, Reddy PY, Rao GS, Balakrishna N, et al. (2003) Antioxidant Defense System and Lipid Peroxidation in Patients with Skeletla Fluorosis and in Fluoride-Intoxicated Rabbits. Toxicol Sci 72(2): 363-368.
2. Fluoride in Drinking-water (2004) Background document for development of WHO Guidelines for Drinking-water Quality.
3. Luo DD, Guan ZZ, Liu YJ, Liu YF, Zhang KL, et al. (2013) The influence of chronic fluorirosis on mitochondrial dynamics morphology and distribution in cortical neurons of the rat brain. Arch Toxicol 87(3): 449-457.
4. DemBsten PK, Thariani H (1992) Biological Mechanisms of Fluorosis and Level and Timing of Systemic Exposure to Fluoride with Respect to Fluorosis. J DENT RES 71(5): 1238-1243.
5. Sarkar C, Pal S, Das N, Dinda RJ (2014) Ameliorative effects of oleic acid on induced metabolic and oxidative dysfunctions in rat brain: Experimental and biochemical studies. Food Chem Toxicol 66: 224-236.
6. Oteiza PL, Koen CL, Han B, Golub MS (1993) A luminum accumulation and neurotoxicity in swiss-webster mice after long-term dietary exposure to aluminum and citrate. Metabolism 42(10): 1296-1300.
7. Tripathi S, Mahdi AA, Navah A, Chander R, Hasan M, et al. (2009) Influence of age on aluminum induced lipid peroxidation and neurolipofuscin in frontal cortex of rat brain: A behavioral, biochemical and ultrastructural study. Brain Research 1253: 107-116.
8. Atmaca N, Atmaca HT, Kancil A, Antepoluglu T (2014) Protective effect of resveratrol on sodium fluoride-induced oxidative stress, hepatotoxicity and neurotoxicity in rats. Food Chem Toxicol 70: 191-197.
9. Montoliu C, Valles S, Renau-Piqueras J, Guerri C (1994) Ethanol-induced oxygen radical formation and lipid peroxidation in rat brain: effect of chronic alcohol consumption. J Neurochem 63(5): 1855-1862.
10. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193(1): 265-275.
11. Liu R, Liu IY, Thompson RF, Doctrow SR, Malfroy B, et al. (2003) Reversal of age related learning and brain oxidative stress in mice with superoxide dismutase/catalase mimetics. Proc Natl Acad Sci USA 100(14): 8526-8531.
12. Okawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissue by thio-barbituric acid reaction. Anal Biochem 95(2): 351-358.
13. Haldebrandt AG, Roots I (1975) Reduced nicotinamide adenine dinucleotide phosphate (NADPH) dependent formation and breakdown of hydrogen peroxide during mixed function oxidation reaction in liver microsome. Arch Biochem Biophys 171(2): 385-397.
14. Racknagel RD, Ghosal AK (1966) Quantitative estimation of peroxidase degeneration of rat liver microsomal and mitochondrial lipids alters carbon tetrachloride poisoning. Exp Mol Pathol 5(5): 413-426.
15. Sohal RS, Donato H (1979) Effect of experimental prolongation of life span on lipofuscin content and lysosomal enzyme activity in the brain of the housefly, Musca domestica. J Gerontol 34(4): 489-496.
16. McCord JM, Fridovich I (1969) SOD enzyme function for erythrocytes in man. J Biol Chem 244(22): 6049-6055.
17. Aebi H (1974) Catalase. In: Bergmeyer HU (Ed.), Methods of Enzymatic Analysis. II: New York: Academic Press Inc, pp. 673-684.
18. Paglia DE, Valentine WN (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. Lab Clin Med 70(1): 158-169.
19. Hazelton GA, Lang CA (1985) Glutathione peroxidase and reductase activities in the aging mouse. Mech Ageing Dev 29(1): 71-81.
20. Ellman GL (1959) Tissue sulfydryl groups. Arch Biochem Biophysics 82(1): 70-77.
21. Folbergrova J, Rehncrona S, Siesj BK (1979) Oxidized and reduced glutathione in the rat brain under normoxic and hypoxic conditions. J Neurochem 32(6): 1621-1627.
22. Sauerheber R (2013) Physiologic Conditions Affect Toxicity of Ingested Industrial Fluoride. Journal of Environmental and Public Health 2013: 439490.
23. Jesberger JA, Richardson JS (1991) Oxygen free radicals and brain dysfunction. Int J Neurosci 57(1-2): 1-17.
24. Fatma EA, Mohamed O El-Badry, Hassan Dina AA, Elraouf AA (2012) Role of Vitamin E in Combination with Methionine and L-carnosine Against Sodium Fluoride-Induced Hematological, Biochemical, DNA Damage, Histological and Immunohistochemical Changes in Pancreas of Albino Rats. Life Science Journal 9(2): 1260-1275.
25. Suh JH, Moreau R, Heath SH, Hagen TM (2005) Dietary supplementation with (R)-α-lipoic acid reverses the age-related accumulation of iron and depletion of antioxidants in the rat cerebral cortex. Redox Report 10(1): 52-60.
26. Mates JM (2000) Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. Toxicology 153(1-3): 83-104.

27. Bains JS, Shaw CA (1997) Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death.

28. Saad El-Dien HM, El Gamal DA, Mubarak HA, Saleh SM (2010) Effect of Fluoride on Rat Cerebellar Cortex: Light and Electron Microscopic Studies. Egypt J Histol 33(2): 245-256.

Citation: Tripathi S, Chauhan DS, Singh VP, Joshi DK, Choudhary M (2015) Age Associated Fluoride Induced Ultra Structural and Biochemical Changes in Rat Brain. MOJ Toxicol 1(5): 00024. DOI: 10.15406/mojt.2015.01.00024