DOSE-DEPENDENT EFFECTS OF FLUORIDE ON NEUROCHEMICAL MILIEU IN THE HIPPOCAMPUS AND NEOCORTEX OF RAT BRAIN

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SUMMARY: Effects of fluoride (F) on lipid peroxidation and enzyme activity levels in the hippocampus and neocortex were studied in 6- to 7-week-old female Wistar rats in five groups of six administered intraperitoneal doses of NaF in physiological saline over the range of 0, 1, 5, 10, and 20 mg NaF/kg bw/day for 14 days. Body weight and brain index decreased significantly ($p<0.05$) as F levels increased in the hippocampus and neocortex. Activities of the free radical enzymes superoxide dismutase (SOD), glutathione S-transferase (GST) and catalase (CAT) likewise decreased significantly ($p<0.05$), whereas the level of lipid peroxidation (LPO) and the activities of glutathione peroxidase (GPX) and xanthine oxidase (XOD) increased compared with those of the control group. The enzymes of secondary signaling, protein kinase C (PKC) and neuronal nitric oxide synthase (nNOS), also increased compared with the control. Dopamine, serotonin, 5-hydroxyindoleacetic acid and homovanillic acid levels likewise increased, whereas norepinephrine and epinephrine levels decreased. The NaF administered groups showed dose-dependent responses with more significant effects in the two fhigher dosage groups. Although NaF treatment produced significant neurochemical alterations in both the hippocampus and neocortex, there was not much difference in the degree of effects in the two organs.

Keywords: Free radical enzymes; Hippocampus; Neocortex; Neurochemical milieu; Neuronal nitric oxide synthase; Neurotransmitters; Protein kinase C; Rat brain and fluoride.

INTRODUCTION

Fluoride (F), as F^- in bound form, is known to cross the blood brain barrier and accumulate in the brain of rats exposed to chronic high F levels.[1-3] Some of the neurotoxic effects of F in rats include DNA damage, oxidative stress, decrease in the nicotinic acetylcholine receptors, and histopathological changes in brain cells of rat with decreased learning and memory ability.[4-9] The hippocampal sub-regions of mice treated with NaF show degenerated nerve cell bodies in the cornu ammonis (CA3, CA4) and dentate gyrus areas of the brain.[10] Except for homovanillic acid (HVA) in the hypothalamus, Tsunoda et al.[11] found no significant difference in levels of the neurotransmitters dopamine, dihydroxyphenylacetic acid (DOPAC), norepinephrine, serotonin, 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in different regions of the brain of mice treated with F. On the other hand, decreased activities of mammalian neurotransmitter enzymes, acetylcholine esterase (AchE) and butyrylcholine esterase (BchE), as well as weakened antioxidant systems and altered nitric oxide synthase (NOS) activity, have been recorded in the brain of rats exposed to high F.[12-14]

Because there is limited information on levels of neurotransmitters and signaling molecules, particularly involving protein kinase C and neuronal NOS, the present study was undertaken to determine the effects of variable doses of F on these

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parameters and F accumulation in the hippocampus and neocortex of rats. Our plan was to correlate these effects on activities of enzymes involved in free radical metabolism with activities of the secondary signaling enzymes protein kinase C and neuronal NOS along with levels of the above-mentioned neurotransmitters including dopamine, norepinephrine, epinephrine, 5-HIAA, HVA, and serotonin.

MATERIALS AND METHODS

Adult female Wistar rats about 6- to 7-weeks old, weighing 100–120 g, were obtained from the National Center for Laboratory Animal Sciences of the National Institute of Nutrition, Hyderabad, India. The animals were housed in polypropylene cages at 25±2ºC on a 12 hr light/dark cycle and were maintained on a standard rat pellet diet (Hindustan Lever Ltd, Lipton India, Bangalore) with water supplied *ad libitum* until autopsy. Rats were divided into 5 groups of 6 each. Each rat was administered intraperitoneally 1 mL of solution containing required quantities of NaF/100 g body weight each day for 14 days. Group I served as controls that were treated with 1 mL of mammalian physiological saline, group II received 1 mg NaF/kg bw, group III 5 mg NaF/kg bw, group IV 10 mg NaF/kg bw, and group V 20 mg NaF/kg bw. (These doses are well below the LD_{50} for rodents, which is reported to be 51.6 mg F/kg bw/day).[15] The NaF solutions were prepared fresh in glass with distilled water prior to use. After 14 days the rats were sacrificed by decapitation. The brains were rapidly removed and dissected out, blotted free of blood. The hippocampus and neocortex were separated, and transferred to small aluminum foil cups at ice temperature, and processed immediately as per the requirement for each parameter to be studied.

Body weight and organo-somatic index: The body weight of each animal was noted before treatment and also on day 14 before decapitation. After the rats were sacrificed the brain weight of each animal was also recorded. From these values the organo-somatic index (OSI) was calculated by the following formula:

$$\text{Organo-somatic index} = \frac{\text{weight (g) of the brain}}{\text{total body weight (g)}} \times 100$$

Estimation of fluoride: F levels in the hippocampus and neocortex of control and treated rats were determined by the method of Birkel[16] with required modifications and are expressed as µg F/g dry tissue. In this method the hippocampus and neocortex were each pooled, homogenized, and dried for 24 hr at 105ºC. In a closed compartment, a weighed 200 mg dry sample was dissolved in 2 mL of a 1:1 mixture of 11.6 M perchloric acid and 14.3 M nitric acid and neutralized with citrate buffer to a pH 5.5 with a mixture of 7.8 M sodium hydroxide and 1.0 M trisodium citrate. The resulting solution sample thus obtained was used after appropriate dilutions for recording the F content with an Orion R 94-09 electrode.

Assay of free radical enzymes: Xanthine oxidase (XOD) activity was assayed by the method described by Govindappa et al.[17] expressed as micromoles of formazan formed/mg protein/hr. Catalase (CAT) activity was measured by the

method of Chance et al.[18] and calculated as Units/mg protein. Superoxide dismutase (SOD) activity was assayed according to the method described by Marklund et al.[19] and expressed as units/mg protein/min. Glutathione transferase (GST) activity was estimated by using the substrate, 1-chloro-2,4-dinitrobenzene (CDNB) by the method of Habig et al.[20] and the activity expressed as nanomoles of GS-CDNB formed/mg protein/minute. The lipid peroxidation (LPO) level as malondialdehyde (MDA) was estimated by the method of Bhuyan et al.[21] and expressed as micromoles of MDA/g wet wt of tissue. Glutathione peroxidase (GPX) activity was measured by the method of Martinez et al.[22] and the activity expressed as nanomoles of NADPH oxidized/mg protein/min.

Estimation of neurotransmitters: The levels of catecholamines and serotonin were determined according to the method of Kari et al.[23] 5-Hydroxyindoleacetic acid (5-HIA) and homovanillic acid (HVA) were estimated by the method of Haubrich et al.[24] using a JASCO FP-750 spectrofluorimeter. The amine content of each tissue sample was expressed as micrograms of amine per gram wet weight of tissue.

Assay for protein kinase C: Assay for PKC was performed using both protamine sulfate and histone III-S as substrates. Protamine sulfate phosphorylation was measured in duplicates (final volume 100 µL) containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 80 µg protamine sulphate, 12.5 µM (γ-^{32}P) – ATP (2 mCi/µM), 4 µg leupeptin. The reaction was initiated by adding PKC at 30ºC and incubating for 10 min. Incorporated radioactivity was determined by histone III-S phosphorylation by the method of Singh et al.[25] using a Beckman LS 1807 liquid scintillation counter. Results are expressed as picomoles of ^{32}P incorporated/min/mg protein.

Nitric oxide synthase estimation: NOS activity in control and experimental samples was determined by enzymatic conversion of [^3H] arginine to [^3H] citrulline as described by Bredt et al.,[26] was quantified by measuring the radioactivity of the flow through fractions containing [^3H] citrulline, and is expressed as picomoles of citrulline/mg protein/min. The protein content in the soluble fractions was determined by the folin-phenol reaction.[27]

Statistical analysis: Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparison test with significance set at $p<0.05$. The analytical data are presented in the tables as Mean±SD.

RESULTS

The animals in groups II–V exposed to NaF for 14 days showed a significant decrease ($p<0.05$) in body weight in a dosage-dependent manner compared with the group I control animals (Figure 1). The organo-somatic brain index value was also significantly reduced ($p<0.05$) in the NaF-exposed groups III, IV, and V (Figure 2). The F content in the hippocampus and neocortex in the exposed rats was significantly higher ($p<0.05$) than in the control group in a dose dependent manner (Figure 3). As seen in Figure 4, the protein kinase C activity increased significantly ($p<0.05$) in the hippocampus as well as the neocortex of groups IV

and V compared with the control animals. Similarly, the neuronal NOS activity in the hippocampus and neocortex of groups IV and V was significantly increased ($p<0.05$) compared with the controls (Figure 5).

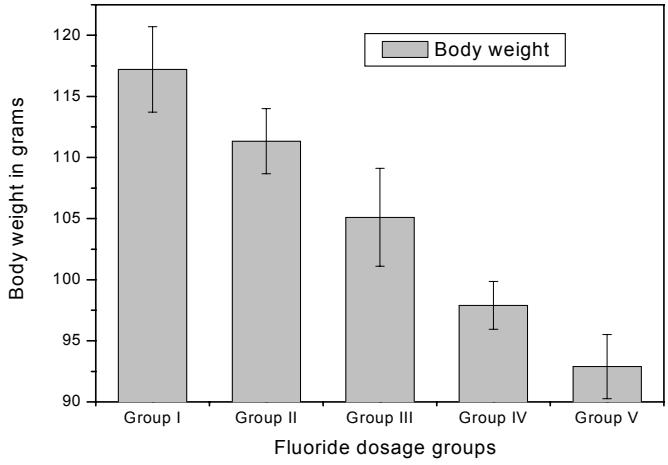

Figure 1. Effect of NaF after 14 days on mean body weight of rats. Values are Mean±SD of six animals per group. The values of multiple comparison test were significant ($p<0.05$) among groups I, II, III, IV, and V.

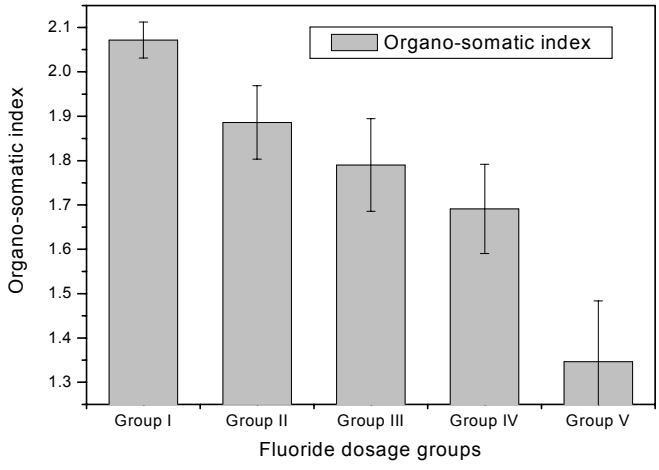

Figure 2. Effect of NaF after 14 days on organo-somatic index of rat brain. The values are Mean±SD of six animals per group. The values of multiple comparison test were significant ($p<0.05$) among groups I, II, III, IV, and V.

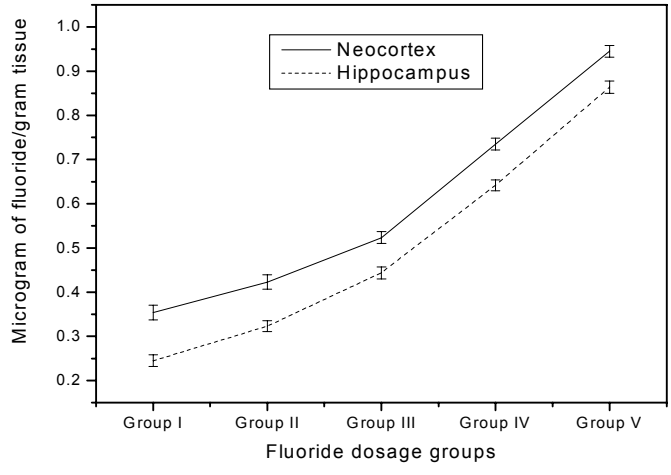

Figure 3. Fluoride levels after 14 days in hippocampus and neocortex of brain in control and NaF-treated rats. Values are expressed as Mean±SD of six animals per group. The values of multiple comparison test were significant ($p<0.05$) among groups I, II, III, IV, and V.

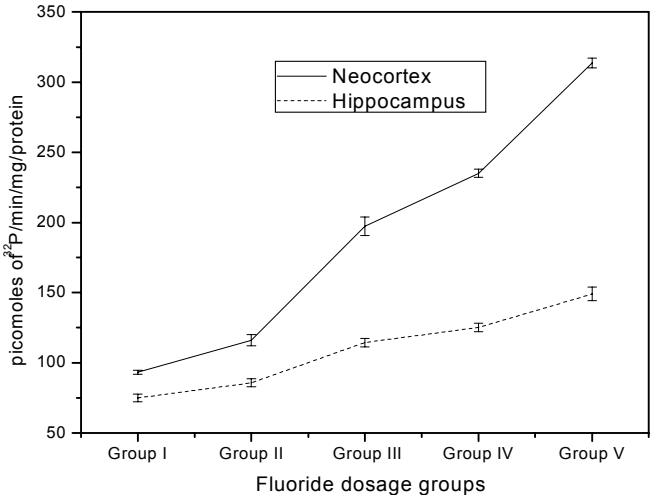

Figure 4. Effect of NaF after 14 days on protein kinase C (PKC) in hippocampus and neocortex of brain in control and NaF-treated rats. PKC values are Mean±SD of six animals per group. The values of multiple comparison tests were significant ($p<0.05$) among groups I, II, III, IV and V.

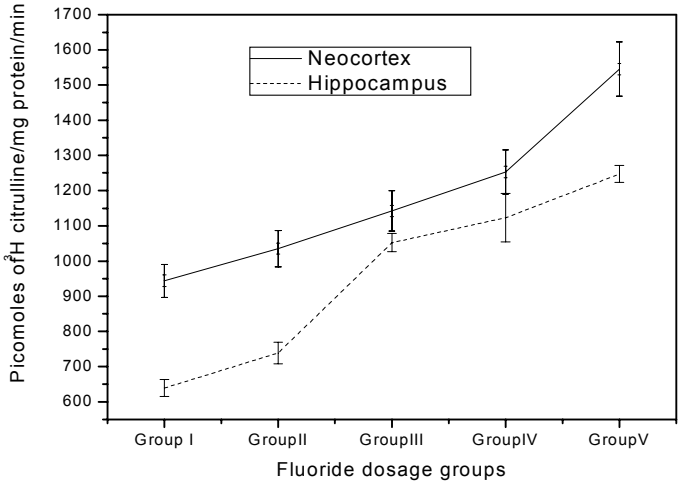

Figure 5. Effect of F after 14 days on neuronal NOS in hippocampus and neocortex of brain in control and NaF-treated rats. nNOS values are Mean±SD of six animals per group. The values of multiple comparison test were significant ($p<0.05$) among groups I, II, III, IV, and V.

As seen in Table 1 the activities of SOD, CAT, and GST enzymes in the hippocampus and neocortex of all four NaF-exposed groups were significantly decreased compared with the controls ($p<0.05$). The activities of XOD and GPX and the level of LPO in the NaF groups were increased significantly ($p<0.05$) in a dosage-dependent manner in comparison with controls. Table 2 shows the significant increase ($p<0.05$) in the levels of dopamine, serotonin, 5-hydroxyindoleacetic acid and homovanillic acid in the hippocampus and neocortex of the NaF treated rats compared with the controls. On the other hand, norepinephrine and epinephrine levels were decreased significantly ($p<0.05$) in the hippocampus and neocortex of the NaF treated rats compared with the controls.

Table 1. Free radical enzymes in hippocampus and neocortex of brain in control and NaF treated rats[a]

Free radical enzymes Mean±SD	Group I (Control)	Group II	Group III	Group IV	Group V
			Neocortex		
Catalase	5.22±0.10	4.73±0.08	4.12±0.10	3.42±0.08	2.88±0.10
GPX	4.06±0.13	4.92±0.10	5.29±0.10	5.72±0.10	6.11±0.13
GST	8.33±0.09	7.69±0.11	7.17±0.10	6.83±0.09	6.10±0.12
LPO(MDA)	4.16±0.11	4.79±0.11	5.18±0.12	5.73±0.09	6.03±0.15
SOD	5.79±0.11	5.18±0.11	4.73±0.10	3.76±0.13	3.30±0.12
XOD	1.43±0.09	2.04±0.14	2.31±0.08	2.82±0.11	3.13±0.11
			Hippocampus		
Catalase	3.73±0.09	3.12±0.12	2.74±0.11	2.17±0.10	1.73±0.12
GPX	2.47±0.42	2.81±0.09	3.23±0.10	3.85±0.12	4.29±0.10
GST	6.73±0.10	5.80±0.10	5.22±0.10	4.83±0.09	4.33±0.09
LPO(MDA)	2.26±0.11	2.85±0.11	3.10±0.12	3.32±0.09	3.69±0.10
SOD	3.37±0.09	2.81±0.09	2.25±0.11	1.80±0.12	0.91±0.10
XOD	0.21±0.08	0.78±0.11	1.05±0.13	1.33±0.09	1.73±0.09

[a]The enzyme activities expressed as: Lipid peroxidation (LPO) (MDA-malondialdehyde) (micromoles of MDA/gm wet wt of tissue), xanthine oxidase (XOD) (micromole of formazan formed/mg protein/hr), superoxide dismutase (SOD) (Units/mg protein/minute), Catalase (Units/mg protein/minute), glutathione-s-transferase (GST) (micromoles of thioether formed/mg protein/min), glutathione peroxidase (GPX) (micromoles of NADPH oxidized/mg protein/min). Values are Mean±SD of six animals per group. Statistical analysis was carried out using one way ANOVA followed by Tukey's multiple comparison test and significance set at $p<0.05$. The values of multiple comparison test were significant ($p<0.05$) among group I, II, III, IV and V.

DISCUSSION

The significant decrease in body weight and organo-somatic brain index of NaF-treated rats in this study is in agreement with earlier reports.[2,28-29] Accumulation of F in brain and in soft tissues has also been reported.[2,30-31] Here we found the proportional increase of F relative to body weight was higher in the hippocampus than in the neocortex.

Under normal conditions the level of free radical production and elimination are in a dynamic balance. However, under conditions of stress, free radicals are pathogenic factors. The present study also revealed alterations in the levels of antioxidants in the hippocampus and neocortex as a response to high F intake. The decrease in antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione transferase (GST), accompanied with an increase in the pro-oxidative markers lipid peroxidation (LPO) and xanthine oxidase (XOD) in the NaF treated rats is suggestive of oxyradical release. Previous studies carried out on mice treated with NaF (20 mg/kg bw) for 14 days revealed decreased SOD, CAT, and GST activities in brain and gastrocnemius muscle.[2] Other studies have also

recorded increased LPO levels in the erythrocytes of fluorotic humans, in brain, liver, kidney and ovary of experimental animals.[29,32-36] The activities of GPX in the brain markedly increased in rats exposed to 30 ppm and 100 ppm F in their drinking water.[29] The present results suggest that F enhances oxidative stress in the brain, thereby disturbing the antioxidant defense, in agreement with earlier studies.[12-13,29,37-38] Increased oxidative stress could therefore be one of the mediating factors in the pathogenesis of F toxicity in the brain as demonstrated here with a dose-dependent effect of NaF on hippocampal oxidative stress.

Table 2. Neurotransmitter levels in hippocampus and neocortex of brain in control and NaF treated rats with variable doses of NaF[a]

Neurotrans-mitters Mean±SD	Group I (Control)	Group II	Group III	Group IV	Group V
			Neocortex		
Dopamine	0.52±0.08	1.10±0.10	1.72±0.10	2.17±0.09	2.90±0.11
NE	0.94±0.08	0.68±0.08	0.52±0.09	0.38±0.09	0.11±0.07
Epinephrine	2.59±0.12	2.23±0.08	1.93±0.08	1.53±0.09	0.82±0.09
5-HIAA	2.32±0.11	2.82±0.10	3.22±0.08	3.69±0.11	4.05±0.12
HVA	0.31±0.10	1.31±0.09	1.74±0.11	2.18±0.11	2.84±0.13
Serotonin	1.20±0.12	1.82±0.09	2.26±0.08	2.78±0.11	3.31±0.08
			Hippocampus		
Dopamine	0.12±0.09	0.78±0.10	1.11±0.09	1.53±0.08	1.89±0.12
NE	1.91±0.10	1.57±0.07	1.07±0.09	0.62±0.09	0.25±0.07
Epinephrine	1.92±0.10	1.62±0.08	1.35±0.10	0.68±0.38	0.49±0.10
5-HIAA	1.48±0.08	1.91±0.09	2.24±0.11	2.74±0.10	2.97±0.10
HVA	0.16±0.10	0.42±0.08	0.92±0.08	1.11±0.10	1.29±0.10
Serotonin	0.33±0.08	0.92±0.09	1.25±0.11	1.81±0.11	2.06±0.12

[a]All the neurotransmitter levels (dopamine, norepinephrine (NE), epinephrine, 5-hydroxy-indoleacetic acid (5-HIAA), homovanillic acid (HVA), and serotonin) are expressed as microgram per gram wet weight. Values are Mean±SD of six animals per group. Statistical analysis was carried out using one way ANOVA followed by Tukey's multiple comparison test and significance set at $p<0.05$. The values of multiple comparison test were significant ($p<0.05$) among group I, II, III, IV and V.

The protein kinase C (PKC) signaling pathway has been implicated in the modulation of motor behavior as well as learning and memory.[39] Earlier reports showed increased PKC activation in rat hippocampus in response to long-term low levels of heavy metal exposure.[40] Exposure to NaF leads to activation of polyphosphoinositide phospholipase C in rat cerebral cortex, resulting in an increase in intracellular free calcium and activation of PKC through the secondary messengers inositol triphosphate (IP_3) and diacylglycerol, respectively.[41-42] The observed increase of PKC activity in hippocampus and neocortex of F treated animals may result in effects on cognitive processes as demonstrated in a previous study.[39]

The present results also corroborate the studies of earlier reports showing higher levels of inducible nitric oxide synthase (iNOS) in the brain of rats exposed to NaF.[14] NOS is activated by calcium. Various oxidants such as superoxide,

hydrogen peroxide, hydroxyl radicals, and lipid peroxides increase intracellular calcium and stimulate tyrosine as well as serine/threonine phosphorylation, suggesting a possible physiological role of oxidants in regulating Ca^{2+} signaling.[43] NaF activates the phosphoinositide cycle to increase intracellular levels of calcium, secondary to IP_3 production and activation of NOS through calmodulin.

Exposure to F also results in decreased acetylcholine esterase (AchE) in the brains of rats and mice.[7,12-13,44] The content of hypothalamic serotonin (5-HT) is reported to increase during subacute fluorosis and decrease during chronic fluorosis.[45] Previous reports of effects of F on dopamine (DA), dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) metabolism suggest paradoxical dose-response effects of F on the central nervous system.[11,46-48] A substantial increase in DA levels, as evidenced by an increase in its acid metabolite HVA suggests a disturbance in catecholamine metabolism following chronic F administration possibly influencing the level of L-tyrosine hydroxylase. Decreased levels of norepinephrine (NE) and epinephrine may be due to the enhanced release of catechol-O-methyl transferase caused by increased neuronal activity. The present results also showed that biogenic amine levels in rat brain were altered in a dose-dependent manner by the administration of F. The observed changes in the biogenic amine levels may be the effect of F on the rates of turnover, which, in turn, play a role in the maintenance of steady state levels of biogenic amines in these tissues or they could be secondary to glutamate induced (glutamanergic) influence on other neurotransmittters.

The present study, as far as we are aware, is the first to report altered levels of PKC, nNOS, and neurotransmitter levels in the hippocampus and neocortex of rat brain following NaF treatment. One mechanism explaining these F-related findings is activation of the excitotoxic cascade.[49] The most abundant neurotransmitter in the brain, and especially in the hippocampus, is glutamate. Yet excess activation of the glutamate receptors is known to be neurotoxic by a process known as excitotoxicity. The ionotropic glutamate receptors (NMDA-N-methyl-D-aspartate, AMPA-alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate) would most likely be involved because of the increase in intraneuronal calcium. Essential to the excitotoxic cascade is the up-regulation of PKC and NOS, again observed in this study with hippocampal exposure to NaF.

It is known that blocking PKC and nNOS significantly attenuates excitotoxicity. It is also known that excitotoxicity, in addition to generating abundant ROS and LPOs, can itself be initiated by free radicals and lipid peroxidation products. A number of studies have clearly shown that F increases brain ROS and LPO activity.[49] By inhibiting antioxidant enzymes, F magnifies the excitotoxic response, since free radicals and LPO act as a positive feedback loop to excitotoxicity. Likewise, F has been shown to inhibit mitochondrial enzymes, which have also been shown to greatly magnify excitotoxicity.[49] Finally, gliosis or overgrowth of astrocytes, has been described in a number of cases of F-induced neurotoxicity in the brain.[49] Activation of brain microglia triggers excitotoxicity

and has been shown to produce chronic neurodegeneration. However, the exact mechanisms of these changes require further study.

In conclusion, this study found that NaF administered through intraperitoneal injection to rats for 14 days at increasing dosages caused dose-dependent neuroexcitotoxicity, triggering lipid peroxidation and free radical generated oxidative stress, and affecting signaling processes including altered levels of PKC, nNOS, and neurotransmitters. These alterations were significant and similar in both the hippocampus and neocortex.

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