Effects of fluoride and ethanol administration on lipid peroxidation systems in rat brain

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Exposure to fluoride and excessive ethanol consumption has been identified as a serious public health problem in many parts of the world, including India. Thus, the effect of co-exposure to fluoride and ethanol for 3-6 weeks was studied on lipid peroxidation (LPO) and oxidative stress related parameters in the rat brain. After 3 weeks, co-treated animals showed 95% increase in LPO levels compared to control. However, the levels of reduced glutathione, total and protein thiols were decreased. These changes were accompanied by a decrease in the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase. Rats exposed to fluoride together with ethanol for 6 weeks resulted in 130% increase in LPO and decrease in the reduced glutathione levels. The activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase were reduced under these conditions. Brain histology revealed excessive lymphocytes, edema and spongeosis in the cortical region after six weeks of fluoride and ethanol treatment. These results suggest that exposure to fluoride together with ethanol enhances lipid peroxidation by affecting antioxidant defence systems in the rat brain.

Keywords: Co-exposure, Ethanol, Fluoride, Lipid peroxidation, Rat brain

Fluorosis, as a consequence of exposure to high amounts of the fluoride1 is a serious public health problem in many parts of the world. Human exposure to fluoride occurs mainly through drinking water and World Health Organization has established 1.5 mg fluoride/L drinking water as the safe limit2. However, in many parts of the world, as in India, fluoride concentration in the groundwater is as high as 27 mg/day2. The detrimental effects of fluoride on skeletal tissues are characterized by dental mottling, crippling deformities, osteoporosis and osteosclerosis3. Exposure to fluoride also affects various soft tissues including brain3. Fluoride can pass through blood brain barrier4 and its accumulation in brain may facilitate the formation of reactive oxygen species (ROS) which are capable of inducing oxidative damage to vital cell components5. In healthy subjects, oxidative damage is largely prevented by a very complex antioxidant system, consisting of several enzymatic and non-enzymatic components, which act cooperatively to provide better protection against free radical attack. The extent to which fluoride can cause oxidative damage is influenced by a number of factors, such as duration of exposure, age, sex, nutrition and simultaneous exposures to other xenobiotics. Recently, increased attention has been paid to interactions of fluoride with other xenobiotics6. However, there are negligible scientific reports about fluoride and ethanol interactions following their combined exposures. Interactions between fluoride and ethanol are important, since both pose a risk to human and animal health. Combined exposures to these toxicants may be commonly noticed in alcoholics who happen to be the inhabitants of high fluoride endemic areas7. Inkielewicz et al.8 have reported the induction of lipid peroxidation in liver, kidney, brain and serum of male rats following co-exposures to fluoride and ethanol for four weeks. A marked elevation in intestinal lipid peroxidation status in rats exposed to fluoride together with ethanol has been reported9. This communication reports the combined effects of fluoride and ethanol administration on lipid peroxidation and oxidative stress related parameters in rat brain.

Materials and Methods

Animals—Six month old female Sprague Dawley rats weighing 190-200 g were procured from the Central Animals House of Panjab University, Chandigarh, India. They were housed in propylene cages and maintained at 22 ± 3 °C, on a 12:12 h light dark cycle and a minimum 40% RH. Standard pellet diet (Ashirwad Industries, India) and water were given ad libitum. The animals

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were acclimatized to the laboratory conditions for 1 week before initiating the experiments. The experimental protocol was approved by the ethical committee of the Institute on the use of laboratory animals. Experiments on the animals were performed in accordance with the guidelines for the use of laboratory animals.

**Experimental protocol**—Rats (32) were randomly distributed into 4 groups of 8 animals each. Untreated animals served as control. Treated groups received intragastric treatment of sodium fluoride (25 mg/kg) as described by Maurer *et al.*, ethanol (30%; 1 mL/kg) comparable to the daily human consumption, and sodium fluoride+30% ethanol (a combination of individual doses) for 6 weeks. The body weight, food and water intakes were recorded during the experimental period. Four overnight fasted animals from each group were sacrificed by decapitation under light ether anesthesia after 3 or 6 weeks.

**Tissue preparation**—Brain were quickly removed and washed in ice-cold 0.9% NaCl solution, dried and weighed. A section of brain was fixed in buffered 10% formaldehyde for histological examination. From other parts, a 10% (w/v) tissue homogenate was prepared in 50 mM phosphate buffered saline (pH 7.4) using Potter-Elvehjem-type glass homogenizer. Non-enzymatic assays were performed using 2 mL of the tissue homogenate and remaining homogenate was centrifuged at 1,000×g for 10 min at 4 °C. The pellet was discarded and supernatant was re-centrifuged at 10,000×g for 30 min to obtain post mitochondrial supernatant.

**Biochemical estimations**—Lipid peroxidation (LPO), superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, reduced glutathione, total and protein thiols were quantified using standard methods. Protein was estimated by the method of Lowry *et al.* using bovine serum albumin as the standard.

**Brain morphology**—Tissue sections from different groups of rats were prepared by the method of Lillie. After fixation in 10% formaldehyde, tissues were embedded in paraffin, solid sections were cut at 5 μm thickness and stained in haematoxylin and eosin. The sections were examined under light microscope and photomicrographs were taken.

**Statistical analysis**—All the data were expressed as mean ± SD. The significance of difference among four groups was analyzed by two-way ANOVA followed by Bonferroni’s correction to multiple comparisons. Values were considered statistically significant at *P* < 0.05. The analyses were done by computer, using SPSS 14.0 software package program.

**Results**

The effect of sodium fluoride and ethanol administration, alone, and in combination for six weeks on the body weight of rats is shown in Fig. 1(a). A reduction in body weight gain was observed, when rats...
were administered ethanol alone and in combination with sodium fluoride. However, rats treated with sodium fluoride alone, revealed body weight pattern similar to the controls. The decline in body weight gain was noticed from the onset of third week of treatments and continued till remaining treatment period. After six weeks, gain in body weight was 29% in control group and 26% in sodium fluoride treated group, whereas animals treated with ethanol alone or co-treated with sodium fluoride showed 18% gain in the body weight. Water intake (Fig. 1c) in treated animals was significantly reduced, whereas food consumption (Fig. 1b) exhibited an irregular pattern, compared to the controls.

The levels of malondialdehyde (MDA), an end product of lipid peroxidation were significantly increased in sodium fluoride or ethanol treated animals (Fig. 1d). However, co-exposure to sodium fluoride and ethanol exhibited additive effect on the tissue lipid peroxidation status, resulting in 95% and 130% increase in the brain MDA levels after three and six weeks of treatment respectively.

The activities of superoxide dismutase and catalase in treated animals were significantly low after three or six weeks, when compared to control rats (Table 1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Duration</th>
<th>Superoxide dismutase activity (units/mg protein)</th>
<th>Catalase activity (millimoles H₂O₂ decomp/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3 weeks</td>
<td>9.23 ± 1.37</td>
<td>3.01 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>6 weeks</td>
<td>8.80 ± 1.27</td>
<td>2.36 ± 0.14</td>
</tr>
<tr>
<td>NaF treated</td>
<td>3 weeks</td>
<td>6.37 ± 0.17</td>
<td>2.24 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>6 weeks</td>
<td>4.10 ± 0.08</td>
<td>1.50 ± 0.12</td>
</tr>
<tr>
<td>EtOH treated</td>
<td>3 weeks</td>
<td>0.17 ± 0.08</td>
<td>0.12 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>6 weeks</td>
<td>0.08 ± 0.08</td>
<td>0.12 ± 0.08</td>
</tr>
<tr>
<td>NaF + EtOH co-treated</td>
<td>3 weeks</td>
<td>5.22 ± 0.21</td>
<td>2.48 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>6 weeks</td>
<td>4.41 ± 0.14</td>
<td>1.80 ± 0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.21 ± 0.14</td>
<td>0.15 ± 0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.14 ± 0.08</td>
<td>0.09 ± 0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.03 ± 0.01</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
</tbody>
</table>

P values: <0.05; *vs controls; †vs NaF treated; ‡vs EtOH treated

Table 1 — The effect of sodium fluoride (NaF) and ethanol (EtOH) administration on superoxide dismutase and catalase activities in the rat brain

[Values are mean ± SD from 4 observations]

There was 72 and 58% decline in the activities of superoxide dismutase and catalase respectively after six weeks sodium fluoride and ethanol co-treatment.

The observed decrease in superoxide dismutase and catalase activities was accompanied by a significant decline in the reduced glutathione, total and protein thiol content in the brain of treated animals, compared to controls (Table 2). After six weeks, reduced glutathione content in brain was decreased by 27% in sodium fluoride treated animals and by 39% in ethanol treated rats. However, administration of sodium fluoride together with ethanol for similar experimental duration showed 55% decline in the brain reduced glutathione content. There was 53% reduction in total and protein thiol levels in brain of rats co-exposed to sodium fluoride and ethanol under these conditions.

The activities of glutathione peroxidase, glutathione reductase and glutathione-s-transferase in treated animals after three or six weeks were significantly low compared to controls (Table 3). However, the observed decrease was more pronounced in animals co-exposed to sodium fluoride and ethanol. Glutathione peroxidase and glutathione reductase activities in the brain of co-treated animals were reduced by 48% after six weeks, whereas glutathione-s-transferase activity showed 52% decline under these conditions.

Brain tissue showed morphological alterations in sodium fluoride or ethanol treated animals, when compared to control rats (Fig. 2). Exposure to sodium fluoride showed excessive lymphocytes and mild spongeosis after three weeks (2b) and edema along with spongeosis after six weeks (2f) in cortical region of the brain. There were similar morphological alterations in ethanol fed rats, showing mild edema and excess of lymphocytes after three weeks (2c) and mild edema, spongeosis and separation of cells after six weeks (2g). Animals administered sodium fluoride along with ethanol showed focal necrosis in the cortical region of the brain (2d, 2h).
Table 3 — The effect of sodium fluoride (NaF) and ethanol (EtOH) administration on glutathione peroxidase, glutathione reductase and glutathione-S-transferase activities in rat brain

<table>
<thead>
<tr>
<th>Duration</th>
<th>Groups ↓</th>
<th>Control</th>
<th>NaF treated</th>
<th>EtOH treated</th>
<th>NaF + EtOH co-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 weeks</td>
<td>15.5±0.37</td>
<td>12.38± 0.27</td>
<td>10.38±0.21</td>
<td>9.51±0.37* #</td>
<td>7.66±0.08* #</td>
</tr>
<tr>
<td>6 weeks</td>
<td>14.49±0.30</td>
<td>9.17±0.16*</td>
<td>8.15±0.17*</td>
<td>7.47±0.23* #</td>
<td>5.53±0.11* #</td>
</tr>
</tbody>
</table>

Values: <0.05; * vs controls; † vs NaF treated; # vs EtOH treated

Discussion

Animal studies have shown a marked difference in xenobiotic toxicity with duration of their exposure. Thus, the effect of fluoride and ethanol administration for 3 or 6 weeks respectively was studied. The data presented herein indicate a reduction in the body weight gain of animals co-treated with sodium fluoride and ethanol as compared to the control group. This is in agreement with previous report, where similar trend was observed in rats following combined exposures to these toxicants. This may be attributed to impaired digestion, absorption, and utilization of nutrients induced by ethanol consumption and excessive breakdown of tissue proteins due to fluoride intake. Irregular feeding pattern observed is presumably due to adaptation of the animals to the fluoride or ethanol intake. However, with longer exposure the animals get familiar to the dietary regimen. The initial decrease in the feed intake is a reflection of the adaptive behaviour of the animals.

Lipid peroxidation represents excessive production of free radicals, which attack cellular biomolecules. Brain contains high concentrations of polyunsaturated fatty acids, and has high oxygen demand, which makes it prone to damage by free radicals. Present findings indicated enhanced MDA levels by fluoride or ethanol treatments to rats, suggesting enhanced lipid peroxidation. Earlier reports have also shown an increase in MDA levels following exposures to fluoride or ethanol. A marked increase in MDA levels in co-treated rats can be attributed to increased oxidative stress in the cell as a result of fluoride induced depletion of antioxidant scavenger system and generation of oxygen radicals during ethanol metabolism.

The observed decrease in the levels of enzymatic as well as non-enzymatic antioxidants in rat brain are in agreement with the earlier reports, where exposure to fluoride or ethanol is reported to cause alterations in the antioxidant defence system in various tissues including brain. However, the observed decrease in superoxide dismutase activity in co-treated rats may be attributed to direct action of fluoride on enzyme activity (competitive inhibition), or may be due to oxidative inactivation of enzyme resulting from excessive ROS production, induced by ethanol metabolism. Reduction in brain catalase activity may be due to fluoride induced inhibition of superoxide dismutase and ethanol related loss of NADPH, or excess of H₂O₂ production, together with enhanced lipid peroxidation or combination of these factors. Impaired scavenging of H₂O₂ and inability of the brain to handle high oxidative stress under these conditions may also contribute this phenomenon.

In the present study, a decrease in reduced glutathione levels upon exposure to fluoride or ethanol individually or in combination was observed. This is presumably due to enhanced utilization of reduced glutathione by glutathione peroxidase in detoxification of H₂O₂ generated by fluoride or ethanol induced oxidative stress or as a consequence of diminished glutathione reductase activity, which is crucial for maintaining reduced/oxidized ratio in the cell. A decrease in reduced glutathione content was also accompanied by a decline in total thiol levels, upon fluoride or ethanol administration, which may contribute to low reduced glutathione levels and/or could be due to reduction in protein thiols as is apparent in this study. Thus, the decreased –SH content may affect –SH/-S-S- ratio in proteins resulting in impaired functioning of enzymes affecting vital metabolic activities of the brain.

A marked decrease in the activities of glutathione peroxidase, glutathione reductase and glutathione-s-transferase was observed in animals...
Fig. 2—Morphology of rat brain exposed to sodium fluoride (NaF) and ethanol (EtOH) for three (a to d) and six (e to h) weeks: (a and e) control, (b and f) NaF treated, (c and g) EtOH treated and (d and h) NaF+EtOH co-treated. Arrows indicate the morphological changes, as explained in the text, [Magnification = 100X].
exposed to fluoride or ethanol, which may be attributed to free radical dependent inactivation of the enzymes or may be solely due to the reduced bioavailability of reduced glutathione under these conditions. Glucose-6-phosphate dehydrogenase is one of the key enzymes of pentose phosphate pathway responsible for maintaining cellular NADPH levels. The activity of this enzyme is impaired directly by fluoride ingestion and indirectly by ethanol consumption, which causes niacin deficiency. This results in diminished levels of NADPH which is required by glutathione reductase to convert oxidized glutathione into reduced glutathione. Thus, the reactive oxygen detoxifying capacity of the brain in co-treated animals is markedly reduced, leading to tissue oxidative stress. These findings indicate the intensified effects of fluoride and ethanol co-exposures in the rat brain. However, Inkielewicz et al. have observed such effects only in the kidney, but not in brain, after four weeks of fluoride and ethanol co-treatment to male rats. This could be attributed to gender specific susceptibility of rats to these toxicants. Deleterious effects of fluoride and ethanol administration were also seen in brain morphology. Fluoride treated animals showed excessive lymphocytes, edema and spongeosis in the cortical region of the brain, which is in agreement with the previous findings. Ethanol administration resulted in mild edema, excess of lymphocytes, spongeosis and separation of cells. Histological alterations were further magnified with fluoride and ethanol co-treatment, resulting in focal necrosis in the cortical region of brain.

**Conclusion**

The present findings suggest that co-administration of fluoride and ethanol elevates lipid peroxidation which affects the antioxidant status and morphology of the rat brain after 3 and 6 weeks of exposure compared to controls. The mechanism by which these toxicants damage brain is presumably mediated by the generation of free radicals, but the primary event initiating the free radical generation following fluoride and ethanol co-exposure needs further investigations.

**Acknowledgement**

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**References**


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