OXIDATIVE STRESS INDICES IN ERYTHROCYTES, LIVER, AND KIDNEYS OF FLUORIDE-EXPOSED RABBITS

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SUMMARY: Changes in oxidative stress indices in soft tissues of rabbits following exposure to fluoride (F) in their drinking water were studied. Twenty-four New Zealand white rabbits, 6–8 weeks old weighing 600–800 g, were divided into four equal groups and given drinking water containing 50, 100, 200, and 0 (control) mg NaF/L ad libitum for 90 days. The level of lipid peroxides (LPO) and activities of superoxide dismutase (SOD) and catalase (CAT) were monitored in erythrocytes before the start of the experiment (day 0) and thereafter on day 45 and day 90, and in liver and kidney tissues on day 90 after sacrifice of the animals. The LPO level increased, and SOD and CAT activities decreased by day 45 in rabbits receiving the F-enriched water. These changes were more pronounced in the two higher F groups of rabbits. Significant (P<0.05) increase in the LPO level in both liver and kidney tissues and decrease in SOD and CAT activities, particularly in kidneys, were observed. These results indicate that oxidative stress appears to play an important role in the pathogenesis of F intoxication.

Keywords: Catalase; Fluorosis in rabbits; Lipid-peroxides; New Zealand white rabbits; Oxidative-stress; Superoxide-dismutase.

INTRODUCTION

Continuous ingestion of small but toxic doses of fluoride (F) results in chronic F toxicity known as fluorosis, manifested by lesions in bones and teeth. Visceral organs like liver and kidneys are also susceptible to toxic effects of F, and pathological changes in these vital organs can occur even before overt clinical signs of F intoxication.1

Although F has been found to induce cytotoxic, genotoxic, immunotoxic, and carcinogenic effects,2,3 the pathophysiology of F intoxication has not been fully elucidated. Free radicals and oxidative stress have been implicated in the pathogenesis of several xenobiotic toxicities, but there are conflicting reports about whether free radicals are directly involved in F toxicity.4,5 Compromise in antioxidant defense and an increase in lipid peroxidation products in experimental fluorosis have been reported by several workers.6-8 On the other hand, supplementation of antioxidant vitamins like vitamin C was found to have little benefit for the prevention of skeletal fluorosis.9 In one study, no change in activity of glutathione peroxidase (GSH-Px) and the level of lipid peroxides (LPO) in pancreas was observed in F-intoxicated rats.10 In another study, no change in status of antioxidant defense systems and LPO was observed in fluorotic human patients and F-intoxicated rabbits, and further investigation was suggested to explore the possible role of oxidative stress in the pathogenesis of fluorosis.11 The

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present investigation was carried out to study the changes in oxidative stress indices in erythrocytes, liver, and kidneys of rabbits following exposure to F in their drinking water.

**MATERIALS AND METHODS**

*Animals and sample collection:* Before beginning the experiment, 24 New Zealand white male rabbits, 4 to 6 weeks old, weighing between 600 and 800 g, were maintained in individual rabbit cages on a standard ration and *ad libitum* access to tap water with a 12-hr photoperiod. During the experiment the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals), Government of India, were followed with the approval of the Institute Animal Ethical Committee (IAEC).

The animals were divided into four equal groups with six rabbits in each group. To the drinking water NaF added at the level of 0 (control), 50, 100, and 200 mg/L was provided *ad libitum* for a period of 90 days. Blood samples were collected on days 0, 45, and 90 by cardiac puncture using heparinized sterile syringes. On day 90, all the animals were sacrificed, and about 5-g samples of liver and kidneys were collected.

*Laboratory estimations:* Blood samples were centrifuged at 200 g for 10 min to separate plasma and packed erythrocytes (red blood cells: RBCs). Packed RBCs were washed three times with NSS (normal saline solution), and chilled distilled water was used to prepare 10% RBC haemolysate. The haemoglobin concentration in the haemolysate was estimated by the cyanomethaemoglobin method.\textsuperscript{12}

Small pieces (about 700–800 mg) of liver and kidneys were quickly chilled in ice-cold NSS, dried, and weighed. Chilled 1.15% Tris-Potassium chloride (KCl) buffer (pH 7.4), approximately ten times the tissue weight, was added to the sample, which was then homogenized. The homogenate was cold centrifuged at 2000 g for 10 min, and the supernatant was collected for further biochemical estimations. Total protein in supernatant was determined by the modified Biuret and Dumas method.\textsuperscript{13}

*LPO:* For these tests the reaction mixture consisted of 0.2 mL haemolysate, 1.3 mL 0.2 M Tris-0.16 M KCl buffer (pH 7.4) and 1.5 mL thiobarbituric acid reagent. It was heated in a boiling water-bath for 10 min. After cooling the mixture, 3 mL of a 3:1 v/v mixture of pyridine and n-butanol and 1 mL 1 N NaOH were added and mixed by vigorous shaking. The absorbance was read at 548 nm and LPO level was calculated\textsuperscript{14,15} For estimation of LPO in liver and kidney, 0.2 mL of the tissue homogenate was added to 0.2 mL 8.1% sodium dodecyl sulphate, 1.5 mL 20% acetic acid (pH 3.5), 1.5 mL 0.8% of thiobarbituric acid, and 0.6 mL distilled water. The mixture was heated at 90ºC for 1 hr in a water bath. After cooling, 1 mL distilled water and 5 mL of a 15:1 v/v mixture of n-butanol and pyridine was added followed by vigorous shaking. The absorbance of organic layer was measured at 532 nm to calculate the LPO level.\textsuperscript{15,16}
SOD: The assay mixture in a total volume of 3 mL consisted of 50 µL 50 mM of Tris-cacodylic acid buffer (pH 8.2), enzyme preparation (sample, 2.9 mL) after suitable dilution with distilled water and 50 µL 0.2 mM pyrogallol. The increase in absorbance was recorded at 420 nm and SOD activity was calculated.17,18

CAT: For this determination, 50 µL of diluted sample was mixed with 2.95 mL phosphate buffer-H2O2 solution. Absorbance of this mixture was read at 240 nm against reference cuvette containing mixture of 50 µL phosphate buffer and 2.95 mL phosphate buffer-H2O2 solution. Time (seconds) required for the fall in the initial absorbance by 0.05 was noted, and CAT activity was calculated.19 CAT activity in supernatant of tissue homogenate was estimated in a similar fashion after appropriate dilution with phosphate buffer.20

Statistical Analysis: The values obtained were expressed as mean ± SE. The data were statistically analyzed using analysis of variance.21

RESULTS AND DISCUSSION

In this work, increased levels of LPO and reduced activities of SOD and CAT in erythrocytes (RBCs) were observed in all F-exposed rabbits (Table 1).

### Table 1. Levels of lipid peroxide (LPO) and activities of superoxide dismutase (SOD) and catalase (CAT) activities in erythrocytes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NaF dosage</th>
<th>Day of observation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>LPO (nmol MDA/mg of Hb)</td>
<td>50 mg/L</td>
<td>3.18±0.19&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>100 mg/L</td>
<td>3.17±0.21&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>200 mg/L</td>
<td>3.12±0.19&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>3.10±0.17&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>3.78±0.33&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td>SOD (units/mg of Hb)</td>
<td>50 mg/L</td>
<td>6.35±0.44&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>100 mg/L</td>
<td>6.55±0.74&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>200 mg/L</td>
<td>5.99±0.59&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>6.55±0.82&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>4.82±0.60&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAT (units/mg of Hb)</td>
<td>50 mg/L</td>
<td>208.35±7.79&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>100 mg/L</td>
<td>205.37±11.32&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>200 mg/L</td>
<td>204.28±3.47&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>203.88±7.98&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>208.46±7.21&lt;sup&gt;aC&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values (mean±S.E.) bearing different superscripts in small letters (a,b,c) in a row and capital letters (A,B,C) in a column differ significantly (p<0.05).

On day 90, the LPO level was positively correlated (r = 0.79, p<0.01), whereas SOD and CAT activities were negatively correlated (r = -0.512, p = 0.01 and -0.409, p = 0.04) with the F exposure dosage. This clearly indicated enhanced oxidative damage of RBCs in the F-exposed animals. Alterations in oxidative stress indices have been reported both in experimental as well as natural cases of F toxicity.22-24 The RBC membrane is rich in polyunsaturated fatty acids and thus prone to oxidative damage by pro-oxidants.25 F is rapidly absorbed following oral exposure to reach systemic circulation. In blood, about 75% of F remains free in plasma and about 5% remains bound to plasma proteins. The rest of the blood F is found mainly inside RBCs or with their membrane.26 Thus, increase in LPO levels
might be due high F concentration in blood and resultant increase in oxidative damage to RBCs.

LPO levels in soft tissues were significantly (p<0.05) higher in the rabbits exposed to 100 and 200 mg NaF/L as compared non-treated control group (Table 2).

Table 2. Levels of lipid peroxides (LPO) and activities of superoxide dismutase (SOD) and catalase (CAT) in liver and kidney tissues

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tissue</th>
<th>NaF dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 mg/L</td>
<td>100 mg/L</td>
</tr>
<tr>
<td>LPO (nmol MDA/mg protein)</td>
<td>Liver</td>
<td>3.07±0.37&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>3.45±0.55&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SOD (units/mg protein)</td>
<td>Liver</td>
<td>19.18±1.71&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>30.92±3.50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAT (units/mg protein)</td>
<td>Liver</td>
<td>323.43±18.88&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>258.96±12.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values (mean±S.E.) bearing different superscripts (a,b,c) in a row differ significantly (p<0.05).

A high degree of positive correlation between LPO level and F exposure was also observed in liver and kidneys (r = 0.90, p <0.01 and 0.89, p <0.01, respectively). Negative correlation was observed between dose of F exposure and activity of SOD (r = −0.609, −0.710; p <0.01) and CAT (r = −0.722, −0.715; p <0.01) both in the liver and kidneys.

Following absorption, F is rapidly distributed to intracellular and extra-cellular space from systemic circulation. A high degree of correlation between F concentration and free radicals in soft tissues from F exposed rats has been reported. Liver is responsible for detoxification of most of the xenobiotic insult and the kidney is the major organ responsible for removal of F from body, thus making them highly vulnerable to toxic effects of F. F accumulates in high concentrations in liver and kidney tissues following multiple administration of NaF in rabbits. Histological and functional alterations in liver and kidneys following elevated F exposure have been reported by several workers. Free radical induced damage may be, at least in part, responsible for tissue damages in these vital organs observed in cases of F intoxication.

F has been implicated in oxidative stress by stimulating the production of free radicals as well as inhibiting antioxidant enzymes action. F stimulates respiratory burst and production of free radicals inside neutrophils. It also inhibits a number of enzymes including those required for cellular respiration at the level of mitochondria. Interruption of cellular respiration is accompanied with decrease in adenosine triphosphate (ATP) concentration, which in turn evokes production of hydrogen peroxide and reactive oxygen species. Decrease in activity of Cu-Zn SOD in pancreas was noted in F intoxicated rats. However, in that study no change in activity of GSH-Px and level of LPO were observed, suggesting an inhibitory action of F on enzyme activity rather than excess production of free radicals. Changes in cellular lipid composition and LPO level in kidneys were
observed in chronic F toxicity in rats. Likewise, considerable changes in membrane lipids were noted in rat liver, which were attributed to increased oxidative stress in F toxicity. Increase in LPO and alterations in antioxidant enzyme status have been reported in males with skeletal fluorosis. An inhibitory effect of F on SOD in germinating mung-bean (Vigna radiata) seedlings supports the above findings and indicates a possible role of free radicals in F toxicity. In F-exposed rats, low level of reduced glutathione and GSH-Px and CAT activities were observed along with increases in LPO in liver, kidney, brain, and blood. Beneficial effects of antioxidant supplementation in F toxicity further substantiates this hypothesis.

From the present study, it can be concluded that excess F exposure is associated with oxidative damage to RBCs, liver, and kidney tissues. Oxidative stress and free radical-induced damage may therefore play an important role in pathogenesis of F toxicity.

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