LIPID PEROXIDATION AND ANTIOXIDANT ENZYME ACTIVITY IN RATS EXPOSED TO FLUORIDE AND ETHANOL

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SUMMARY: The aim of this study was to investigate the impact of fluoride (F) and ethanol (EtOH) administered separately or together on free radical mediated parameters and on F accumulation and excretion in rats in a 4-week experiment. Thirty adult male Wistar rats were divided into five equal groups: 1. controls drinking tap water (0.3 mg F/L); 2. controls drinking tap water and for the last 14 days of the experiment dosed intragastrically with 1 mL of tap water containing 0.3 mg F/L (twice/day); 3. animals receiving 25 mg F/L in drinking water; 4. animals receiving 5 g ethanol/kg bw/day for the last 14 days of the experiment; 5. animals receiving 25 mg F/L in drinking water and 5 g ethanol/kg bw/day for the last 14 days of the experiment. In rats treated only with F a significant increase in F concentration was found in liver, kidney, brain, and serum, and the F excretion in urine increased in an exposure time-dependent manner. The activity of catalase (CAT) and the concentration of sulfhydryl (SH) groups decreased, whereas the concentration of thiobarbituric acid reactive substances (TBARS) increased in all investigated tissues. In animals treated with EtOH alone, the F content in liver and kidney, CAT activity, and TBARS concentration increased, and the concentration of SH groups decreased in all investigated tissues. In rats co-exposed to F and EtOH, the F concentration significantly increased in brain, and the CAT activity and the concentration of SH groups increased in all investigated tissues, whereas the TBARS concentration was lower (except in kidney) than in animals given only F. The results of this study indicate that both F and EtOH can induce lipid peroxidation in rats under separate or combined exposure.

Keywords: Antioxidant enzyme activity; Ethanol treatment; Fluoride in urine; Fluoride in rats; Lipid peroxidation.

INTRODUCTION

In their natural and occupational environments, humans are frequently exposed to many unsuspected chemical substances. Recently, increased attention has been paid to interactions of xenobiotics with one another or with dietary factors. It is well known that uptake, accumulation, and toxicity of many xenobiotics can be modified by dietary factors.1-6 Particularly important are interactions between xenobiotics to which exposure is quite common. Examples of such substances are fluoride (F) and ethanol (EtOH). Interactions between F and EtOH are an important problem in modern toxicology since both pose a risk to human and animal health.

Exposure to F can occur in the workplace and in the environment because F is utilized in a number of industrial practices and is a ubiquitous ingredient of drinking water, foodstuffs, and dental products.7 Likewise, widespread alcoholism is a serious problem in modern society. Excessive consumption of EtOH in the form of alcoholic beverages may be common among industrial workers and inhabitants of areas with elevated water fluoride.8,9 Because ethanol increases the

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permeability of biological membranes it can make alcoholics more susceptible to the effects of other xenobiotics including ethanol compared to non-alcoholics.

One of the effects of F exposure in organisms is the impact of free radical parameters on antioxidant defense systems, such as the activity of antioxidant enzymes and nonenzymatic species leading to an increase in free radical mediated tissue impairments such as lipid peroxidation. Exposure to EtOH can also interfere with free radical parameters in the organism, especially by increasing lipid peroxidation. Induction of oxidative stress by ethanol is well established experimentally.

The aim of this study was to investigate the impact of F and EtOH given separately or together to rats in a short-term experiment on free radical mediated parameters and on F accumulation in organs and excretion with urine.

MATERIALS AND METHODS

Animals and experimental design: The study was carried out for a period of 4 weeks on 30 adult male Wistar rats weighing approximately 162 ± 8.2 g at the beginning of the experiment. The animals were kept under standard laboratory conditions (temperature 22±2°C in a natural light-dark cycle). All animals were fed a standard laboratory pellet diet containing 0.7 mg water-extractable F/kg. The animals were randomly allocated to five groups of six rats each:

I. Controls, drinking tap water containing 0.3 mg F/L
II. Controls, drinking tap water containing 0.3 mg F/L and given tap water containing 0.3 mg F/L intragastrically (1 mL twice/day by a stomach tube) for the final 14 days of the experiment
III. Exposed, drinking water containing 25 mg F (from NaF)/L
IV. Exposed, receiving ethanol intragastrically (1 mL) in a total dose of 5 g/kg bw/day divided into two equal doses administered at 8:00 am and 2:00 pm for the final 14 days of the experiment
V. Exposed, receiving NaF as in Group III and ethanol as in Group IV

At the end of the experiment animals were sacrificed by exsanguination under ether narcosis, and samples of blood, kidney, liver, and brain were collected. Blood was taken with and without anticoagulant by cardiac puncture.

Analytical procedures: In blood, kidney, liver, and brain, catalase (CAT) activity was determined by the method of Aebi. The concentration of TBARS (thiobarbituric acid reactive substances) was determined by the method of Rice-Evans et al., and the level of SH (sulfhydryl) groups was determined according to Ellman et al.

Protein content in blood, kidney, liver, and brain was determined by the method of Lowry et al. Hemoglobin content in the blood was determined by the method of Drabkin.

Each week the concentration of F in urine was determined directly after dilution with equal volumes of TISAB buffer by a fluoride ion-specific electrode (Orion) and Ag/AgCl reference electrode. Urinary creatinine was determined by the method of Folin-Morris. Urinary F is reported as mg F/g creatinine.

The concentration of F in soft tissues was determined after dry combustion of the sample according to the method described recently by us. The accuracy of measurements was assessed with reference materials – in serum and soft tissues with Serum Control (Clin Check, Munich, Germany), and in urine with Seronorm.
Control Urine (Nycomed Pharma AC, Oslo, Norway). Mean F recovery was 99.6% from urine, 97.9% from serum, and 103% from soft tissues.

Statistical analysis: Statistical analysis was performed using the Fisher-Snedecor and Student’s t-test.

RESULTS

Results of water consumption and fluoride intake by the five groups of rats are presented in Table 1.

Table 1. Water consumption and F intake

<table>
<thead>
<tr>
<th>Animals</th>
<th>Water consumption mL/24 hr mean±SD</th>
<th>F intake mg F/24 hr/rat mean range</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Controls</td>
<td>34.5±2.5</td>
<td>0.010 0.009–0.011</td>
</tr>
<tr>
<td>II Controls (i.g.)</td>
<td>33.8±3.6</td>
<td>0.010 0.009–0.011</td>
</tr>
<tr>
<td>III F</td>
<td>35.3±3.9</td>
<td>0.882 0.780–0.980</td>
</tr>
<tr>
<td>IV EtOH</td>
<td>30.2±4.2</td>
<td>0.009 0.010–0.008</td>
</tr>
<tr>
<td>V F + EtOH</td>
<td>35.9±4.3</td>
<td>0.897 0.790–1.000</td>
</tr>
</tbody>
</table>

F concentrations in soft tissues, serum, and urine are given in Tables 2 and 3.

Table 2. Fluoride content in soft tissues (µg F/g) and serum (µg F/mL)

<table>
<thead>
<tr>
<th>Animals</th>
<th>Kidney mean±SD (n)</th>
<th>Liver mean±SD (n)</th>
<th>Brain mean±SD (n)</th>
<th>Serum mean±SD (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Controls</td>
<td>0.848±0.037 (6)</td>
<td>0.708±0.028 (6)</td>
<td>0.632±0.033(5)</td>
<td>0.051±0.006 (5)</td>
</tr>
<tr>
<td>II Controls (i.g.)</td>
<td>0.784±0.054 (6)</td>
<td>0.666±0.023 (6)</td>
<td>0.586±0.054(4)</td>
<td>0.056±0.007 (5)</td>
</tr>
<tr>
<td>III F</td>
<td>5.380±0.464 (6)</td>
<td>3.940±0.134 (6)</td>
<td>3.350±0.252(5)</td>
<td>0.095±0.049 (4)</td>
</tr>
<tr>
<td>IV EtOH</td>
<td>0.863±0.081 (6)</td>
<td>0.721±0.052 (6)</td>
<td>0.616±0.046(5)</td>
<td>0.053±0.007 (4)</td>
</tr>
<tr>
<td>V F + EtOH</td>
<td>5.770±0.395 (6)</td>
<td>3.720±0.507 (5)</td>
<td>3.660±0.227(5)</td>
<td>0.090±0.017 (5)</td>
</tr>
</tbody>
</table>

Statistical significance:

I vs. III p<0.001
II vs. IV p<0.05
I vs. V p<0.01
II vs. V p<0.01
III vs. V n.s.
IV vs. V p<0.001

n=number of animals.

Table 3. Fluoride concentration in urine (mg F/g creatinine ± SD)

<table>
<thead>
<tr>
<th>Animals</th>
<th>Exposure time in weeks</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Controls</td>
<td>2.12±0.078</td>
<td>2.26±0.610</td>
<td>1.76±0.161</td>
<td>1.83±0.009</td>
<td></td>
</tr>
<tr>
<td>II Controls (i.g.)</td>
<td>1.79±0.152</td>
<td>1.62±0.218</td>
<td>1.95±0.092</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III F</td>
<td>4.02±0.912</td>
<td>5.34±0.036</td>
<td>6.62±1.720</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV EtOH</td>
<td>2.07±0.129</td>
<td>1.95±0.161</td>
<td>2.21±0.349</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V F + EtOH</td>
<td>4.51±0.250</td>
<td>5.58±0.611</td>
<td>6.21±0.318</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistical significance:

I vs. III p<0.001
II vs. IV n.s.
I vs. V p<0.001
II vs. V p<0.001
III vs. V n.s.
IV vs. V p<0.001

n=number of animals.

Values are means±SD for 4 animals in each group.
Catalase (CAT) activity in soft tissues and erythrocytes is presented in Table 4.

| Table 4. Catalase (CAT) activity in soft tissues (k/mg protein) and erythrocytes (k/g Hb) |
|---------------------------------|-------------|-------------|-------------|------------|
| Group | Liver | Kidney | Brain | Erythrocyte |
|       | mean±SD | mean±SD | mean±SD | mean±SD |
| I Controls | 0.225±0.028 | 5.26·10⁻²±4.90·10⁻³ | 1.01·10⁻³±8.56·10⁻⁵ | 32.82±3.58 |
| II Controls (i.g.) | 0.213±0.018 | 4.59·10⁻²±1.89·10⁻³ | 9.22·10⁻⁴±1.81·10⁻⁴ | 29.84±3.94 |
| III F | 0.153±0.019 | 3.69·10⁻²±1.89·10⁻³ | 6.35·10⁻⁴±7.38·10⁻⁵ | 36.36±2.99 |
| IV EtOH | 0.252±0.019 | 7.82·10⁻²±7.73·10⁻³ | 1.12·10⁻³±4.15·10⁻⁵ | 39.82±3.34 |
| V F + EtOH | 0.239±0.015 | 5.76·10⁻²±2.27·10⁻³ | 7.64·10⁻⁴±8.16·10⁻⁵ | 40.12±1.90 |

Statistical significance:
- I vs. III p<0.01 ↓
- II vs. IV p<0.01 ↑
- I vs. V n.s. ↑
- II vs. V p<0.05 ↑
- III vs. V p<0.001 ↑
- IV vs. V n.s. ↓

Values are for 6 animals in each group.

Concentrations of thiobarbituric acid reactive substances (TBARS) and sulfhydryl (SH) groups in soft tissues and plasma are given in Tables 5 and 6, respectively.

| Table 5. TBARS concentration in soft tissues and plasma (nM/g protein) |
|---------------------------------|-------------|-------------|-------------|-------------|
| Group | Liver | Kidney | Brain | Plasma |
|       | mean±SD | mean±SD | mean±SD | mean±SD |
| I Controls | 409.8±19.23 | 692.2±12.7 | 781.0±24.9 | 305.8±11.7 |
| II Controls (i.g.) | 350.7±18.02 | 621.5±3.4 | 744.7±7.0 | 244.7±5.9 |
| III F | 588.8±15.94 | 795.0±23.3 | 889.8±13.6 | 410.7±13.0 |
| IV EtOH | 417.3±10.11 | 811.5±15.1 | 804.8±8.9 | 320.2±24.1 |
| V F + EtOH | 539.2±8.86 | 858.8±19.4 | 873.2±20.1 | 363.3±18.6 |

Statistical significance:
- I vs. III p<0.001 ↓
- II vs. IV p<0.001 ↓
- I vs. V p<0.05 ↓
- II vs. V p<0.001 ↑
- III vs. V p<0.001 ↑
- IV vs. V n.s. ↓

Values are for 6 animals in each group.

| Table 6. Concentration of sulfhydryl (SH) groups in liver and kidney (mM/g protein) and in brain and plasma (µM/g protein) |
|---------------------------------|-------------|-------------|-------------|-------------|
| Group | Liver | Kidney | Brain | Plasma |
|       | mean±SD | mean±SD | mean±SD | mean±SD |
| I Controls | 0.183±0.051 | 0.129±0.022 | 3.29±0.26 | 7.23±0.64 |
| II Controls (i.g.) | 0.192±0.019 | 0.126±0.015 | 2.48±0.25 | 7.06±0.71 |
| III F | 0.116±0.020 | 0.079±0.013 | 1.80±0.18 | 4.72±0.62 |
| IV EtOH | 0.158±0.036 | 0.113±0.014 | 2.10±0.16 | 6.12±1.27 |
| V F + EtOH | 0.146±0.046 | 0.110±0.006 | 2.01±0.10 | 5.89±0.51 |

Statistical significance:
- I vs. III p<0.001 ↓
- II vs. IV p<0.05 ↓
- I vs. V n.s. ↓
- II vs. V p<0.05 ↓
- III vs. V p<0.01 ↑
- IV vs. V n.s. ↓

Values are for 6 animals in each group.
DISCUSSION

The present study was undertaken to assess the oxidative status in liver, kidney, brain, and blood of rats during co-exposure to F and EtOH. For this purpose, the activity of the antioxidant enzyme CAT, concentration of TBARS as an indicator of lipid peroxidation, and the concentration of SH groups that protect the cell from oxidative stress were determined in these tissues. The exposure of rats to 25 mg F/L corresponds to human environmental exposure in areas with high F level in drinking water or in occupational conditions. Intragastric administration of 5 g ethanol/kg bw/24 hr corresponds to ethanol abuse in man.

Decreased consumption of water was noticed only in the EtOH group. In the F group water consumption was lower, but not insignificantly lower. The intake of water in the F and the F + EtOH groups was at the same level.

As expected, exposure to F resulted in a marked increase of F concentration in kidney, liver, brain, and serum. Ethanol alone increased the F content in kidney and liver, but had no effect on other tissues. This may be due to increased permeability of cellular membranes caused by ethanol. In rats co-exposed to F and EtOH the F concentration increased only in brain compared to animals treated with F alone.

Also, a significant increase in F excretion in urine occurred following administration of NaF in the drinking water. The F increase changed in an exposure-time dependent manner. In animals exposed only to EtOH, urinary F excretion increased insignificantly. Co-exposure to F and EtOH did not significantly affect the rate urinary F excretion.

Enhanced peroxidation of lipids in intracellular and extracellular membranes is known to cause damage to tissues and organs. Antioxidant enzymes (SOD, CAT, GPx) are very important in protecting organisms from reactive oxygen species (ROS). CAT is a hemoprotein found in the peroxisomes of eukaryotic cells that catalyses the conversion of hydrogen peroxide to water and oxygen. The activity of this enzyme also can be induced in response to cellular hydrogen peroxide exposure.

In the rats treated with sodium fluoride, CAT activity was significantly depressed in liver, kidney, and brain. On the other hand, EtOH caused a significant increase in the activity of this antioxidant enzyme in the investigated tissues. Co-exposure to F and EtOH resulted in intermediate activity. In co-exposed animals catalase activity was higher than in animals exposed only to F, whereas it was lower in rats treated only with EtOH.

As noted by various authors, F can stimulate lipid peroxidation in membranous structures. However, the mechanism of fluoride-induced lipid peroxidation is not fully clarified. Available data indicate that the mechanism is multidirectional and may involve a decrease in the level of glutathione and the total pool of SH groups and changes in the activity of antioxidant enzymes. These can induce a peroxidative state in biological systems and, in turn, lead to peroxidation of polyunsaturated fatty acids.

SH groups occur in living organisms as constituents of enzymes, tissue and serum proteins, peptides (glutathione), coenzymes, and single amino acids. SH
groups easily react with free radicals, notably with hydroxyl radicals, and protect the cell against oxidative attack by chemicals. Apart from antioxidant defense enzymes and other low-molecular-antioxidants, SH groups are important components of the antioxidant defense system of organisms.  

In the present study, exposure of the rats to F decreased the concentration of SH groups in all investigated tissues. A similar pattern of changes was observed in the ethanol group. In animals co-exposed to F and EtOH, however, the level of SH groups increased compared to those treated only with F, but the level decreased significantly compared to animals exposed only to EtOH.

TBARS concentration increased significantly in all investigated tissues of animals exposed to F, thereby indicating an enhancement of lipid peroxidation. The EtOH induced escalation of lipid peroxidation might be the consequence of increased formation of free radicals as well as the inhibition of CAT and SOD (superoxide dismutase). The present study confirmed the involvement of EtOH in lipid peroxidation. Ethanol is able to induce the formation of free radicals. During EtOH biotransformation occurring with the involvement of the microsomal ethanol-oxidizing system (MEOS), xanthine oxidase and aldehyde oxidase, reactive oxygen species (ROS) and hydroxyethyl radicals are generated.  

EtOH, apart from being a source of free radicals, can also disturb the antioxidant defense system. Although significant, the increase of TBARS in rats treated only with EtOH is lower than in animals exposed to F. In animals co-exposed to F and EtOH the concentration of TBARS was in intermediate level. Only in the kidney, probably due to a less effective antioxidant defense system compared to the liver, the TBARS concentration increased very distinctly indicating a synergistic effect.

A very important finding of this study is that, despite the ability of F and EtOH to induce oxidative stress, the effect is not intensified (except in the kidney) by simultaneous exposure to both substances. The disturbances in the oxidative status observed in our study indicate a risk of organ damage during exposure to F and EtOH via free radical mechanism.

The results concerning the activity of CAT, the concentration of SH groups, and concentration of TBARS in the investigated tissues clearly indicate that F and EtOH are able to induce the oxidative stress during repeated administration singly or together during co-exposure.

Acknowledgement

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