INFLUENCE OF FLUORIDE ON CASPASE-3 EXPRESSION IN KIDNEY OF RATS FED A NORMAL OR A LOW CALCIUM AND PROTEIN DIET AND ITS REVERSAL BY PROTEIN AND CALCIUM SUPPLEMENTATION

Huacheng Chen, Jianhua Hong, Ruiyan Niu, Zilong Sun, Jundong Wang
Shanxi, China

SUMMARY: Gene and protein expression levels of caspase-3 in the kidney of rats treated with 150 mg/L fluoride ion (F, from NaF) in their drinking water and fed a normal or a low calcium (Ca) and low protein (Pr) diet were studied. On the 60th and 120th days, caspase-3 expression levels and renal apoptosis increased significantly. Supplementation with Pr and Ca reduced the level of caspase-3 and the amount of apoptosis, with the effect of Pr being greater than that of Ca.

Keywords: Apoptosis; Calcium supplementation; Caspase-3; Protein supplementation; Rat kidney.

INTRODUCTION

By proteomic analysis of kidney, a primary organ for excretion and retention of fluoride (F), Xu et al, have shown that F induces oxidative stress and dysfunction of cell proliferation, which can result in apoptosis. His group also reported that excessive F exposure resulted in apoptosis in kidney. In fact, various investigations have demonstrated apoptosis induced by F in many other organs including lungs, liver, bone tissues, and brain, but the underlying mechanisms are still unclear because of the intricate signal pathways involved.

Accumulated data suggest that the mitochondria-initiated death pathway plays an important role in triggering apoptosis. Recently, Lee et al. found that F-induced apoptosis in human gingival fibroblasts through the mitochondria-mediated pathways. Accordingly, it appears desirable to measure the changes in caspase-3, a key and common protease in mitochondria-mediated pathways.

The objective of the present study was to investigate the toxic effects of F on the expression of caspase-3 in kidney of rats fed a normal or a calcium (Ca) and protein (Pr) deficient diet and the ameliorating effects of Ca and Pr supplementation.

MATERIALS AND METHODS

Experimental animals: One hundred and forty-four 30-day-old Wistar albino rats weighing 58±7.8g were obtained from the Experimental Animal Center of Shanxi Medical University. The study design was approved by the Institutional Animal Care and Use Committee of China.

Establishment of animal model: The rats were allotted randomly to six groups of twenty-four. Each group was maintained on the diets and water regimens shown in Table 1 under standard temperature (22–25°C), ventilation, and hygienic conditions.
conditions. On the 60th and 120th day of the treatment, twelve rats were selected randomly in each group deprived of food for 12 hr. Afterward the rats were anesthetized with 20% urethane (ethyl carbamate, NH₂COOC₂H₅) solution, and the kidneys from six rats in each group were carefully removed and stored in liquid nitrogen for gene expression tests. The kidneys from the other six rats were fixed, dehydrated, and embedded by paraffin for immunohistochemistry study.

**Table 1.** Fluoride in the drinking water (mg F⁻/L) and diet (mg F⁻/kg), protein (Pr), and calcium (Ca) levels (%), and energy density (ED as MJ/kg) in the diet of rats

<table>
<thead>
<tr>
<th></th>
<th>Pr</th>
<th>Ca</th>
<th>Fluoride in diet</th>
<th>Fluoride in water</th>
<th>ED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control (NC)</td>
<td>17.92</td>
<td>0.58</td>
<td>24.73</td>
<td>&lt;0.6</td>
<td>12.6</td>
</tr>
<tr>
<td>Normal control+NaF (NC+F)</td>
<td>17.92</td>
<td>0.58</td>
<td>24.73</td>
<td>150</td>
<td>12.6</td>
</tr>
<tr>
<td>Low protein and low calcium (LPrLCa)</td>
<td>10.01</td>
<td>0.24</td>
<td>23.81</td>
<td>&lt;0.6</td>
<td>12.6</td>
</tr>
<tr>
<td>Low protein and low calcium+NaF (LPrLCa+F)</td>
<td>10.01</td>
<td>0.24</td>
<td>23.81</td>
<td>150</td>
<td>12.6</td>
</tr>
<tr>
<td>High protein and low calcium+NaF (HPrLCa+F)</td>
<td>25.52</td>
<td>0.25</td>
<td>23.69</td>
<td>150</td>
<td>12.6</td>
</tr>
<tr>
<td>Low protein and high calcium+NaF (LPrHCa+F)</td>
<td>10.60</td>
<td>1.93</td>
<td>23.77</td>
<td>150</td>
<td>12.6</td>
</tr>
</tbody>
</table>

* From 338 mg/L NaF.

**Total RNA extraction and QRT-PCR:** Total cellular RNA was extracted from the kidneys by a modified technique for cartilage using Trizol Reagent (invitrogen, USA) and XHF-1 High-speed Dispersator (Scietz, China). The RNA extracts were treated with RNase-free DNase I to remove contaminating DNA, quantified on a spectrophotometer (Eppendorf, Germany), and stored at −80°C.

According to the alignments of the published cDNA sequences of caspase-3 and β-actin genes in rats, two pairs of specific primers (Table 2.) were designed by the primer premier 5.0 software. The primers of caspase-3 gene were designed to amplify a 137-base pairs (bp) transcript. The endogenous house-keeping gene β-actin was used as control to normalize the quantity of caspase-3 transcripts with its primers designed to amplify a 115 bp transcript.

**Table 2.** Primer sequences with their corresponding PCR product size and position

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5'→3')</th>
<th>Primer location</th>
<th>Product (bp)</th>
<th>Genbank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>AGCCATGTACGTAGCATTCC ACCCTCATAATTGGGACACAG</td>
<td>471-585</td>
<td>115</td>
<td>NM_031144.2</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>CTCAGTGTGACATGACG TCAACAATTGGACGCTCAG</td>
<td>1563-1699</td>
<td>137</td>
<td>NM_012922.2</td>
</tr>
</tbody>
</table>

The expression level of caspase-3 gene was quantified by real-time amplification of caspase-3 gene and the house-keeping gene β-actin as control from the above RNA preparation using the Mx3000™ real-time PCR system (Stratagene, USA) and One-Step SYBR® RT-PCR kit (Takara, China). The final PCR mixture volume was 20 µL. The RT-PCR protocol included reverse transcription at 42°C for 15 min and an initial denaturation at 95°C for 5 sec. This was followed by 40 PCR cycles consisting of a denaturation step at 95°C for 5 sec, an annealing step at 60°C for 20 sec, and an extension step at 72°C for 6 sec. Finally, the melting curve analysis was performed at 95°C for 15 sec, at 60°C for 1
Influence of F on caspase-3 expression in kidney of rats fed a normal or a low Ca and protein diet and its reversal by protein and calcium supplementation
Chen, Hong, Niu, Sun, Wang

...min, and at 95°C for 15 sec as in the protocol for the three reaction steps. The amplified products were analyzed by agarose gel electrophoresis.

**Immunohistochemistry for caspase-3**: The procedures were processed according to the protocol recommended for the Caspase-3 immunohistochemistry kit. Following deparaffinization and rehydration, sections were irradiated in 0.1 mol/L sodium citrate buffer (pH 6.0) in a microwave oven (medium low temperature) for 12 minutes. Then the sections were exposed to 3% H₂O₂ for 10 min to bleach endogenous peroxidases, followed by rinsing 3 times in phosphate-buffered saline (PBS) for 10 minutes. Sections were selectively incubated using an anti-caspase-3 polyclonal antibody (1:100, Wuhan boster Biotechnology Co. LTD) for 1 hr at 37°C, washed 3 times in PBS. Next, a biotinlabeled (=biotin-labeled) goat antirabbit secondary antibody (Wuhan boster Biotechnology Co. LTD) was introduced at a dilution of 1:1000, and incubation was conducted at 30 min at 37°C. The specificity of the antibodies was tested by omission of the primary antibodies. After washing in PBS, tissues were visualized with 3,3’-diaminobenzidine (DAB) and counterstained with hematoxyline. Finally, the sections were dehydrated in xylene and coverslipped.

**Statistical analysis**: An independent sample t-test (Statistical Package for the Social Sciences, SPSS 11.5) was performed to analyze differences in caspase-3 gene and protein expression levels among the experimental six groups. Differences with p<0.05 were considered statistically significance.

**RESULTS**

**Caspase-3 gene expression**: The relative expression level of caspase-3 mRNA in kidney from rats is shown in Figure 1.

![Figure 1](image_url)

*Figure 1*. The relative expression level of caspase-3 gene in kidney of rats on day 60 and 120 under normal nutrition (A). The relative expression level of caspase-3 gene in kidney of rats on day 60 and 120 with different conditions of Pr and Ca intake (B).
In comparison with the NC (normal control) group, the expression level of caspase-3 gene in the NC+F group was increased by 31% and 28% on the 60th and 120th day, respectively. When compared with the LPrLCa (low calcium/low protein) group, the expression levels of caspase-3 in the LPrLCa+F group were increased by 49% on the 60th day and 48% on the 120th day, respectively. Compared with the LPrLCa+F group, the expression level of caspase-3 was decreased in the HPrLCa (high protein/low calcium) +F group and the LPrHCa (low protein/high calcium) +F group by 8.1% and 3.4% on the 60th day, and by 6.8% and 4.7% on the 120th day, respectively.

**Immunohistochemistry for caspase-3:** The caspase-3 protein expression levels by immunohistochemical detection in the renal tissue of the rats recorded in Table 3.

| Table 3. The expression of caspase-3 measured as optical density in kidney of rats at day 60 and 120 (mean±SD, n = 6) |
|---|---|---|---|---|---|---|
| Day | NC | NC+F | LPrLCa | LPrLCa+F | HPrLCa+F | LPrHCa+F |
| 60  | 30.70±1.40 | 32.25±2.19* | 34.10±2.26 | 36.20±2.14* | 34.95±2.45 | 34.86±1.78 |
| 120 | 30.05±1.63 | 31.96±1.83* | 31.64±2.99 | 36.45±1.98* | 34.01±3.66† | 35.08±3.05 |

* p<0.05 NC+F group compared with NC group; LPrLCa+F group compared with LPrLCa group; † p<0.05 HPrLCa+F group or LPrHCa+F group compared with LPrLCa+F group, respectively.

The immunohistochemistry image results for the 60th and 120th day are presented in Figures 2 and 3.

Figure 2. Photomicrographs (×400) showing the caspase-3 expression in kidney of rats on the 60th day in the NC group (A), NC+F group (B), LPrLCa group (C), LPrLCa+F group (D), HPrLCa+F group (E), and LPrHCa+F group (F). The brown granules around the renal tubule are regarded as the protein expression of caspase-3. Compared with the LPrLCa group (C), the LPrLCa+F (D), HPrLCa+F (E), and LPrHCa+F (F) groups exhibit stronger staining intensity, accounting for the increase in caspase-3 expression, as found in the comparison between the NC group (A) and the NC+F group (B).
DISCUSSION

There is now extensive information available on the relationship between fluoride and reactive oxygen species (ROS), lipid peroxidation, oxidative stress, and DNA damage. Recent data show that ROS play an important role in mediating apoptosis by inducing the activation of caspases, in turn resulting in a high frequency of DNA strand breaks. One of the molecular hallmarks of apoptosis is the activation of caspases, which are cysteine proteases that cleave a critical set of cellular proteins to initiate the apoptotic signals. Among all the caspase members, caspase-3 in particular is an essential apoptotic effector leading to cytoskeletal breakdown, nuclear demise, and other cell changes associated with apoptosis. Therefore, to gain initial insight into potential mechanisms involved in F-induced renal apoptosis, the effect of F on kidney mRNA and protein for caspase-3 was determined. Our results demonstrate that the relative expression levels of both protein and mRNA for caspase-3 were increased. To our knowledge, effects of F on caspase-3 abundance in both protein and gene in kidney of rats fed a low Ca and low protein (Pr) diet have not been reported previously.

Earlier studies from our laboratory indicate that Pr or Ca supplementation can alleviate fluorosis in goats, rabbits, and rats. The present study also revealed an ameliorative effect of Pr or Ca supplementation. Results showed that the expression level of caspase-3 was reduced in the HPrLCa+F and LPrHCa+F groups in comparison with the LPrLCa+F group. According to the extent of the expression level decrease, Pr was superior to Ca. We therefore postulate that Pr
supplementation may be due to a greater strengthening of physiological function and resistance to F than Ca, which has been well-documented.\(^\text{20}\)

In summary, this study demonstrated the negative effect of F on caspase-3 expression in kidney of rats fed with either a normal or a Ca and Pr deficient diet. Additionally, Pr or Ca supplementation significantly alleviated the F toxicity, with a greater effect by Pr than by Ca.

**ACKNOWLEDGEMENTS**

This research was sponsored by the China National Natural Science Foundation (Grant No. 30871899) and the Shanxi Province Key Laboratory Open Foundation (Grant No. 20081057).

**REFERENCES**