EFFECTS OF SODIUM FLUORIDE ON JNK SIGNALING PATHWAY IN THE KIDNEYS OF A FRESHWATER TELEOST, *CYPRINUS CARPIO*

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SUMMARY: Chronic exposure to fluoride (F) can lead to various negative effects in fish. Previously we found that F resulted in damage and apoptosis in the kidneys of *Cyprinus carpio*. In this study, the effects of F on protein expression and location in the JNK signaling pathway were determined using western blotting and immunohistochemistry methods in the kidneys of carp exposed to 0, 40, 80, and 120 mg F/L, respectively. Western blotting analysis showed that, compared with the control, the expression of JNK protein was relatively stable in F-exposed fish, while p-JNK protein levels were enhanced significantly with the increase of F exposure concentrations. The immunohistochemistry analysis illustrated the proteins of JNK and p-JNK were predominantly localized in the cytoplasm of the kidney of *Cyprinus carpio*. Compared with the control group, the level of JNK protein was relatively constant, yet the p-JNK expression and p-JNK/JNK ratio were elevated with increased F concentrations. These findings indicate that the damage of kidney in carp exposed to F is mediated via the JNK pathway. F exposure may active JNK through phosphorylation, promote the expression of p-JNK protein, and thus induce damage to the kidneys in *Cyprinus carpio*.

Keywords: Carp; Fluoride; JNK signaling pathway; Kidney.

INTRODUCTION

Fluoride (F) is not an essential nutrient and, in excess, can produce toxicity in both humans and other mammals.[1-3] Natural (i.e., weathering of F-containing ores) and anthropogenic activities (such as metal mining and smelting, and fertilizer production) has resulted in the accumulation of F compounds in freshwater ecosystems.[4] Recently, an increasing number of studies on fish have shown that a high concentration of F may have negative effects, such as slower growth and development, increased mortality, metabolic disorders, pathological changes in tissues, F accumulation in and deformity of bone tissue, and ecological pressure on fish.[4-11] However, the mechanisms on F toxicity to fish have not been investigated in detail and the mechanisms for F-induced damage and renal apoptosis have still not been clarified.

C-Jun N-terminal kinases (JNK) is one of the members of the Mitogen-activated protein kinases (MAPKs) family, which can regulate cell proliferation, differentiation, and apoptosis and be activated by many internal and external factors.[12-14] Activated JNK, once transferred to the nucleus, can activate a variety of transcription factors (c-jun, c-fos, and Elk-1, etc.) to initiate the apoptosis by the death receptor pathway. Our previous study indicated that chronic exposure to F led to damage and apoptosis in the kidneys of *Cyprinus carpio*.[15] In the present study, protein expression and location of JNK and p-JNK in the kidneys of carp

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chronically exposed to F were determined to further investigate the underlying mechanisms for the observed adverse effects in the F-exposed kidneys of carp.

MATERIALS AND METHODS

Experimental animals and F exposure: Healthy carp juveniles were obtained from Taiyuan Fish Hatcheries, Shanxi, China, with a mean length 12.0 ± 0.38 cm. They were acclimatized for 15 days in the laboratory before the experimental processing. Then, the juveniles were divided randomly into 4 groups of 150 fishes each which were exposed to nominal concentrations of F: (1) control group without NaF; (2) 40 mg F/L; (3) 80 mg F/L; (4) 120 mg F/L. Each group was subdivided into three subgroups of 50 to provide a sample subgroup and two replicate subgroups. During the experiment, the juvenile carp were fed with a commercial carp diet, the exposure conditions were maintained as follows: water temperature, 22.0–24.0ºC; pH, 6.8–7.2; dissolved oxygen, 5.0–7.0 mg/L; hardness, 20.0 mg/L (as $CaCO_3$). All animal work was authorized by the Institutional Animal Care and Use Committee of China.

Tissue preparation: After 90 days of exposure, eighteen juvenile fish from each group (six juvenile fish from each subgroup) were anesthetized and dissected on ice. Kidney tissues were collected and part of them were stored at –80ºC for western blotting while the remainder were fixed in 4% paraformaldehyde for immunohistochemical analysis.

Western blotting: Proteins of kidney stored at –80ºC were extracted and measured by BCA Protein Assay Kit. Samples containing equivalent amounts of protein were applied to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose (NC) membrane for 80 min at 120V. Membranes were blocked with 5% fat-free milk powder in TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl and 0.1% Tween 20) for 2 hr at room temperature and then incubated with anti-JNK and anti-p-JNK (1:1000, Bioworld, USA) overnight at 4ºC. After washing, NC membranes were incubated at room temperature for 1.5 hr with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, Bioworld, USA). The immunoreactive protein on the membrane was visualized with Western Blot diaminobenzidine (DAB) detection Kit (Boster, China) and quantified by Image-Pro Plus 6 software (Media Cybernetics, USA).

Immunohistochemistry (IHC): The procedures were conducted based on the manufacturer's manual of Strept Avidin-Biotin-peroxidase Complex (SABC) kit (Boster, China). After being deparaffinized and rehydrated, the sections were incubated with the rabbit anti-JNK or anti-p-JNK polyclonal antibodiy (diluted 1:300) overnight at 4ºC. After being washed 3 times in PBS, the slides were then incubated with Biotin-labeled anti-rabbit secondary antibody (Bioworld, USA) (1:500) for 0.5 hr at room temperature. The specificity of the antibody was examined by omission of the primary antibody. Then the sections were added to SABC followed by storage at 37ºC for 20 min and washing 4 times by PBS for 5 min. After the positive proteins were visualized with DAB and counterstained with

hematoxylin, the sections were observed under the optical microscope. The color intensity was then determined as an index of target protein expression. Finally, photomicrographs were taken of the sections using a Leica 2500 microscope (Germany).

Statistical analysis: Experimental data were expressed as mean values ± standard deviation (SD). With SPASS 11.5 statistical software, differences between groups were evaluated by one-way ANOVA, and the significance between pairs was determined by Tukey's Multiple Comparison Test. Differences were considered to be statistically significant when $p<0.05$.

RESULTS

Expression of JNK and p-JNK proteins in the kidneys of carp after exposure to F: The effects of F on the expression of the JNK and p-JNK proteins in the kidney of *Cyprinus carpio* were measured by western blotting. The expression of JNK protein was relatively stable in F-exposed fish, while p-JNK protein levels were enhanced significantly with the increase of F exposure concentrations, with a maximal increase of 17.4% observed in fish exposed to 120 mg F/L (Figure 1).

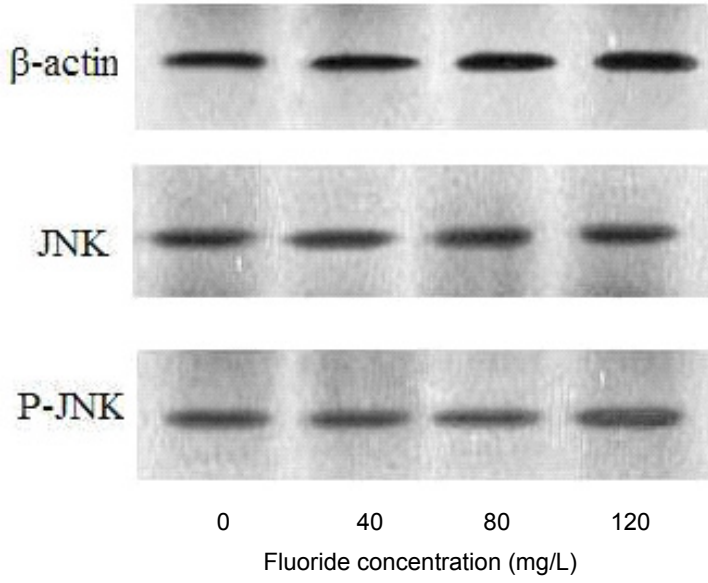


Figure 1. Effects of fluoride on the levels of JNK and P-JNK proteins in the kidney of *Cyprinus carpio*. The intensities of JNK and p-JNK proteins were normalized to that of β-actin.

Consistent with these results, the p-JNK/JNK ratio increased remarkably in fish exposed to 40, 80, and 120 mg F/L (Figure 2).

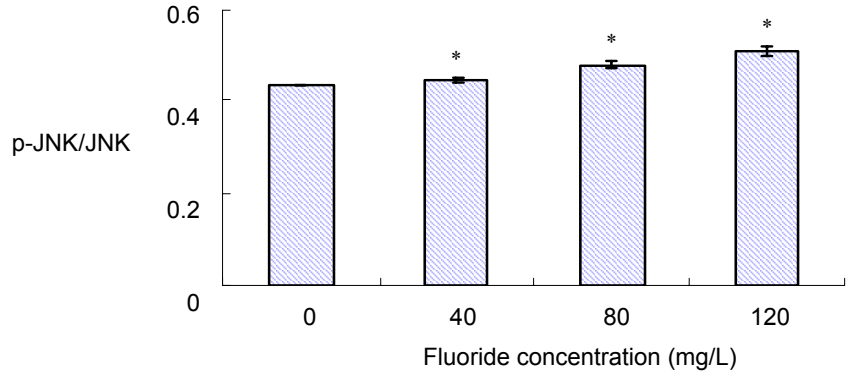
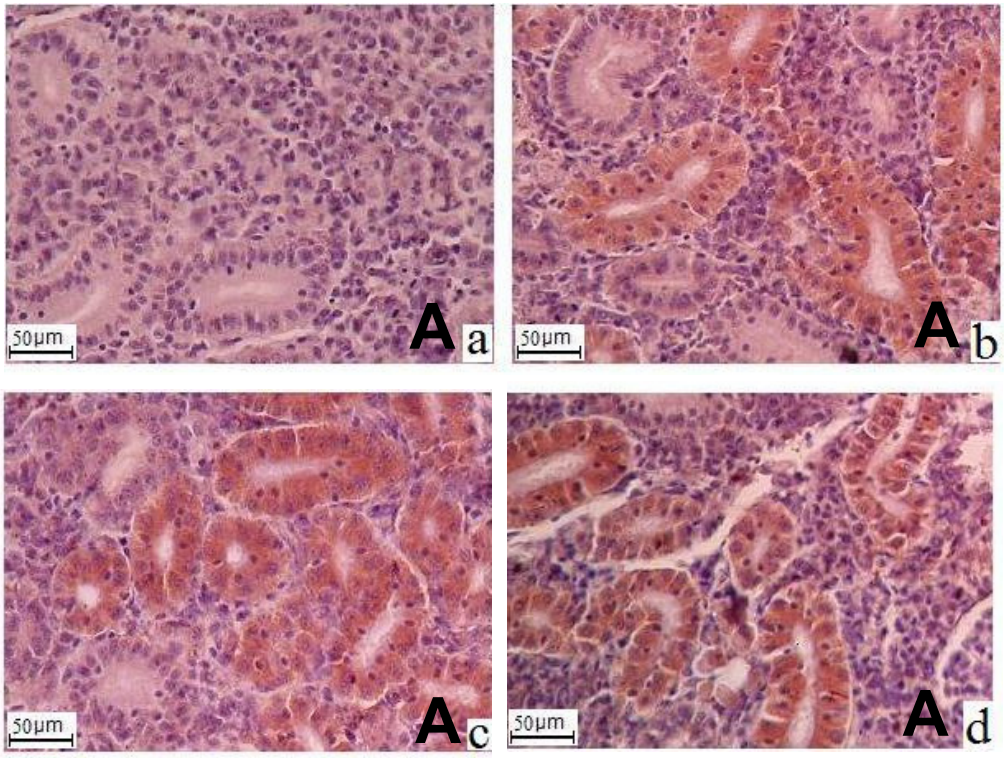


Figure 2. Effects of fluoride on the p-JNK/JNK ratio in the kidney of *Cyprinus carpio*. Compared with the control (0 mg F/L), the expressions of JNK and P-JNK proteins were significantly increased in fish exposed to 40, 80, and 120 mg F/L. Compared to control: *p<0.05.

Cellular localization of JNK and p-JNK proteins in the kidneys of carp after exposed to F: The immunohistochemistry analysis (Figure 3) illustrated that the positive proteins were dyed brown and the proteins of JNK and p-JNK were predominantly localized in the cytoplasm of the kidney of *Cyprinus carpio*.



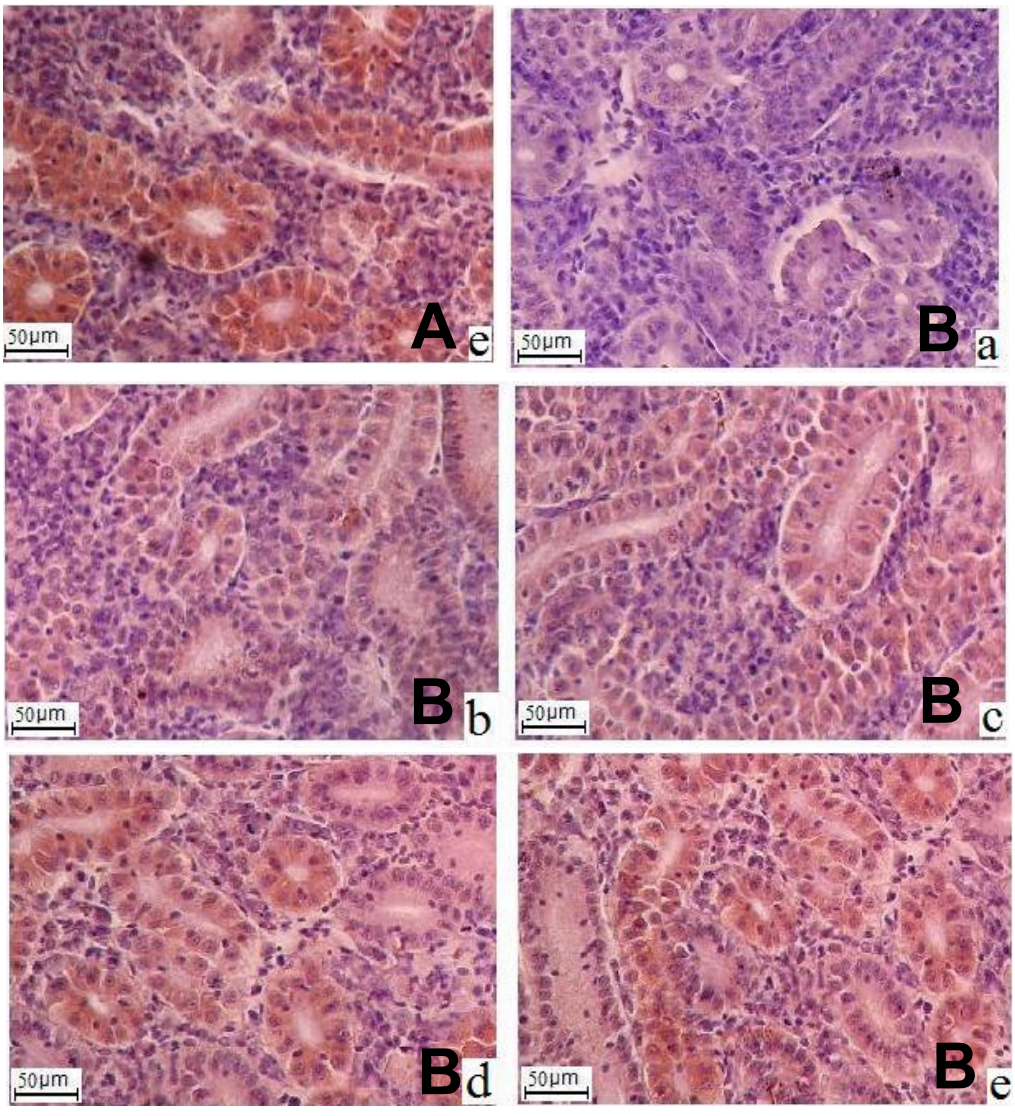


Figure 3. Cellular localization of JNK and p-JNK proteins in the kidneys of carp, *Cyprinus carpio,* after exposure to F. A: JNK; B: p-JNK; a: negative control; b: kidneys of control fish; c: kidneys of fish exposed to 40 mg F/L; d: kidneys of fish exposed to 80 mg F/L; e; kidneys of fish exposed to 120 mg F/L.

Compared with the control group, the level of JNK protein in the kidneys was relatively constant in all the F-exposed fish, yet the p-JNK expression and the p-JNK/JNK ratio were elevated with increased F concentrations (Figure 4).

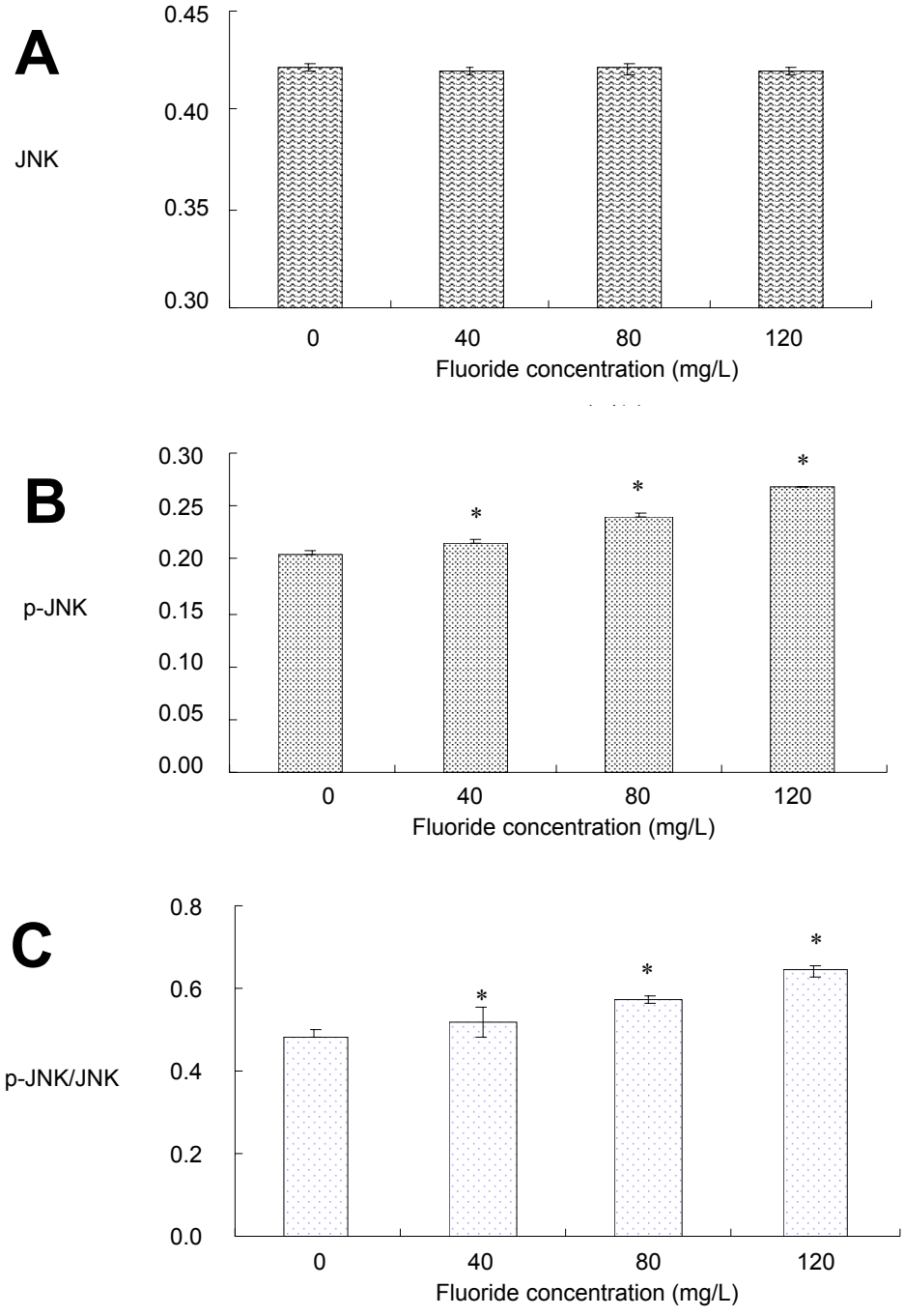


Figure 4. Quantified data, from immunohistochemistry, presenting the fluoride-induced JNK phosphorylation levels relative to the amount of total proteins in the kidneys of *Cyprinus carpio* after chronic exposure to fluoride. A: the protein expression of JNK; B: the protein expression of p-JNK; C: the ratio of p-JNK/JNK. Compared to control: *p<0.05.

DISCUSSION

The JNK is one of the members of the Mitogen-activated protein kinases (MAPKs) family. It is generally responsive to cell stressors such as hypertonicity, ultraviolet light, heat shock, and proinflammatory cytokines.[16] After being phosphorylated, activated JNK (p-JNK) can catalyse downstream kinases. A few studies have indicated that F can induce apoptosis via activation of JNK. For example, Liu et al. confirmed the positive correlation between the expression of p-JNK protein and the apoptotic death rate.[17] Li et al. also indicated that F could increase phosphorylation of JNK, and notably induced apoptosis.[18] Exposure to F induced apoptosis in odontoblast-like cells depending on the activation of JNK.[19] In the present study, although the levels of JNK protein remained stable after chronic exposure to F, the remarkable elevation of p-JNK protein and the increased ratio of p-JNK/JNK indicated that F affected JNK phosphorylation. The levels of p-JNK protein increased with the F exposure concentration, implying that the activation of JNK signal pathway possibly played an important role in the renal deterioration of F-exposed carp.

Previous study has found that JNK kinase can be activated by increased oxidative stress levels. The activation of JNK kinase could cause apoptosis. For example, the expression of apoptotic proteins were elevated by activation of JNK in the hippocampal neurons during brain injury.[20] Increased level of apoptosis in rat brains and SH-SY5Y cells exposed to excessive F positively correlated with activating JNK phosphorylation.[17] Our previous study showed that F can reduce the activity of superoxide dismutase and catalase, and the capacity of total antioxidant, enhance the content of reactive oxygen species and MDA, damage the kidney, and induce apoptosis in kidney of F-exposed carp.[15] Xiong et al. found that drinking water F levels over 2.0 mg/L could cause damage to renal function in children.[21] Based on all above results, we hypothesize that increased ROS and decreased antioxidant capacity result in the activation of the JNK signaling pathway in the kidneys of chronically F-exposed fish as described previously.[15]

In conclusion, our data indicate that the kidney damage in carp exposed to F is mediated via the JNK pathway. F exposure may active JNK through phosphorylation, promote the expression of p-JNK protein, and thus induce the damage of kidneys in *Cyprinus carpio*.

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