

*Full Length Research Paper*

# Ameliorative effect of protein and calcium on fluoride-induced hepatotoxicity in rabbits

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To investigate whether protein (Pr) or calcium (Ca) supplementation could ameliorate hepatic damage induced by excessive fluoride (F); thirty-two 30-day-old healthy New Zealand rabbits were randomly divided into four groups (female: male = 1:1). The four groups were maintained on distilled water and fed the following diets for 120 days: (1) a malnutrition control (MC) diet (8.58% Pr, 0.49% Ca); (2) the MC diet plus HiF (high fluoride in their diet, 200 mg F ion/kg from NaF); (3) a Ca deficient MC diet plus HiPr+HiF (0.46% Ca, 18.41% Pr, plus HiF); and (4) a Pr deficient MC diet plus HiCa+HiF (2.23% Ca, 8.35% Pr, plus HiF). Results show that in HiF group, the serum total Pr (TPr) and albumin (ALB) content significantly decreased, whereas both Pr and Ca rich diets significantly enhanced their levels. In liver, low superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities, high malondialdehyde (MDA) content, and evident mitochondria lesions in HiF group indicated a significant oxidative stress, while Pr or Ca supplementation brought an ultrastructural repair and a recovery antioxidant defense in liver. The findings in the present work implied the ameliorative effects of Pr or Ca supplementation on F-induced hepatotoxicity in rabbits.

**Key words:** Fluoride, hepatotoxicity, malnutrition, calcium supplementation, protein supplementation.

## INTRODUCTION

Natural and artificial fluoride (F) sources, including fluoridated foodstuffs, groundwater, toothpaste, and dentifrices, lead to an excessive F exposure in daily life (Barbier et al., 2011). Besides skeletal and dental tissues, high F permeability is known to allow F ion penetrate cell

membranes and accumulate in diverse soft tissues such as stomach, small intestine, liver, kidney, and brain pyruvic transaminase (Lech, 2011), threatening the health of human and animal. Further investigation revealed F tendentiously alters the activity of many mitochondria-rich cells like hepatocyte (Dabrowska et al., 2004), which accorded with the result of a study on organ-specific toxicological response to F by Chattopadhyay et al. (2011).

Actually, accumulated studies demonstrated the adverse effects of F on liver in different animal models. Severe alterations in liver architectures were observed in mice exposed to 15 mg NaF/L for 30 days. Proteomic analysis of fish liver showed that 35 mg/L NaF for 3 days resulted in 51 functional proteins in liver expressed

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**Abbreviations:** F, Fluoride; Pr, protein; OSI, organo-somatic index; TPr, total protein; ALB, albumin; AKP, alkaline phosphatase; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; SGOT, serum glutamic-oxaloacetic transaminase; SGPT, serum glutamic.

**Table 1.** F ion (mg/kg), protein (Pr) and calcium (Ca) level (%), and energy density (ED, MJ/kg) in the diet of the rabbits.

Parameter	Malnutrition control (MC)	High fluoride (HiF)	High fluoride and high protein (HiF+HiPr)	High fluoride and high calcium (HiF+HiCa)
F ion in diet	20.1	200 <sup>a</sup>	200 <sup>a</sup>	200 <sup>a</sup>
Pr in diet	8.58	8.58	18.41	8.35
Ca in diet	0.49	0.49	0.46	2.23
ED in diet	9.84	9.84	10.37	9.84

<sup>a</sup>F ion from 442 mg/kg NaF. (A standard rabbit diet contains 12–16% Pr and 1% Ca.).

differentially (Lu et al., 2010). In rat liver, enzymes of the antioxidative system, such as superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH), were significantly inhibited after NaF exposure (Blaszczyk et al., 2011). Due to the importance of liver health for body metabolism, prevention and treatment of F-induced liver lesion have become a matter of major concern. However, the effective therapy still need further study.

Epidemiological investigations showed that endemic fluorosis is mainly prevalent in underdeveloped countries, particularly in the malnutritional areas (Hassan and Yousef, 2009; Massler and Schour, 1952; Michael et al., 1996; Moudgil et al., 1986; Murray and Wilson, 1948; Rugg-Gunn et al., 1997). This finding was corroborated by laboratory experiments which demonstrated that low levels of dietary nutrition intensify F intoxication, but nutritional supplementation counteracts it (Chinoy et al., 2006; Wang et al., 2009; Yan et al., 2009). However, potential therapeutic effects of nutrient (Ca and Pr supplementation) on indices of hepatic health in animals with excessive F intake have not been investigated in detail. Hence, the aim of this study was to determine whether feeding a diet containing additional Pr or Ca can ameliorate F-induced hepatotoxicity in rabbits fed a Pr and Ca deficient diet.

## MATERIALS AND METHODS

### Experimental materials

Thirty-two 30-day-old healthy New Zealand rabbits weighing  $1.07 \pm 0.25$  kg were housed in a spacious animal unit with good ventilation and hygienic conditions at 22 to 25°C on a 12 h light/dark cycle. The study design was approved by the Institutional Animal Care and Use Committee in Shanxi Medical University, Shanxi, China.

### Establishment of animal model

As shown in Table 1, all rabbits were randomly divided into four groups of four females and four males each, and had free access to the diets with a similar energy content *ad libitum* and distilled water with low-F. Notably, the diets were prepared according to physical circumstances during the dry grass season (Table 1). The F level in this study was chosen according to our previous investigation in Batou, a high F polluted area in China, where the average grass F content was up to  $204.4 \pm 66.5$  mg/kg during dry grass season (Wang et al., 1995).

### Organo-somatic index

After 120 days treatment, the body and liver weight of each animal was recorded. Based on these values, the organo-somatic index (OSI) was calculated by the following formula:

Organo-somatic index = Weight (g) of the liver / Weight (kg) of the body on Day 120

### Biochemical examination

Followed by weighing, blood samples were immediately collected by heart puncture. Serum was gotten, centrifuged at 3000 rpm for 10 min, and stored at  $-70^{\circ}\text{C}$  for further analysis. Then rabbits were anesthetized with 20% urethane (ethyl carbamate,  $\text{NH}_2\text{COOC}_2\text{H}_5$ ) solution, and livers were quickly obtained. One gram (1 g) of each liver sample was homogenized with 9 ml 0.9% saline solution at  $4^{\circ}\text{C}$ . The levels of serum total Pr (TPr) and albumin (ALB), and the activity of serum alkaline phosphatase (AKP), serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were determined with the reagent kits provided by the Nanjing Jianchen Biotech Company Limited (Nanjing, China). Additionally, the content of malondialdehyde (MDA), and activities of SOD and glutathione peroxidase (GSH-Px) in liver were also examined using the biochemical reagent kits (Nanjing Jianchen Biotech Company Limited China) prepared according to the manufacturer's instructions.

### Transmission electron microscopy studies

Five liver samples were randomly selected from each group for transmission electron microscopy (TEM) studies. In order to maintain the consistency and homogeneity of the detected sample, the lower edge of right liver lobe was chosen. The samples were cut into 1 mm thick sections and fixed in 3% glutaraldehyde in phosphate buffer (PH7.4) and post fixed in 2% osmium tetroxide in phosphate buffer. Following by fixation, samples were dehydrated at increasing concentrations of ethanol. They were then embedded in araldite resin ultrathin. About 50 nm thick sections were cut by an ultratome, and stained by uranyl acetate saturated in 70% ethanol and lead citrate, then observed and photographed using JEM-1400 (JEOL Limited, Tokyo, Japan) transmission electron microscope.

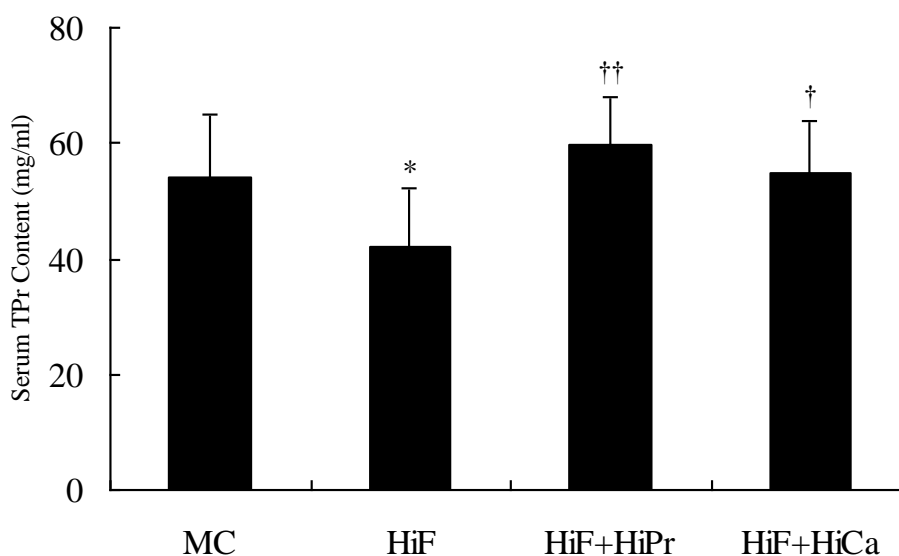
### Statistical analysis

Experimental data were expressed as mean  $\pm$  SD. Differences between groups were evaluated by independent-samples t-test using the SPSS 11.5 statistical software (SPSS Inc., Chicago, Illinois). Values of  $P < 0.05$  were considered statistically significant.

**Table 2.** Effects on body weight, organo-somatic liver index (g/kg), the serum ALB content (mg/ml), and the activity of AKP (U/100 ml) following exposure to HiF, HiF+HiPr, and HiF+HiCa for 120 days in rabbits (mean±SD; n=8).

Parameter	MC	HiF	HiF+HiPr	HiF+HiCa
Body weight	1.672 ± 0.173	1.457 ± 0.184*	1.891 ± 0.285††	1.495 ± 0.231
Organo-somatic liver index	30.30 ± 3.846	27.28 ± 3.861	29.64 ± 3.645	28.25 ± 3.051
ALB	31.21 ± 2.310	28.34 ± 2.94*	31.72 ± 2.82†	29.07 ± 2.43
AKP	6.274 ± 0.653	8.723 ± 1.074	11.870 ± 0.864	7.631 ± 0.695

\*p<0.05, (HiF group compared with MC group); †p<0.05, (HiF+HiPr group compared with HiF group); ††p<0.01, (HiF+HiPr group compared with HiF group).

**Figure 1.** Effects on serum TPr content (mg/ml) following exposure to HiF, HiF+HiPr, and HiF+HiCa for 120 days in rabbits (mean±SD; n=8). \*p<0.05, (HiF group compared with MC group); †p<0.05, ††p<0.01 (HiF+HiPr group compared with HiF group; HiF+HiCa group compared with HiF group).

## RESULTS

### Body weight and organo-somatic liver index

Exposure to HiF for 120 days significantly reduced ( $p<0.05$ ) the body weight of rabbits in comparison to the MC group, while body weights increased by Pr supplementation with statistical significance ( $p<0.01$ ). However, Ca supplementation showed no effect compared with the HiF group ( $p>0.05$ ). For organo-somatic liver index, after 120 days exposure, no significant difference was observed in HiF, HiF+HiPr and HiF+HiCa groups (Table 2).

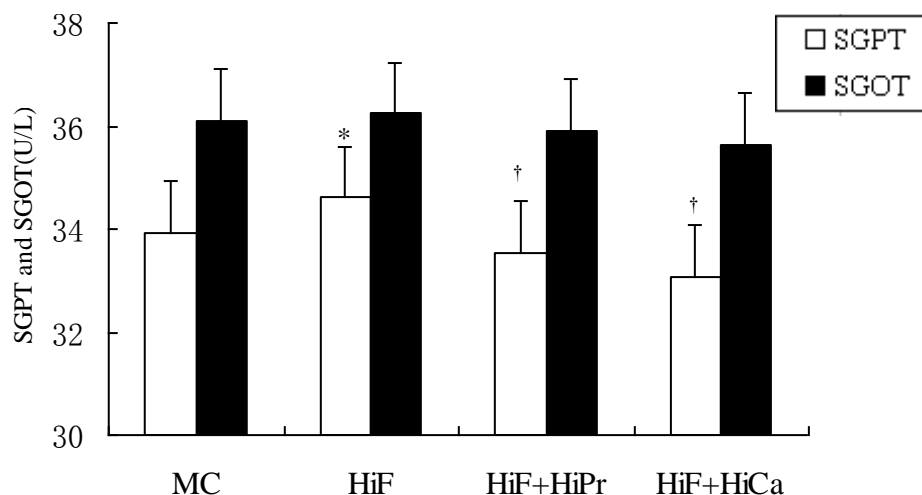
### The serum contents of total protein (TPr) and albumin (ALB)

As seen in Figure 1 and Table 2, compared to MC group, the contents of TPr and ALB in serum decreased

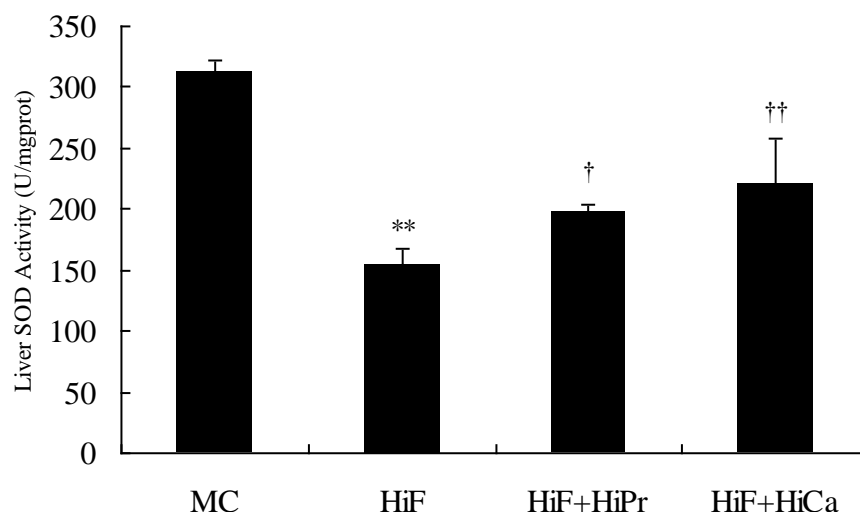
significantly ( $p<0.05$ ) in HiF group. In contrast with the HiF group, Pr supplementation increased significantly TPr ( $p<0.01$ ) and ALB ( $p<0.05$ ) levels. However, Ca only exhibited ameliorative roles in TPr ( $p<0.05$ ) but not in ALB, when compared with the HiF group ( $p>0.05$ ).

### Alkaline phosphatase (ALP), serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) activities in serum

No statistical difference in ALP was noted between MC group and HiF group, and between HiF group and HiF+HiPr or HiF+HiCa groups (Table 2). Compared with the MC group, high fluoride significantly increased the activity of SGPT. Supplementation of Pr or Ca decreased SGPT activity, with the comparison to HiF group. As for SGOT activity, no statistical difference was observed between MC group and HiF group, and between HiF



**Figure 2.** Effects on SGOT and SGPT (U/L) following exposure to HiF, HiF+HiPr, and HiF+HiCa for 120 days in rabbits (mean±SD; n=8). \*p<0.05, (HiF group compared with MC group); †p<0.05, (HiF+HiPr group compared with HiF group; HiF+HiCa group compared with HiF group).



**Figure 3.** Effects on liver SOD activity (U/mgprot) following exposure to HiF, HiF+HiPr, and HiF+HiCa for 120 days in rabbits (mean±SD; n=8).\*\*p<0.01, (HiF group compared with MC group); †p<0.05, ††p<0.01(HiF+HiPr group compared with HiF group; HiF+HiCa group compared with HiF group).

group and HiF+HiPr and HiF+HiCa groups (Figure 2).

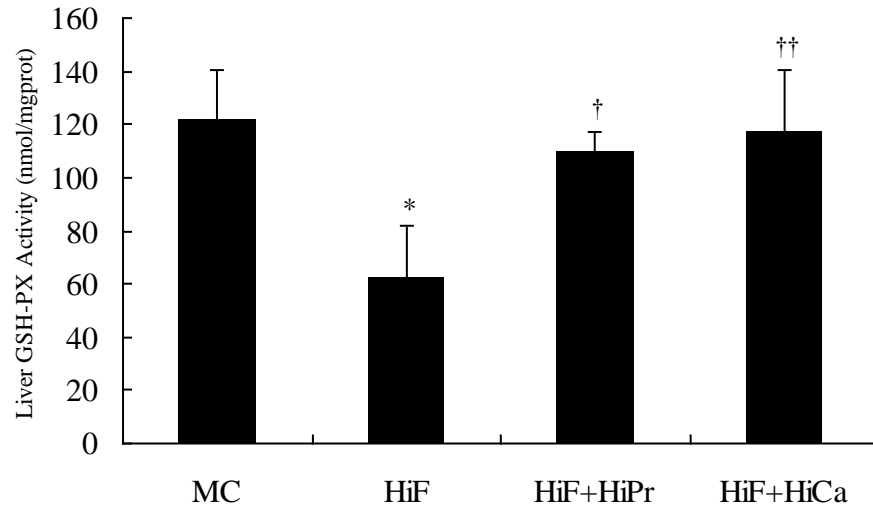
#### Superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) activities, malondialdehyde (MDA) content in liver

As seen in Figures 3 and 4, excessive F significantly inhibited the SOD (p<0.05) and GSH-PX (p<0.01) activities and content in liver, compared with the MC group. Interestingly, the two aforementioned indexes increased significantly in HiF+HiPr group (p<0.05) and

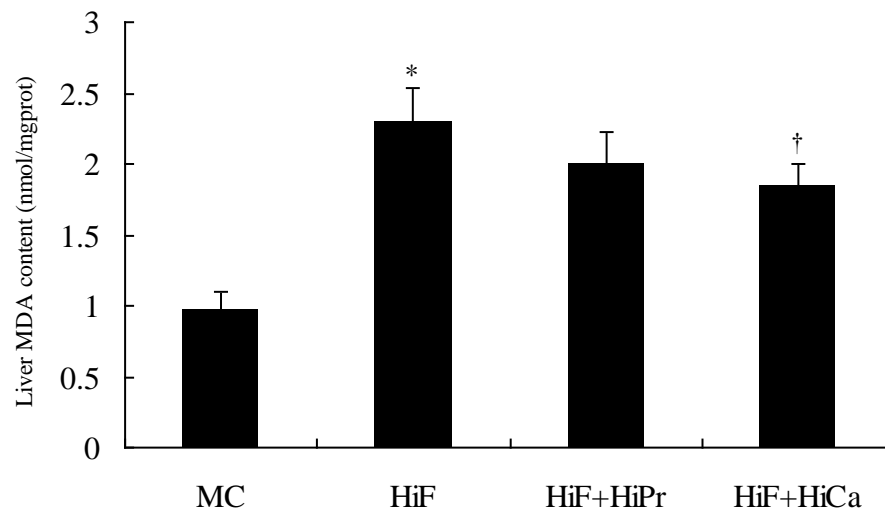
HiF+HiCa group (p<0.01). Compared to animals in MC group, MDA level in liver increased significantly (p<0.05) in HiF group, while Ca supplementation significantly reduced MDA level in comparison to HiF group (Figure 5). Pr supplementation exhibited little effect on MDA content.

#### Transmission electron microscopy observations

In Figure 6, the MC group showed mitochondria were abundant, polymorphic, with their cristae oriented



**Figure 4.** Effects on liver GSH-PX activity (nmol/mgprot) following exposure to HiF, HiF+HiPr, and HiF+HiCa for 120 days in rabbits (mean±SD; n=8).  $p < 0.05$ , (HiF group compared with MC group);  $^{\dagger}p < 0.05$ ,  $^{\dagger\dagger}p < 0.01$  (HiF+HiPr group compared with HiF group; HiF+HiCa group compared with HiF group).



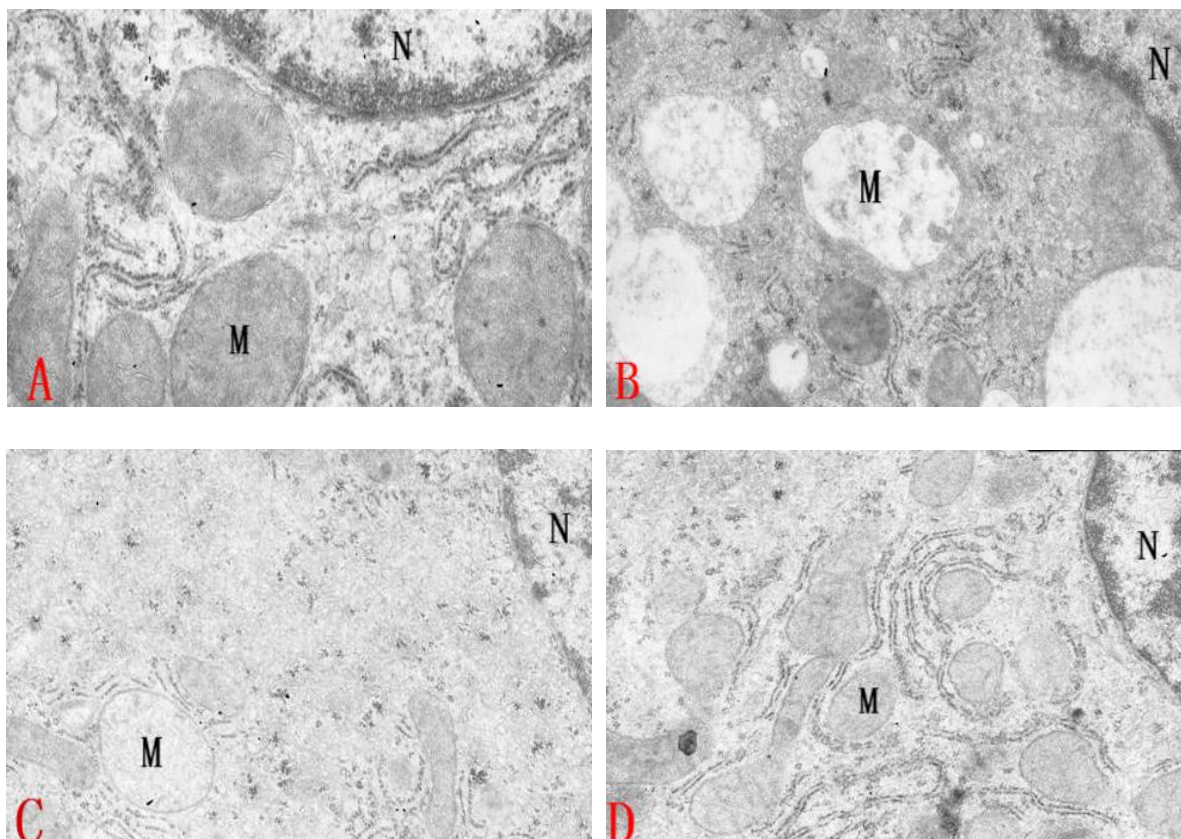
**Figure 5.** Effects on liver MDA content (nmol/mgprot) following exposure to HiF, HiF+HiPr, and HiF+HiCa for 120 days in rabbits (mean±SD; n=8).  $*p < 0.05$ , (HiF group compared with MC group);  $^{\dagger}p < 0.05$ , (HiF+HiCa group compared with HiF group).

transversely. Matrix granules were numerous, revealing that high electron density, rough endoplasmic reticulum, and glycogen granules were all discernible. In HiF group, the prominent alterations were that numerous mitochondria were swollen and enlarged. Mitochondrial matrix was of low electron density and appeared transparent. Many mitochondrial cristae were broken, with their membrane ruptured or disintegrated. In some areas, the outer and inner mitochondrial membranes were damaged. Administration of Pr during the F exposure period showed pronounced recovery in liver ultrastructural changes, although low electron density of

mitochondria were still visible in some part region. Ca supplementation along with F administration showed apparent recovery in liver ultrastructural as compared to F treatment alone. In HiF+HiCa group, mitochondria were abundant and polymorphic with their cristae oriented transversely. Matrix granules were numerous reflecting high electron densities.

## DISCUSSION

The primary findings in the present study are mainly as



**Figure 6.** TEM micrographs (X15000.) in liver of rabbits on the 120th day. (A), (B), (C), (D) represents results of liver from the MC, HiF, HiF+HiPr, and HiF+HiCa groups, respectively. nucleus (N), mitochondria (M).

follows. Firstly, HiF exposure obviously inhibited the growth and serum TPr level in rabbit, which were significantly resisted by Pr supplementation. Secondly, severe mitochondria lesions in hepatocytes, including matrix vacuolar degeneration, mitochondrial cristae broken, membranes damage were observed in HiF group. However, in HiF+HiPr and HiF+HiCa groups, evident liver ultrastructural repair occur. Thirdly, Pr or Ca supplementation significantly reduced the F-induced oxidative stress in rabbit liver.

In this study, organo-somatic liver in HiF group showed slight decrease with no statistical significance; however, after Pr or Ca supplementation, this index presented a recovery, especially for HiPr diet. Previous studies demonstrated that F inhibits Pr synthesis by weakening the beginning of the peptide chain and preventing the production of peptide chains in ribosomes (Moudgil et al., 1986). In the present study, serum TPr and ALB contents decreased significantly in the HiF group, compared with the MC group, which are similar to earlier reports about children and animal (Cenesiz et al., 2005; Shivashankara et al., 2000). Collectively, data from our and other laboratories suggested that excessive F could result in a low body weight of animals through adversely affecting Pr metabolism. In addition, serum TPr and ALB contents

exhibited obvious recovery in rabbits fed Pr or Ca supplemented diets, revealing that Pr or Ca could interfere with the F toxic action.

Zhang et al. (2004) reported that Ca malnutrition correlated with endemic fluorosis, and Ca supplementation can alleviate it. Another studies showed that high F intake can accelerate the formation of insoluble calcium fluoride in the intestines which was then excreted through the feces, thus causing low blood calcium in cases of insufficient dietary intake (Shivashankara et al., 2000). Therefore, it is evident that supplementation of Ca could reduce the F toxic effect.

Results in this study also indicated that Ca supplementation inhibited F toxicity by decreasing serum AKP activity. However, such change did not occur in HiF+HiPr group. The possible reason may be that protein, as a biomacromolecule, cannot permeate the cytomembrane, and therefore show little or no effect on the intracellular fluorion. Thus Pr supplement in the diet may not alter the serum AKP activity. As a sensitive index for liver pathological changes, SGPT activity was significantly increased in serum of rabbits treated with high F, which is coincident with results of previous investigation (Blaszczyk et al., 2011; Kanbur et al., 2009). Meanwhile, Pr or Ca supplementation significantly

decreased the activity of SGPT, exerting a protective role. The changes in biochemical indexes could be correlated with ultrastructural alterations. Ultrastructural examinations showed that elevated F caused changes in the size and shape of the mitochondria, including blurred mitochondrial crests and distorted membranes, which were depicted in previous studies (Chattopadhyay et al., 2011; Dabrowska et al., 2004, 2006). After Pr or Ca supplementation, the structural damages in liver were alleviated. It appears that remission of most pathomorphological changes by Pr or Ca may provide further evidence for their alleviative role in F-induced hepatotoxicity.

Accumulated literatures demonstrated that the enhanced lipid peroxidation and decreased activities of antioxidant enzymes play crucial roles in membrane structure lesion, systemic dysfunctions, and cell apoptosis. Interestingly, excessive F is highly related to the increased superoxide free radicals and lipid peroxidation. Numerous studies have reported that activities of antioxidant enzymes including SOD and GSH-Px were significantly decreased in animals exposed to F (Burgstahle, 2009; Chattopadhyay et al., 2011; Ghosh et al., 2008; Mittal and Flora, 2007; Ranjan et al., 2009). Similar results were also obtained in rabbit liver in the present experiment. On the other hand, MDA, as a main product during oxidative reactions, is considered an important biomarker for reflecting the level of lipid peroxidation. In the current study, F exposure for 120 days obviously enhanced the MDA content in liver of rabbits, which was consistent with previous investigations (Cenesiz et al., 2005; Zhan et al., 2005). In normal cases, there is a dynamic balance between free radical production and elimination. The increased MDA content and the decreased activities of antioxidant enzymes in this study suggested that the balance between the oxidative system and antioxidant system was broken during F exposure.

Our results also showed that Pr or Ca supplementation significantly alleviated the activities of SOD and GSH-Px, and inhibited MDA level, indicating a recovery in liver antioxidant defense. To our knowledge, protective effects of nutrition supplementation such as Pr or Ca on F-induced changes in SOD and GSH-Px, and MDA in liver have not been reported previously.

In conclusion, we observed that F resulted in morphological alteration and dysfunctions of liver under the malnutrition diet. Feeding Pr-rich or Ca-rich diets may be beneficial in reducing F-induced toxicity in rabbit liver. This investigation further confirmed earlier epidemiological survey. Furthermore, these findings could provide important clues in the reversal of F induced toxicity in endemic fluorosis regions in the world, especially in underdeveloped countries where Pr or Ca deficient. Administration of nutrient such as Pr or Ca may be beneficial in decreasing the liver damage caused by free oxygen radicals in fluoride-ingested persons.

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