ABSTRACT — Emerging evidence has demonstrated that iron overload plays an important role in oxidative stress in the liver. This study aimed to explore whether fluoride-induced hepatic oxidative stress is associated with iron overload and whether grape seed proanthocyanidin extract (GSPE) alleviates oxidative stress by reducing iron overload. Forty Kunming male mice were randomly divided into 4 groups and treated for 5 weeks with distilled water (control), sodium fluoride (NaF) (100 mg/L), GSPE (400 mg/kg bw), or NaF (100 mg/L) + GSPE (400 mg/kg bw). Mice exposed to NaF showed typical poisoning changes of morphology, increased aspartate aminotransferase and alanine aminotransferase activities in the liver, increased MDA accumulation, decreased GSH-Px, SOD and T-AOC levels in liver, indicative of oxidative stress. Intriguingly, all these detrimental effects were alleviated by GSPE. Further study revealed that NaF induced disorders of iron metabolism, as manifested by elevated iron level with increased hepcidin but decreased ferroportin expression, which contributed to hepatic oxidative stress. Importantly, the iron dysregulation induced by NaF could be normalized by GSPE. Collectively, these data provide a novel insight into mechanisms underlying fluorosis and highlight the potential of GSPE as a naturally occurring prophylactic treatment for fluoride-induced hepatotoxicity associated with iron overload.

Key words: Fluoride, Liver, Oxidative stress, Iron overload, Grape seed proanthocyanidin extract

INTRODUCTION

Excessive consumption of fluoride over a long period of time may result in a serious global public health problem, known as fluorosis. A substantial body of evidence has confirmed that chronic fluorosis produces harmful effects on teeth and bones, as well as on other organs including the heart, liver, and kidneys (Gazzano et al., 2010; Perumal et al., 2013). Hepatotoxicity is of particular concern to the public as the liver is the main organ responsible for the metabolism of fluoride ingested through the gastrointestinal tract. in vivo and in vitro studies have identified biochemical and cytotoxic alterations in the liver following excessive fluoride exposure (Agalakova and Gusev 2011; Chattopadhyay et al., 2011). Although the mechanisms by which fluoride elicits these toxic effects have not been fully elucidated, increasing evidence shows that fluoride can stimulate reactive oxygen species (ROS) generation and disturb the normal antioxidant systems in the liver, indicating that oxidative stress plays a vital role in fluoride-induced hepatotoxicity (J et al., 2013). Nonetheless, the detailed molecular mechanisms underlying oxidative stress induced by fluoride still remain largely unknown.

Mammalian cells require sufficient amounts of iron to satisfy their metabolic needs and also to accomplish specialized functions, including oxygen transport, electron transport, and xenobiotic metabolism (Bayeva et al., 2013; Lawen and Lane, 2013). Under normal circumstance, iron readily cycles between two redox states: ferrous and ferric ions; this provides electrons for enzymatic and free radical reactions (Pereira et al., 2012). However,
er, abnormally increased iron becomes cytotoxic via Fenton reactions (Fe$^{2+}$+H$_2$O$_2$→Fe$^{3+}$+OH·+·OH), producing powerful hydroxyl free radicals, thus leading to oxidative stress in tissues (Wydra et al., 2015). To combat this, intracellular iron concentrations are regulated by elaborate homeostatic systems. It is suggested that the hepcidin-ferroportin axis makes a major contribution to iron homeostasis in a host of organisms (Ganz and Nemeth, 2011b; Arezes and Nemeth, 2015). Hepcidin (encoded by HAMP), the hepatic peptide hormone, is a principal regulator of iron metabolism (Ganz and Nemeth, 2011b). Hepcidin binds to ferroportin (FPN), an only known iron efflux transporter bound to the cell membrane of hepatocytes and macrophages, where it induces FPN internalization and eventual degradation. Therefore, upregulation of hepcidin would reduce the level of FPN, in turn preventing iron efflux, causing iron accumulation, thus leading to oxidative damage via Fenton reactions (Ward and Kaplan, 2012; Ganz and Nemeth, 2011b; Ganz and Vaulont, 2012). This role of the hepcidin-ferroportin axis as the principal regulator of extracellular iron metabolism in health and disease makes it a promising target for the treatment of iron disorders (Ganz and Nemeth, 2011a).

Recently, we have found that iron overload is implicated in fluoride-induced oxidative injury in human embryo hepatocytes, and that this iron overload associated oxidative stress can be rescued by a naturally occurring botanicals called grape seed proanthocyanidin extract (GSPE) (Niu et al., 2016), whose antioxidant capacity is closely related to its iron-chelating effects (Bagchi et al., 2014; Weber et al., 2007). However, the in vivo evidence of fluoride-induced iron overload correlated with oxidative stress and the protective effect of GSPE in the liver is still lacking. Therefore, this study aimed to elucidate the role of iron overload in fluoride-induced oxidative stress in mouse liver and whether GSPE alleviates the oxidative damage by reducing iron overload.

MATERIALS AND METHODS

Chemicals

GSPE (> 99% purity) and NaF were purchased from Beijing Chemical Reagent Corp. (Beijing, China). Taq DNA polymerase, dNTP mix, and SYBR PrimeScript RT-PCR Kits were purchased from Takara Corp. (Dalian, China). The primers for HAMP, FPN, and β-actin were synthesized and purified by Invitrogen Corp. (Shanghai, China). The anti-GAPDH antibody was purchased from Kangche Bio-Tech (Shanghai, China). Hepcidin and FPN antibodies were purchased from Abcam Inc. (Cambridge, MA, USA). The assay kits for iron, aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), and malondialdehyde (MDA) were obtained from the Nanjing Jiancheng Bio-engineering Research Institute (Nanjing, China). All other chemicals were of analytical grade and obtained commercially.

Animals and treatment

The experimental protocol was approved by the Ethics Review Committee for Animal Research at Huazhong University of Science and Technology. Forty healthy Kunming male mice weighing 20-25 g were obtained from the laboratory center at Huazhong University of Science and Technology (Wuhan, China). Animals were kept in well-ventilated cages under specific pathogen-free conditions in a temperature-controlled room at 22-25°C, with a 12-hr light-dark cycle. They received a standard laboratory diet composed of 60% ground corn meal, 15% ground beans, 10% bran, 10% corn oil, 3% casein, 1% mineral mixture, and 1% vitamin mixture and were supplied with water ad libitum throughout the experimental period.

After 1 week of acclimation, the animals were randomly divided into four groups and each group consisted of 10 animals. Group 1 was exposed to distilled water as the untreated control group. Group 2 was given drinking water containing NaF (100 mg/L). Group 3 was intra-gastrically administrated GSPE (400 mg/kg body weight). Group 4 was given drinking water containing NaF (100 mg/L) and intra-gastrically administrated GSPE (400 mg/kg body weight). These dosages were chosen on the basis of previous studies (Zhou et al., 2015; Niu et al., 2015). All of the mice were treated daily for 5 weeks.

After 5 weeks treatment, the mice were weighed prior to sacrifice. The liver was then dissected and weighed to the nearest milligram on an electronic balance after removing the blood and other adherent tissue. After weighing, three of liver sections were created. One section was fixed in Bouin’s solution for histopathological analysis, another section was used for the preparation of tissue homogenates, and the remaining section was frozen immediately in liquid nitrogen and stored at -80°C for RNA isolation and protein extraction.

Preparation of liver homogenates

Tissue homogenates were prepared by placing liver tissue in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, and the protein inhibitors, sodium pyrophosphate, β-glycerophosphate, ethylenediaminetetraacetic acid (EDTA), Na$_2$VO$_4$, and leupeptin (Beyotime Biotechnology, Shanghai, China), and...
then homogenizing with a TissueLyser (Qiagen, Hilden, Germany). The protein concentrations of these homogenates were measured with a Bradford protein assay kit (Beyotime Biotechnology).

**Histopathological examination**
Liver tissues were taken and washed with the normal saline solution immediately after sacrifice. The tissues were fixed in 10% neutral formalin for 24 hr, dehydrated in different grades of alcohol, and processed for paraffin embedding. Sections that were 5 μm thick were cut using a rotary microtome. The sections were processed and passed through a graded alcohol series, stained with hematoxylin and eosin, cleared in xylene, and examined microscopically.

**Oxidative stress**
GSH-Px, SOD, T-AOC, and MDA levels were detected using commercially available kits, strictly according to the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute). Results were normalized to the total amount of protein, measured by bicinchoninic acid assay (BCA).

**Liver function**
AST and ALT levels were assayed using commercially available kits, strictly according to the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute).

**Iron content**
The liver iron content was determined by a commercially available kit from the Nanjing Jiancheng Bioengineering Institute. Briefly, Fe³⁺ was reduced to Fe²⁺ in an acidic buffer solution, after which Ferene S reacted with Fe²⁺ to produce a blue color that was measured by colorimetry at 593 nm.

**Real-time PCR**
RNA was extracted from liver tissues using the TRIzol method. The A260/280 ratio was in the range of 1.8-2.0 and RNA integrity was verified by agarose gel electrophoresis. cDNA was synthesized by reverse transcribing 4 μg of total RNA in a final reaction volume of 20 μL using a First-Strand cDNA Synthesis Kit, according to the manufacturer’s instructions. Primer oligonucleotide sequences specific for quantitative real-time PCR (qPCR) are shown in Table 1 and were designed and synthesized by Sangon Biotech Inc. (Shanghai, China). qPCR was conducted using the SYBR PrimeScript RT-PCR Kit, following the manufacturer’s protocol, on an ABI Prism 7900 Sequence Detection System. The PCR mixtures contained 1 μL cDNA, 10 μL SYBR Premix Ex Taq 2 ×, and 0.25 μM forward and reverse primers, in a final volume of 20 μL. Triplicate reactions were performed, starting with a polymerase activation step for 10 sec at 95°C and followed by 40 cycles of 5 sec at 95°C and 20 sec at 60°C. Fluorescence data were acquired after each cycle and β-actin was used as an endogenous control to calculate relative quantitative gene expression values. The absence of primer dimers and unspecific products was verified after every run by a melting curve analysis (72-95°C) and agarose gel electrophoresis.

**Western blotting**
Liver tissues were homogenized in RIPA lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM Phenylmethanesulfonyl fluoride (PMSF), and 1 × Roche complete mini protease inhibitor cocktail). The supernatants were collected after centrifugation at 10,000 × g at 4°C for 15 min. The protein concentrations were determined using a BCA assay kit. Equal amounts of protein were separated by 10% SDS polyacrylamide gel electrophoresis under denaturing and non-reducing conditions prior to transferring them to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in TBST at room temperature for 1 hr and then incubated with the indicated primary antibody at 4°C overnight. After washing in TBST, the blots were incubated with a horseradish peroxidase-coupled secondary antibody and the protein bands were visualized using enhanced chemi-
luminescence and analyzed using Gel-Pro Analyzer version 3.0 software.

**Statistical analysis**

Results were expressed as the mean ± standard deviation (SD) of at least three experiments, performed in triplicate. Data were evaluated statistically using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for the comparison of independent means with SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). The level of significance was set at $P < 0.05$.

**RESULTS**

There were no clinical signs of dysfunction in the treated rats over the course of the experimental period, nor were there any significant deviations in body weight gain or the liver index in the rats.

**NaF induced liver histology damage that was attenuated by GSPE**

As shown in Fig. 1, the control group showed normal histological features. GSPE treatment did not cause noticeable morphological changes to the liver, as compared with the control group. The liver histology in NaF-treated mice showed signs that were typical of poisoning, such as steatosis, dim boundary, dismissed cell membrane, edema and cytoplasm disintegrating pieces; in contrast, all of these changes were mitigated by co-administration of GSPE.

**NaF impaired liver function that was alleviated by GSPE**

As shown in Fig. 2, the activities of AST and ALT were significantly higher in the NaF group than in the control group ($P < 0.05$). However, the activities of AST and ALT in the NaF + GSPE group were markedly lower than those in the NaF group ($P < 0.05$).

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**Fig. 1.** NaF induced liver histology damage that was attenuated by GSPE. Mice were orally exposed via drinking water containing NaF (100 mg/L) and/or intragastrically administrated GSPE (400 mg/kg body weight) for 5 weeks. Fixed liver sections of 1 mouse were stained with hematoxylin and eosin (HE) and observed by light microscopy (magnification 200 ×). The histological changes in the liver of NaF-treated mice showed steatosis (black arrows), dim boundary (red arrows), dismissed cell membrane (yellow arrows), edema (blue arrows) and cytoplasm disintegrating pieces (green arrows).
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Fig. 2. NaF impaired liver function that was alleviated by GSPE. Mice were treated with NaF and/or GSPE in indicated doses for 5 weeks. The liver tissue homogenate of 3 mice was prepared as for evaluation of ALT (a) and AST (b) activities. Values were represented as the mean ± S.D. of three independent determinations, assayed in triplicate. Significant differences were detected by one-way ANOVA, followed by the SNK test: *P < 0.05 vs. the control group, #P < 0.05 vs. the NaF group.

DISCUSSION

Chronic fluoride intoxication has been reported to cause toxicity to the liver (Gazzano et al., 2010; Perumal et al., 2013). In the present study, daily exposure to 100 mg/L NaF for 5 weeks was found to cause liver toxicity, as evidenced by strong increases in AST and ALT levels, and hepatocyte degeneration. This is in agreement with the observation that fluoride caused significant increases in AST and ALT activities due to hepatocyte damage in mice and rats (Bouaziz et al., 2006; He et al., 2014). It is suggested that oxidative stress contributes to fluoride-induced liver toxicity (J et al., 2013). In agreement with this, the present study showed a significant increase in the liver MDA level and decreased levels of GSH-Px, T-AOC, and SOD activities in fluoride-treated mice, as compared to controls. Our results are consistent with a previous report showing an elevated liver MDA level in mice experimentally intoxicated with fluoride (Nabavi et al., 2013). Similarly, Bharti et al. (2014) demonstrated that the levels of GSH-Px, T-AOC, and SOD activities in fluoride-treated mice were significantly lower than those observed in the NaF group (P < 0.05 for both comparisons).
overload is able to induce oxidative stress (Handa et al., 2016; Marques et al., 2015). Free radical-mediated hepatocyte injury is one of the most thoroughly studied mechanisms proposed to explain the liver toxicity of excessive iron (Zhang et al., 2012). In iron overload patients or animals, accumulated iron facilitates the formation of free radical that disrupt the redox balance of the cells, thus causing oxidative stress (Toyokuni, 2011). Although previously we have confirmed that iron overload is involved in fluoride-induced oxidative injury in human embryo hepatocytes (Niu et al., 2016), the in vivo evidence of fluoride-induced oxidative stress resulting from iron overload is still obscure. The results of present study showed an increased total iron content in fluoride-treated mouse liver, suggestive of iron overload. Furthermore, significant increases in hepcidin mRNA and protein expression, accompanied by decreased FPN expression in fluoride-treated mouse liver were also observed. The production of hepcidin is normally regulated by iron stores, where-

Fig. 3. NaF induced liver oxidative stress that was mitigated by GSPE. Mice were treated with NaF and/or GSPE with indicated doses for 5 weeks. The liver tissue homogenate of 3 mice was prepared for evaluation of oxidative stress markers: (a) GSH-Px, (b) MDA, (c) T-AOC, (d) SOD. Values were represented as the mean ± S.D. of three independent determinations, assayed in triplicate. Significant differences were detected by one-way ANOVA, followed by the SNK test: *P < 0.05 vs. the control group, #P < 0.05 vs. the NaF group.

Fig. 4. NaF increased liver iron content that was reduced by GSPE. Mice were treated with NaF and/or GSPE in indicated doses for 5 weeks. The liver tissue homogenate of 3 mice was prepared for evaluation of iron content. Values were represented as the mean ± S.D. of three independent determinations, assayed in triplicate. Significant differences were detected by one-way ANOVA, followed by the SNK test: *P < 0.05 vs. the control group, #P < 0.05 vs. the NaF group.
by more hepcidin is produced by hepatocytes when the iron is abundant, thus limiting further iron absorption or release from stores (Ganz and Nemeth, 2011b). Hepcidin regulates iron homeostasis by binding to the iron receptor, FPN, causing its internalization and lysosomal degradation. The removal of FPN results in decreased iron export from hepatocytes, inducing iron accumulation in the liver (Ward and Kaplan, 2012; Ganz and Nemeth, 2011b; Ganz and Vaulont, 2012). In this study, fluoride may exert a certain impact on iron metabolism, cause iron accumulation in the liver (Ward and Kaplan, 2012; Ganz and Nemeth, 2011b; Ganz and Vaulont, 2012). In this study, fluoride may exert a certain impact on iron metabolism, cause iron accumulation in the liver of mouse, eventually leading to liver iron overload. This is supported by our previous in vitro study that fluoride induced iron overload accompanied by increased Hepcidin but decreased FPN expression (Niu et al., 2016). Similarly, Lee et al. (2015) demonstrated that excessive iron induced hepcidin activation and FPN degradation in human liver SK-HEP-1 cells, thus resulting in reactive oxygen species (ROS) generation. Taken together, it is conceivable that fluoride-elicited iron increase could lead to the production of highly toxic hydroxyl radicals via the Fenton reactions, thereby elevating the level of oxidative stress in liver.

A number of in vitro and in vivo studies have indicated that GSPE possesses a broad spectrum of pharmacological and medicinal properties that include antagonizing oxidative stress (Bagchi et al., 2014; Weber et al., 2007). The current study identified significantly less hepatotoxicity in mice co-treated with NaF and GSPE, with markedly fewer histopathological and liver enzymes (AST and ALT) changes, as compared to those treated with NaF.
alone. Furthermore, the liver MDA accumulation was markedly decreased in co-treated animals, whereas the liver levels of GSH-Px, T-AOC, and SOD were significantly increased. These results were consistent with the preventive effects of proanthocyanidin on plasma MDA accumulation in fluoride-treated mice (Attia et al., 2010). Moreover, decreased iron content and hepcidin mRNA and protein expression, but increased FPN mRNA and protein expression were observed in NaF and GSPE co-treated group when compared to NaF group. A substantial body of work has confirmed that polyphenol compounds produce cytoprotective effects via iron chelation, and that both iron binding and lipophilicity are important determinants of their overall antioxidant activities (Nazimabashir et al., 2014). The catechol-containing polyphenols in GSPE can chelate iron ions, preventing the production of free radicals normally caused by iron overload, triggering a reduction in hepcidin levels and an increase in FPN levels, activating iron efflux from hepatocytes and reducing liver oxidative stress. The present results reflect the iron chelating effect of GSPE on relieving of fluoride-induced oxidative stress. A similar study by Wu et al. (2010) also exhibited that GSPE chelated iron and protected against neuronal death in a wide range of cellular and animal models of neurological disorders. Overall, these results showed that GSPE could protect the liver from the fluoride-induced oxidative damage by alleviating iron overload.

In conclusion, the results of the present study showed that iron overload plays a vital role in fluoride-mediated hepatic oxidative damage in mouse. Furthermore, GSPE is capable of mitigating this oxidative stress by alleviating iron overload. These data provide a novel insight into mechanisms underlying fluorosis and highlight the potential of GSPE as a naturally occurring prophylactic treatment for fluoride-induced hepatotoxicity associated with iron overload.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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