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PROTECTIVE EFFECT OF GRAPE SEED PROCYANIDIN EXTRACT AGAINST FLUORIDE-INDUCED HEPATIC INJURY IN RATS

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ABSTRACT: This study aimed to determine the effects of grape seed procyanidin extract (GSPE) in alleviating sodium fluoride (NaF)-induced hepatic injury. Rats were randomly divided into four groups: control, NaF, NaF+GSPE, and NaF+Vc (vitamin C). After 28 days of intervention, key liver function-related biochemical parameters, oxidative stress markers, and histopathological changes in the hepatic tissue were analyzed. The oxidative stress was closely related to hepatic cell apoptosis as measured using the TUNEL method. In addition, the levels of apoptosis-related proteins Bax and Bcl-2 were determined by western blot. Compared with the control group, the NaF group showed reduced levels of antioxidant enzymes GSH, CAT, and SOD, with concomitantly increased MDA content, apoptosis, and histopathological damage in the liver tissues. However, GSPE administration significantly attenuated the oxidative stress and apoptosis in hepatic tissue and recovered histopathological damage. In addition, GSPE supplementation up-regulated anti-apoptotic Bcl-2 level and decreased pro-apoptotic Bax level. In conclusion, our results suggest that GSPE can exert antioxidant and anti-apoptotic protective effects against fluoride-induced hepatic injury in a rat model.

Key words: Grape seed procyanidin extract; Sodium fluoride; Oxidative stress; Hepatic apoptosis.

INTRODUCTION

Fluoride is widely distributed in the environment and accumulates in the human body mainly through food and water intake. However, excessive fluoride intake can result in fluorosis,[1,2] that in addition to its well-documented effects on teeth and bones, also causes toxic damage in many other organs and tissues.[3] For instance, the liver as a highly metabolically active organ is especially vulnerable to these types of toxicants.[4] Various studies have been published on fluoride-induced hepatotoxicity in both humans and animals,[5-7] that indicated the role of fluoride in impairing hepatic antioxidant enzyme activity and inducing oxidative stress. The oxidative stress can trigger apoptotic cell death in affected hepatocytes;[8-10] however, antioxidant treatment can prevent such apoptosis and alleviate hepatic damage.[2,11] While the specific mechanisms governing fluoride-induced hepatotoxicity remain to be fully clarified, they are nonetheless clearly linked to oxidative stress and hepatocyte apoptosis.

Fluorosis is an irreversible process but can be treated with antioxidants such as quercetin, gallic acid, and anthocyanins to prevent its development.[2, 5, 6] Grape seed proanthocyanins extract (GSPE) is a bioactive derivative of grape seeds that have been shown to exhibit diverse pharmacological and biochemical properties,[12,13] exerting both antioxidant and anti-apoptotic effects in multiple studies.[14-16] Thus, GSPE represents a promising therapeutic agent for the treatment of fluoride-induced hepatic injury, yet no studies to date have examined its ability to protect against hepatic oxidative stress or associated apoptotic cell death following fluoride

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exposure. Lipid peroxidation has been well established as a mechanism of cellular injury, and usually plays an important role in the induction of oxidative stress. On the one hand, malondialdehyde (MDA) can be used as an indicator and terminal product of lipid peroxidation. Furthermore, catalase (CAT), superoxide dismutase (SOD), and reduced glutathione (GSH) are recognized as important parameters that reflect the antioxidant capacity of the body. Therefore, these parameters were evaluated in the current study as markers of lipid peroxidation and antioxidant potential. This study aimed to explore the *in vivo* protective effects of GSPE in a fluoride-induced hepatic injury rat model. Specific focus was placed on its ability to modulate the expression of the key apoptosis-related proteins Bcl-2 and Bax to clarify the potential hepatoprotective mechanisms.

MATERIALS AND METHODS

Chemicals and Reagents

GSPE (≥95% pure) was purchased from Tianjin Peak Natural Product Research Development (China). The compositional analysis by the manufacturer demonstrated that the procyanidin extract was composed of oligomeric procyanidins (82.5%) and procyanidin B2 (1.94%). The MDA (S0131) and SOD (S0101) assay kits were purchased from Beyotime Biotechnology (Shanghai, China). The GSH (BC1170) and CAT (BC0200) assay kits were obtained from Solar Bioscience & Technology (Beijing, China). A total protein assay kit (A045-4-1) was purchased from Jian Cheng Bioengineering Institute (Nanjing, China). A TUNEL assay kit (KGA703) was obtained from KeyGen Biotechnology (Nanjing, China). Anti-Bcl2 (ab196495), anti-Bax (ab53154), and anti-GAPDH (ab181602) were purchased from Abcam (Cambridge, UK).

Animals and Treatment

Male Sprague-Dawley rats (240–260 g) were purchased from Sippr-BK Laboratory Animal (Shanghai, China). In total, 32 rats were randomized into four treatment groups: control, NaF (sodium fluoride), NaF+GSPE, and NaF+Vc (vitamin C). NaF was administered over 21 days through their drinking water (600 ppm). Rats in the latter two treatment groups initially underwent a 7-day pre-treatment with GSPE (100 mg/kg) or vitamin C (100 mg/kg) via oral gavage before initiating NaF treatment. After the 21 days, liver tissues were collected and stored at –80 °C before analysis. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of the Animal Management Rules of the Health Ministry of the People's Republic of China (documentation number 55, 2001, China), and the study was approved by the Animal Care and Use Committees of Xinxiang Medical University (approval no, XXLL20170108).

Oxidative Stress Marker Analyses

Hepatic MDA content, GSH levels, and CAT/SOD activity were quantified using commercial kits based on the manufacturer’s instructions to evaluate the impact of NaF treatment on oxidative stress in the liver.

Serum Biochemistry

Blood samples were collected from all rats and were centrifuged at 3,000 rpm for 10 min. Supernatant serum was then stored at –20 °C for further analysis. Serum biochemical parameters, including alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST), albumin (ALB), direct bilirubin (DBIL), total bilirubin (TBIL), total protein (TP), total cholesterol (TC), triacylglycerol (TG), and high-density lipoprotein (HDL) were quantified with commercially available kits.

Tissue Histology and TUNEL Staining

Samples of collected hepatic tissue were fixed with 4% paraformaldehyde and paraffin-embedded. These tissues were then either subjected to hematoxylin and eosin (H&E) staining to assess tissue histology or stained with a TUNEL assay kit (KGA703; Solar Bio) to quantify hepatocyte apoptosis. Following TUNEL staining, cells with brown nuclear granules were considered to be TUNEL-positive and thus apoptotic. The apoptotic index was calculated by counting the number of apoptotic cells in each of five random (200) fields of view as follows: TUNEL-positive cells / total cells 100%.

Western Blotting

Liver tissue samples were homogenized by incubating them for 30 min in total protein extraction lysis buffer supplemented with phenylmethanesulfonyl fluoride (PMSF), protease inhibitors, and a phosphatase inhibitor. Lysates were then centrifuged for 10 min at 12,000 rpm at 4 °C, and the protein levels were measured with a protein assay kit. A total of 30 µg per protein sample was then separated via 10% SDS-PAGE and transferred to a PVDF membrane which was subsequently blocked for 1.5 h using 5% non-fat milk in TBST. Blots were then probed overnight with anti-Bcl-2 (1:500), anti-Bax (1:500), or polyclonal anti-GAPDH (1:1,000) at 4 °C. After being washed with TBST, blots were then probed with an HRP-conjugated secondary antibody (1:6,000) for 1 h, washed thrice in TBST, and analyzed via enhanced chemiluminescence with an ECL Western blotting detection system (NCI5079; Thermo Fisher Scientific, Waltham, MA, USA). Quantity One v4.6.6 was used to quantify the protein levels of the analyzed samples.

Statistical Analysis

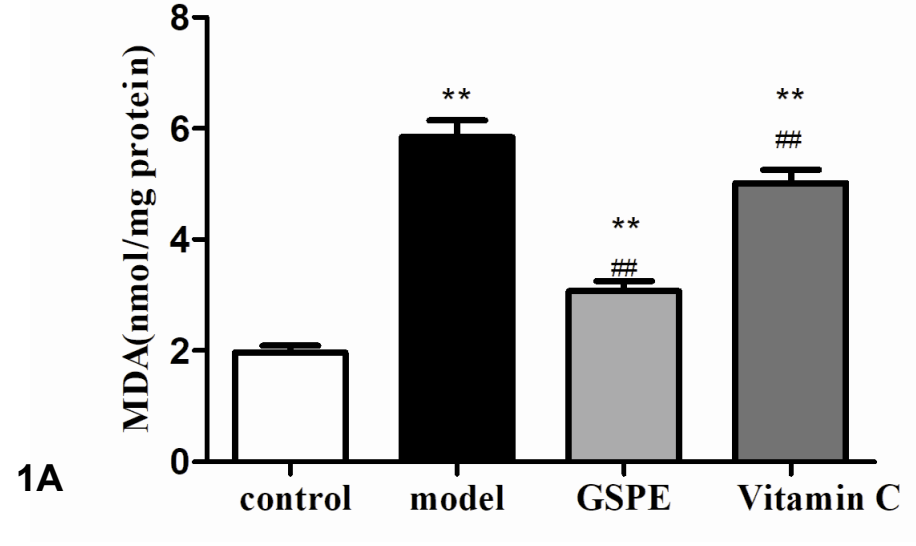
All statistical analyses were conducted using SPSS v11.5. The data are expressed as mean ± standard deviation (SD). One-way ANOVA was used for statistical inference. The LSD test was used to determine significance between groups. $P<0.05$ was considered statistically significant.

RESULTS

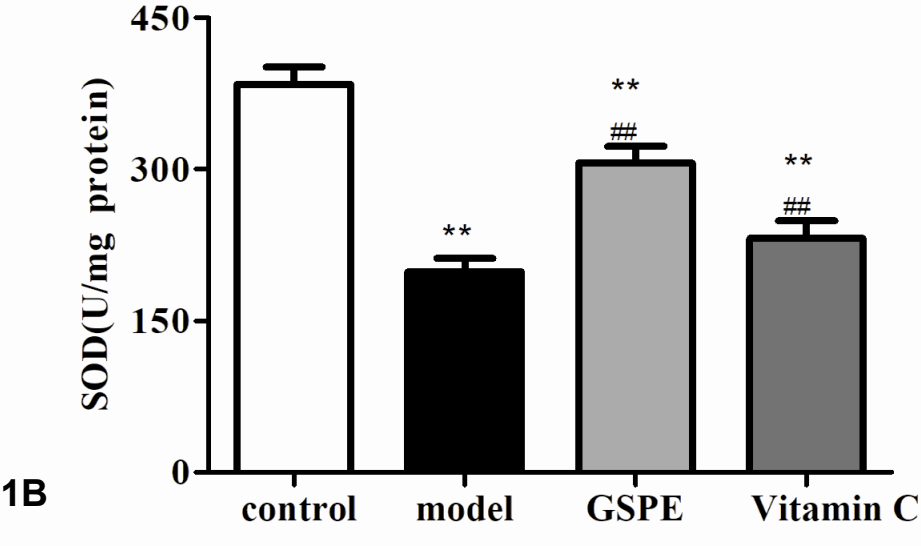
GSPE Reduces the Severity of Fluoride-Induced Liver Oxidative Damage

Oxidative damage analysis results revealed that NaF treatment significantly increased the MDA content in the NaF rats relative to controls ($P<0.01$), whereas the hepatic GSH, SOD, and CAT levels were significantly decreased ($P<0.01$) (Figures 1A–1D). In contrast, GSPE treatment was associated with a significant reduction in MDA levels and an increase in GSH, SOD, and CAT activity relative to the NaF

group (P<0.01). Vitamin C treatment resulted in similar changes in these oxidative stress indicators compared to the GSPE group, suggesting that GSPE can protect against NaF-induced oxidative stress in the liver.

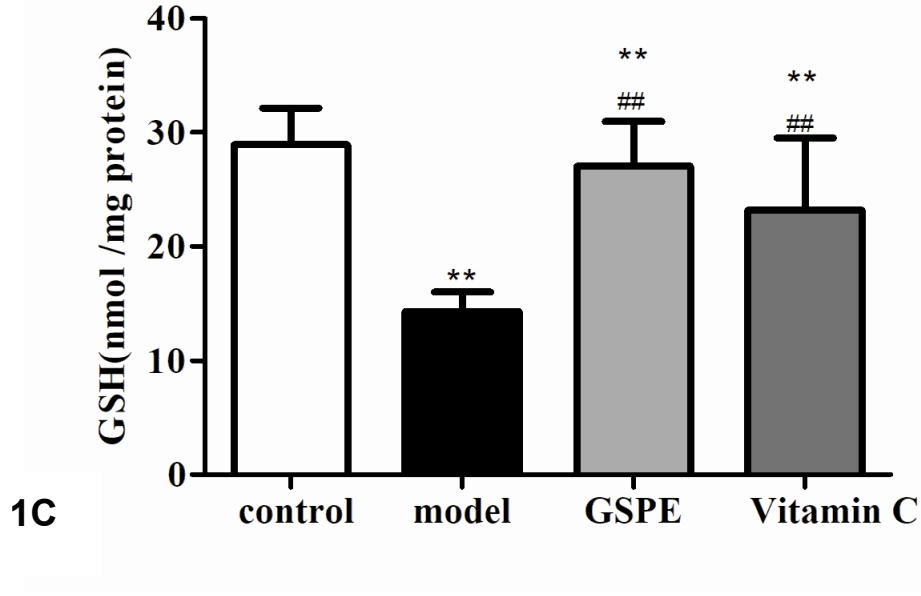


1A

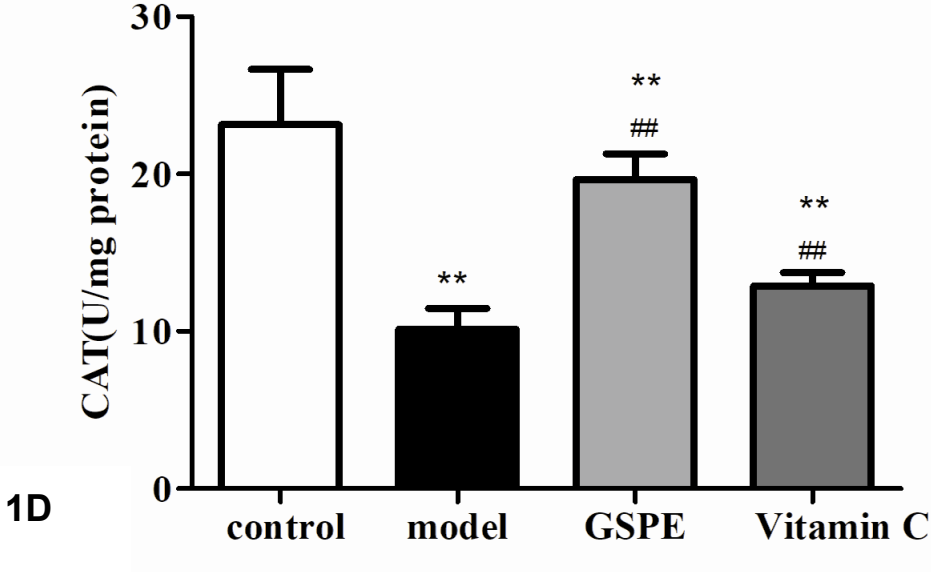


1B

Figures 1A and 1B. GSPE attenuates fluoride-induced liver oxidative damage. The results were presented as means±SD (n=8). **P<0.01, vs. control group, #P<0.05, ##P<0.01, vs. model group. IA: Effect of GSPE and vitamin C on MDA (ng/mg protein); 1B: Effect of GSPE and vitamin C on SOD (U/mg protein).



1C



1D

Figures 1C and 1D. GSPE attenuates fluoride-induced liver oxidative damage. The results were presented as means±SD (n=8). **P<0.01, vs. control group, #P<0.05, ##P<0.01, vs. model group. ID Effect of GSPE and vitamin C on GSH (ng/mg protein); 1D: Effect of GSPE and vitamin C on CAT (U/mg protein).

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GSPE Suppresses Fluoride-Induced Disruption of Liver Function

To directly examine the effects of GSPE on fluoride-induced changes in liver functionality, we measured a series of biochemical parameters in the rat model. Following NaF treatment for 21 days, significant changes in these biochemical parameters were detected in NaF-treated rats (Table 1), whereas GSPE co-treatment was sufficient to reverse these abnormalities, consistent with the ability of this extract to prevent NaF-induced disruption of liver function ($P<0.01$).

Table 1. Changes in the levels of serum biochemical parameters in rats of each groups. The results were presented as means ± SD (n=8)

Biochemical parameters	Control	Model	GSPE	Vitamin C
ALP(U/L)	18.83±2.41	50.98±5.35**	23.93±0.98##	38.01±2.11##
AST(U/L)	162.27±5.97	358.46±21.45**	211.46±15.85##	336.62±16.86#
ALT(U/L)	68.62±8.52	149.65±18.69**	82.96±7.08##	106.37±5.13##
ALB (g/dl)	4.84±0.57	3.17±0.35**	4.55±0.34##	3.74±0.25##
DIBL (mg/dl)	0.48±0.03	2.40±0.12**	1.02±0.05##	2.04±1.02##
TBIL (mg/dl)	1.19±0.14	2.95±0.19**	1.66±0.15##	2.46±0.14##
TP (mg/dl)	8.32±0.32	6.91±0.43**	7.94±0.28#	7.26±0.39
TC (mg/dl)	97.54±8.70	158.26±21.30	118.29±8.16	130.29±8.04
TG (mg/dl)	97.54±8.70	158.26±21.30**	118.29±8.16##	130.29±8.04##
HDL(mg/dl)	49.27±3.64	33.14±3.96**	43.25±5.17##	37.11±1.57#

**P < 0.01, vs. control group: #P <0.05, ##P <0.01, vs. model group

GSPE Ameliorates Fluoride-Induced Liver Damage

Histopathology analyses of liver tissues in different rat groups revealed that the hepatocytes of control animals were intact and regularly arranged without any evidence of necrosis or degeneration (Figures 2A–2H). In contrast, NaF treatment was associated with clear signs of damage and inflammatory cell infiltration. Importantly, the administration of both GSPE and vitamin C was sufficient to substantially ameliorate this NaF-induced liver damage. We then examined hepatocyte apoptosis via a TUNEL staining approach (Figures 3A–3I), revealing

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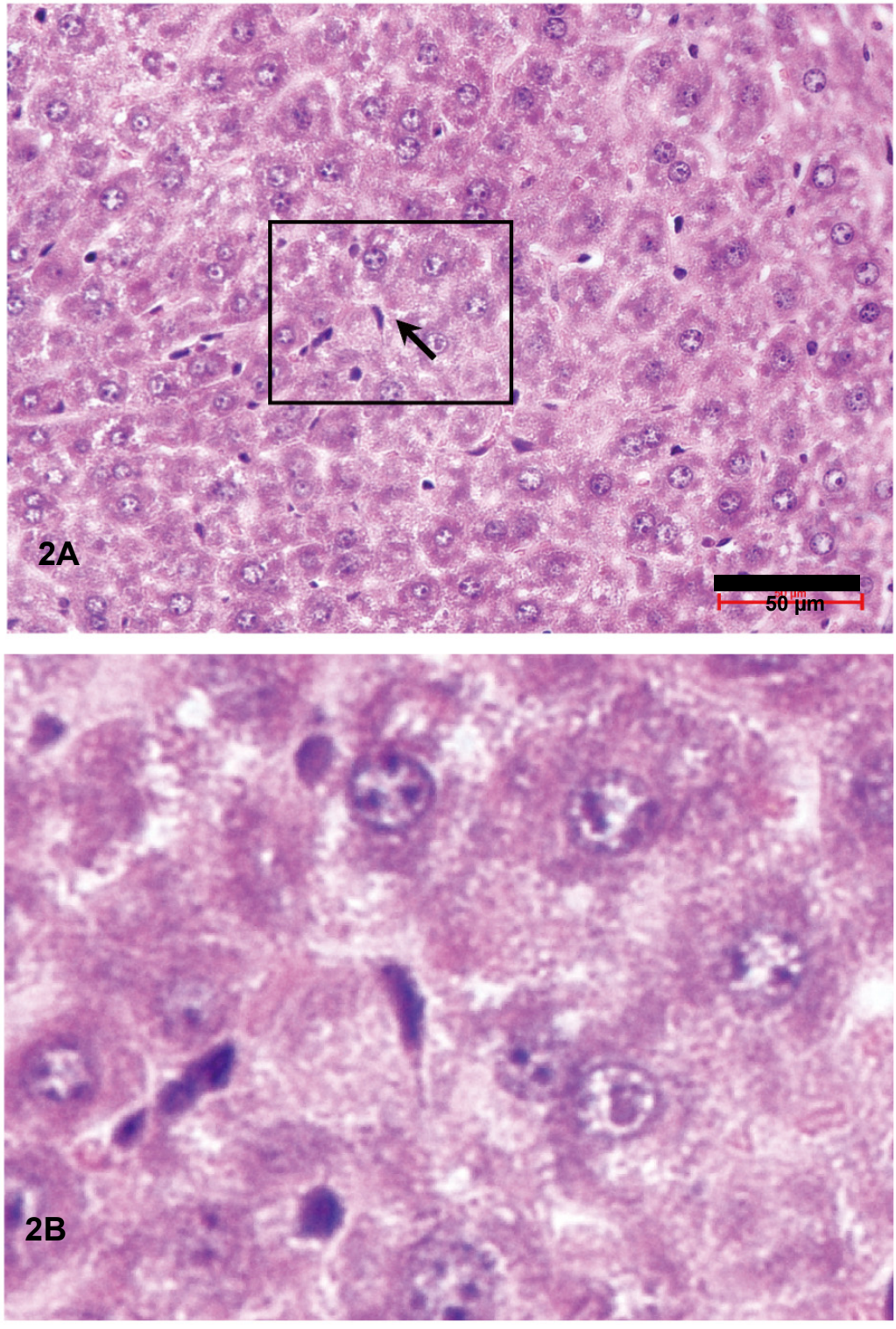
significantly higher rates of apoptotic cell death in the NaF group relative to the control group, whereas GSPE and vitamin C administration both significantly alleviated this apoptotic phenotype (P<0.05; Fig. 3).



Figures 2A and 2B. GSPE improves fluoride-induced liver pathological damage (n=8). 2A: Control; 2B: Control with high magnification. Haematoxylin and eosin staining.

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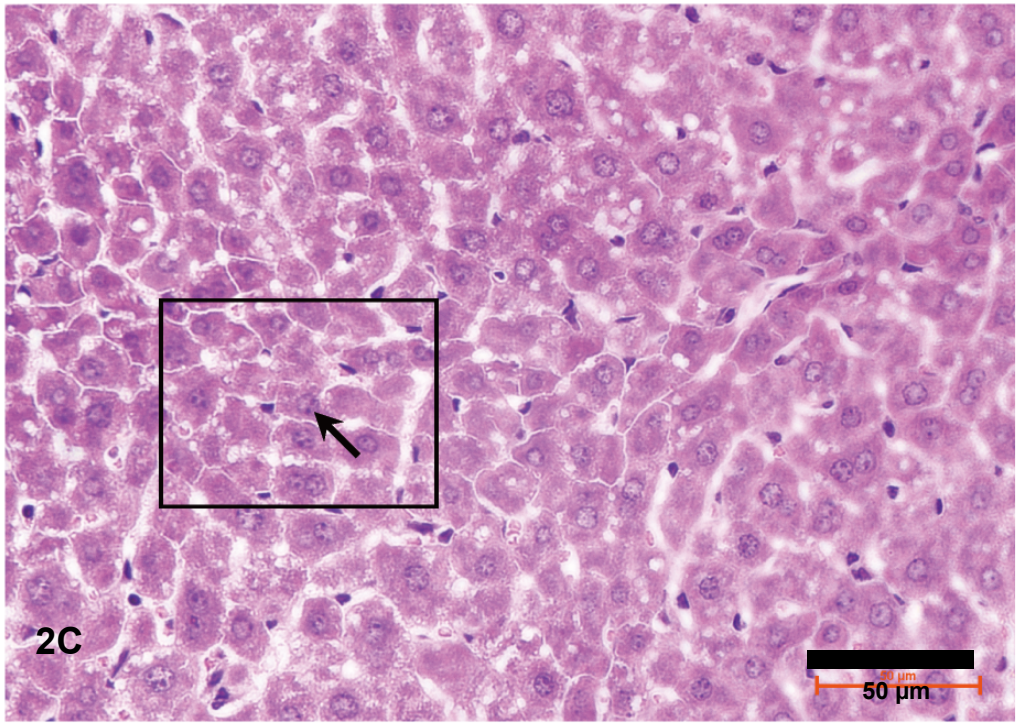
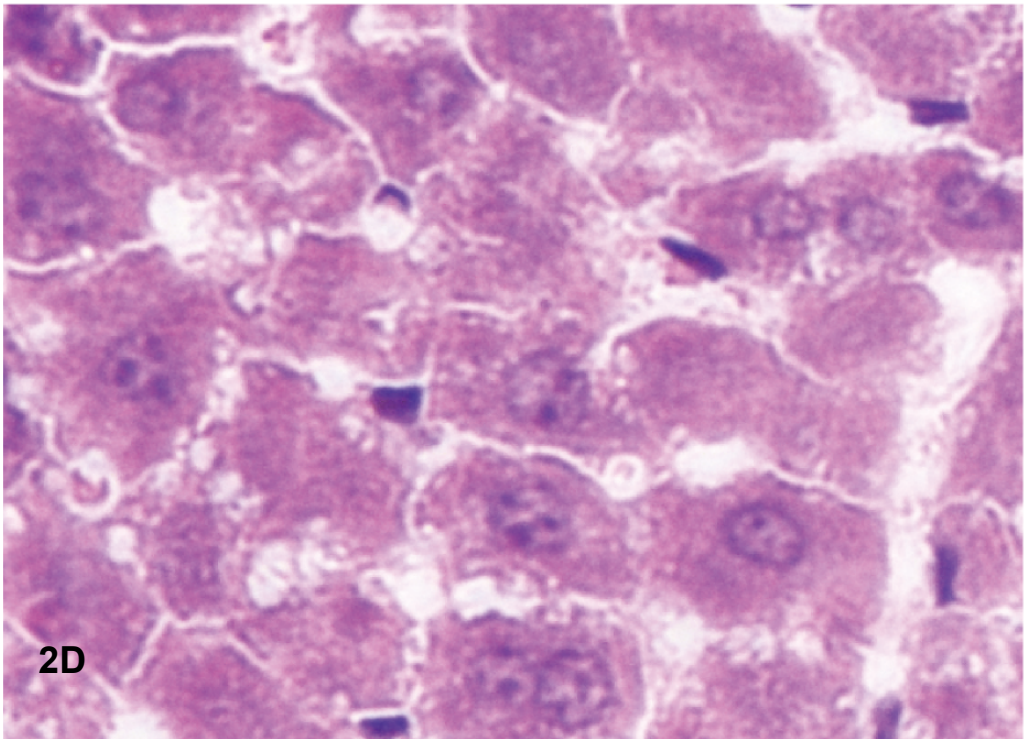
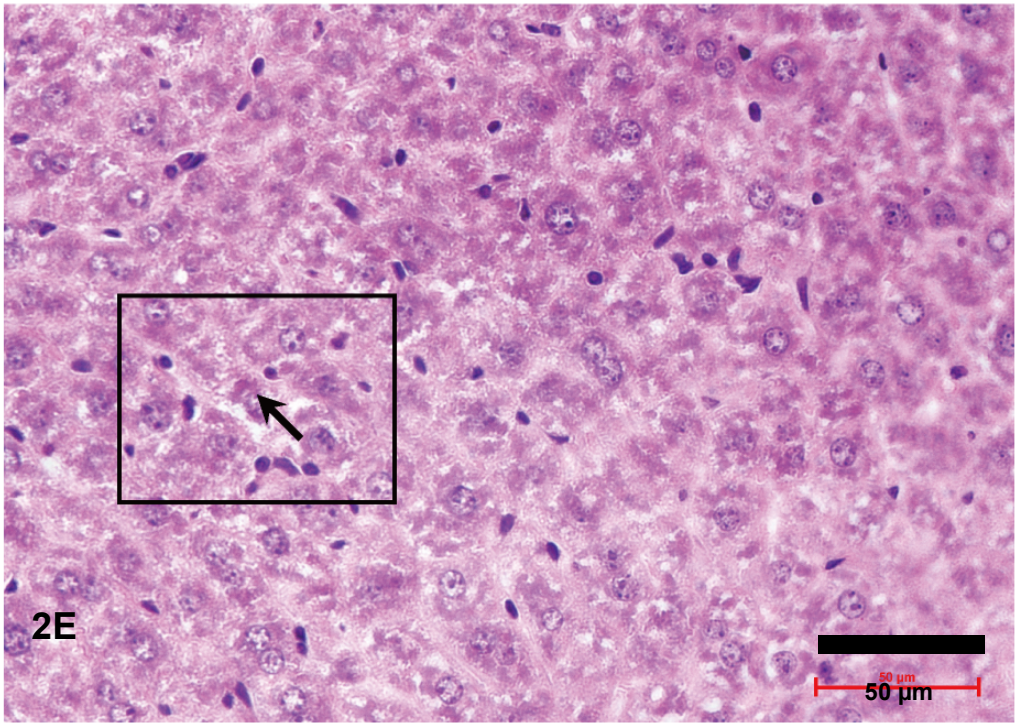
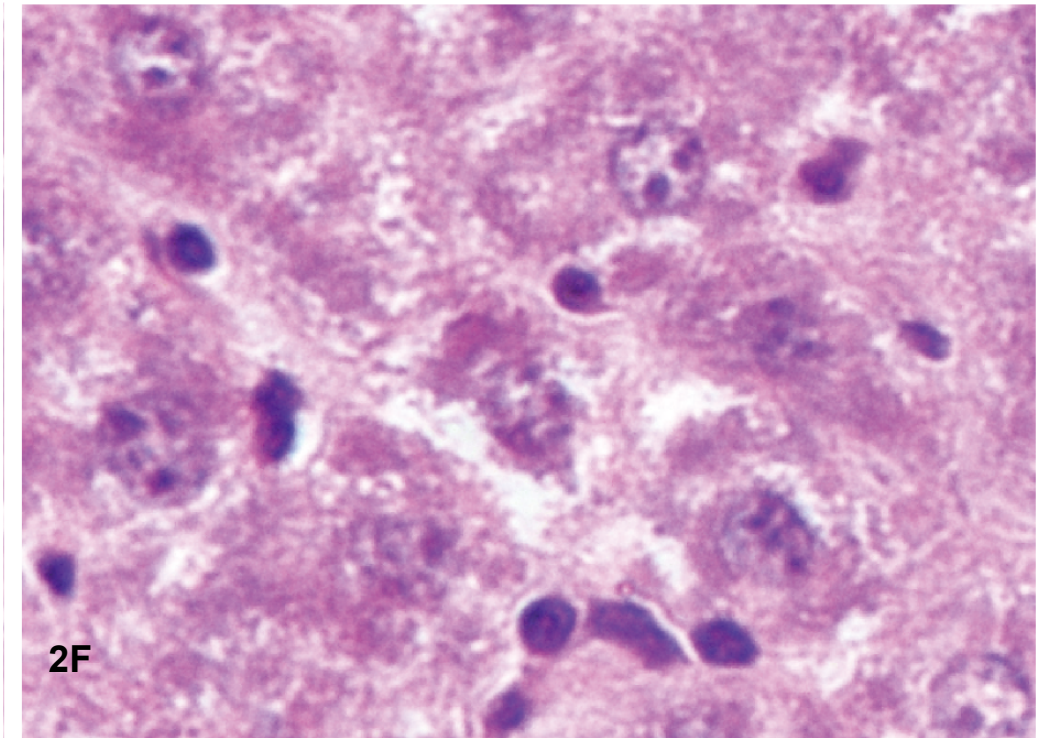




Figures 2C and 2D. GSPE improves fluoride-induced liver pathological damage (n=8). 2C: NaF; 2D: NaF with high magnification. Haematoxylin and eosin staining.





Figures 2E and 2F. GSPE improves fluoride-induced liver pathological damage (n=8). 2E: NaF + GSPE; 2F: NaF + GSPE with high magnification. Haematoxylin and eosin staining.

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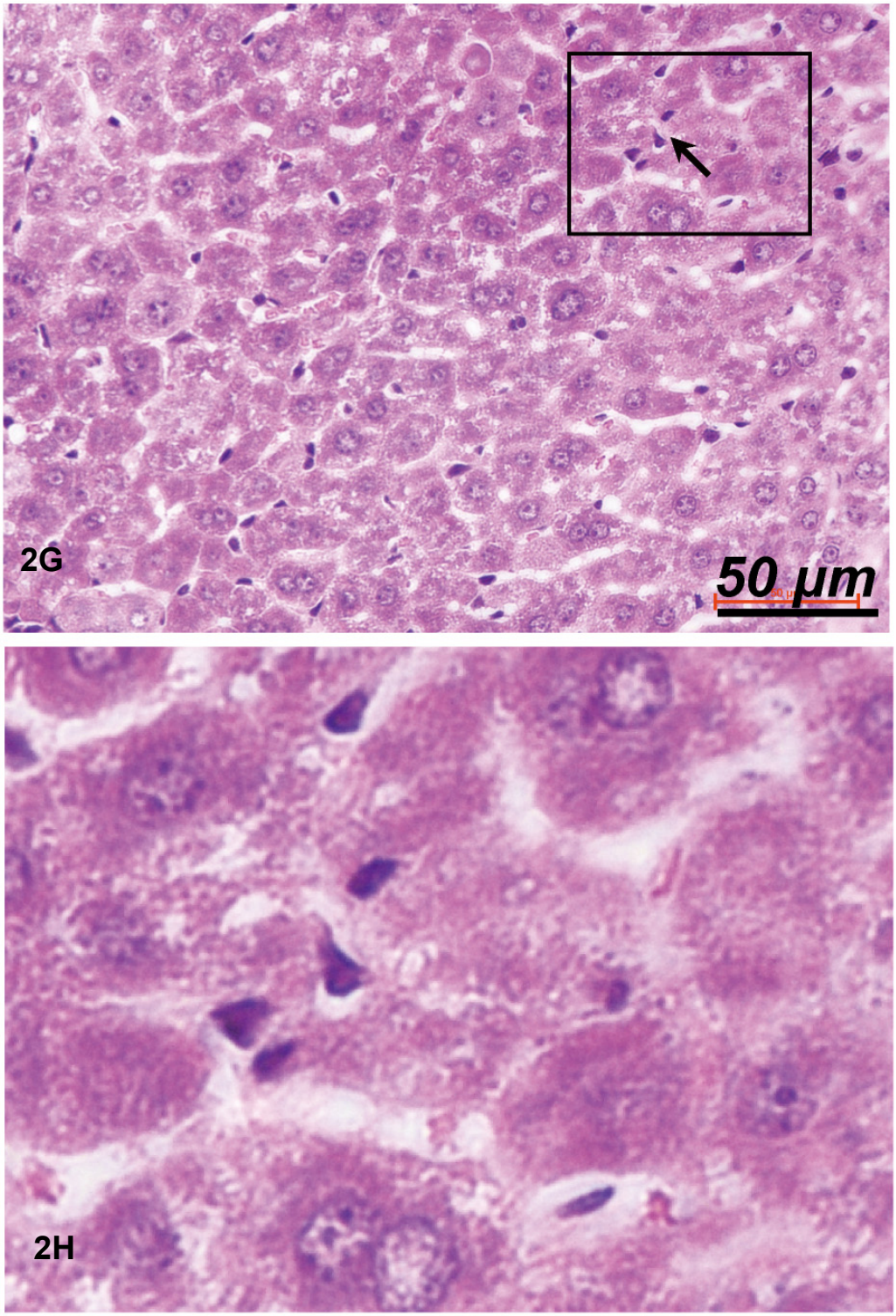
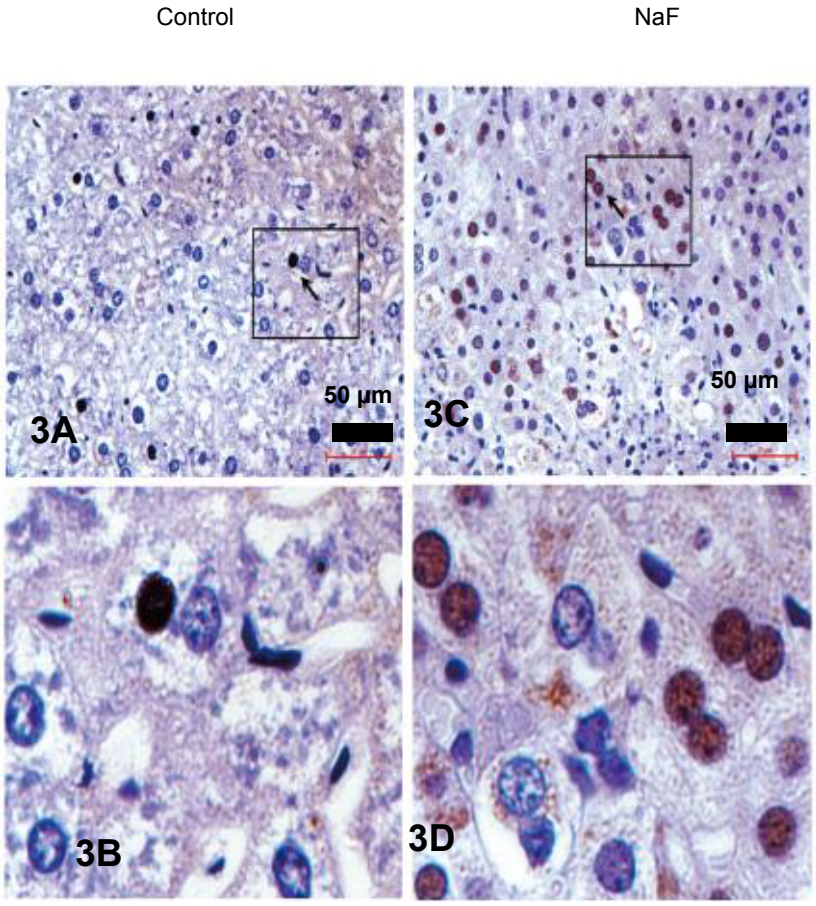


Figures 2G and 2H. GSPE improves fluoride-induced liver pathological damage (n=8). 2G: NaF + vitamin C; 2F: NaF+ vitamin C with high magnification. Haematoxylin and eosin staining.



Figures 3A–3D. Immunohistochemistry studies of liver tissue by in situ terminal deoxynucleotidyl transferase diated dUTP nick end labeling (TUNEL) reaction. Representative photomicrographs of TUNEL positive cells. Quantification of TUNEL-positive cells in each group. 3A: Control; 3B: Control with high magnification; 3C: NaF; 3D: NaF with high magnification.

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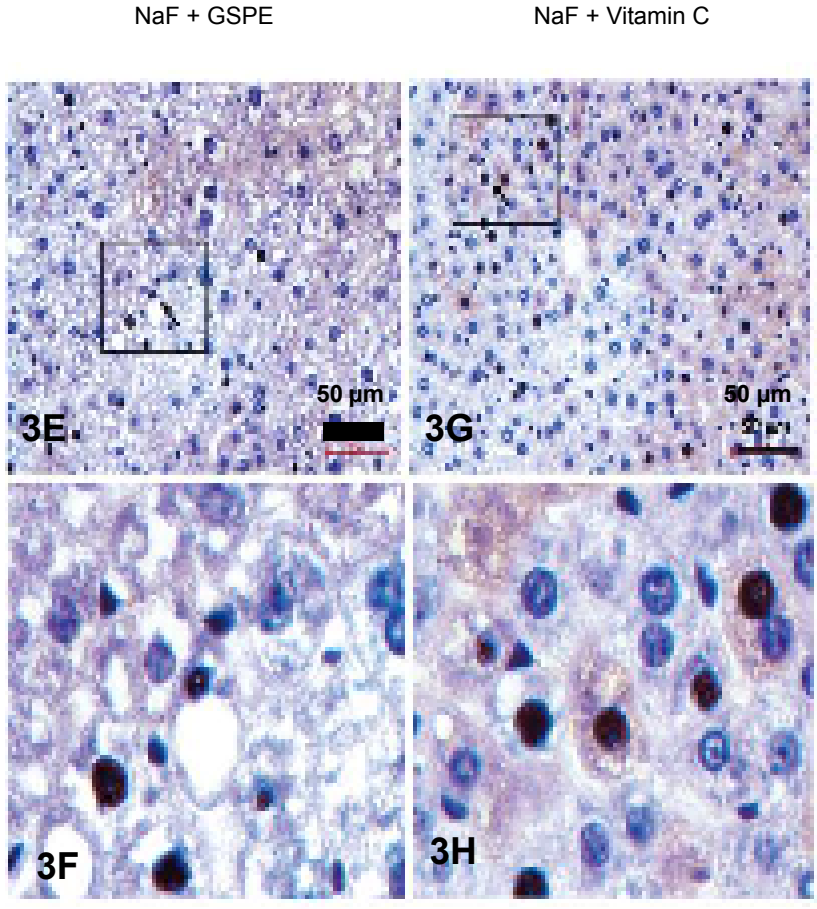

Figures 3E–3H. Immunohistochemistry studies of liver tissue by in situ terminal deoxynucleotidyl transferase diated dUTP nick end labeling (TUNEL) reaction. Representative photomicrographs of TUNEL positive cells. Quantification of TUNEL-positive cells in each group. 3E: NaF + GSPE; 3F: NaF + GSPE with high magnification; 3G: NaF + vitamin C; 3H: NaF + vitamin C with high magnification.

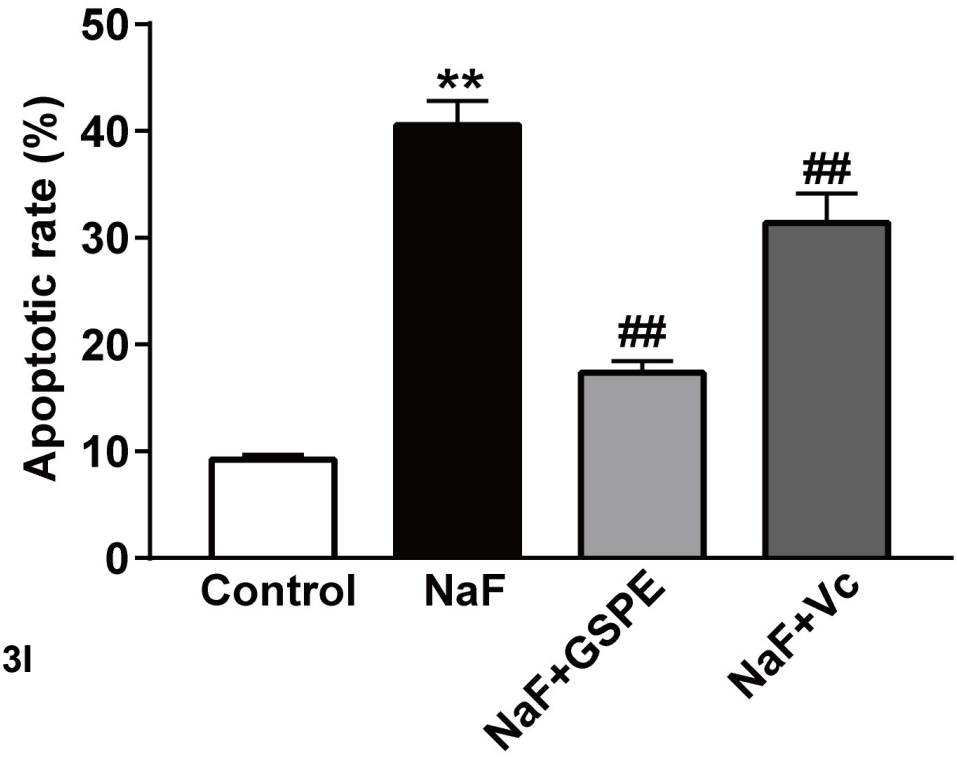

Figure 3I. Immunohistochemistry studies of liver tissue by in situ terminal deoxynucleotidyl transferase diated dUTP nick end labeling (TUNEL) reaction. 3I: Quantification of TUNEL-positive cells in each group. The results were presented as means±SD (n=8). **P<0.01, vs. control group?#P<0.05, ##P<0.01, vs. model group.

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GSPE Mitigate Fluoride-Induced Apoptotic Signaling

The effects of NaF and GSPE on apoptotic signaling in rat hepatocytes were examined. Relative to the control rats, the liver tissue samples of NaF-treated rats exhibited increased Bax protein levels (P<0.05) and decreased Bcl-2 protein levels (P<0.05) (Figures 4A–4C). In contrast, GSPE pre-treatment was sufficient to prevent such Bax upregulation and Bcl-2 downregulation, with vitamin C exhibiting similar effects (Figures 4A–4C). Together, these data suggest that GSPE can alter Bax and Bcl-2 protein levels in hepatocytes *in vivo,* potentially contributing to the anti-apoptotic properties of this extract.

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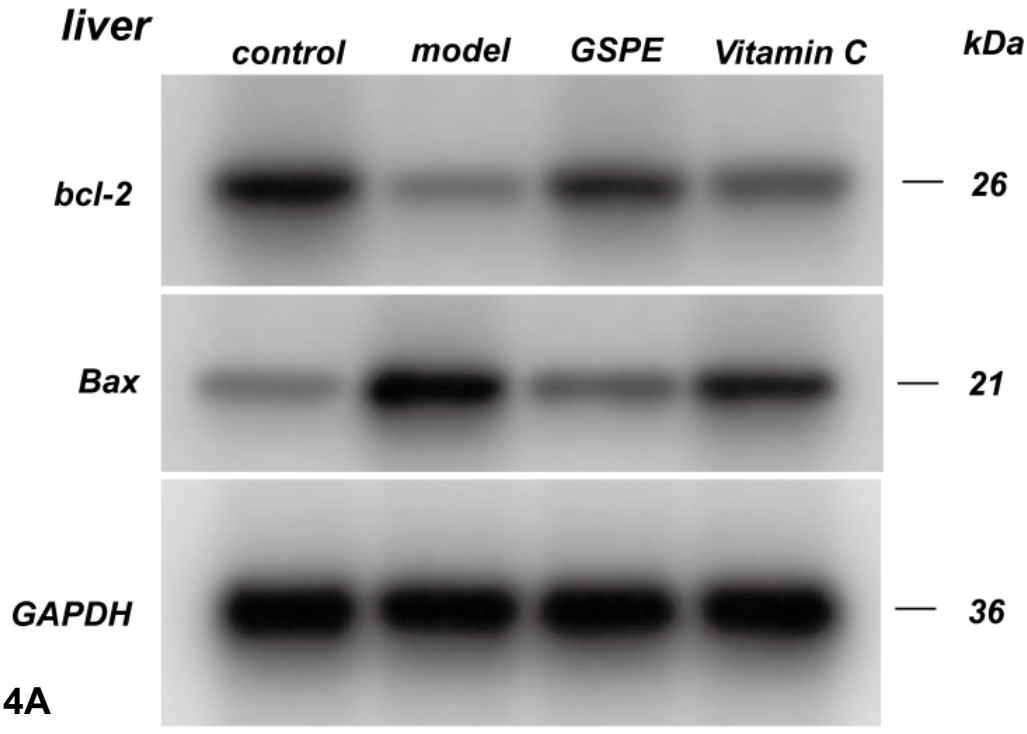

Figure 4A. GSPE increasing antiapoptotic Bcl-2 but decreasing pro-apoptotic Bax in livers of fluoride treated rats. After indicated treatment, the levels of Bcl-2 and Bax expression in hepatic from different groups were detected by western blot assay. 4A: Representative bands.

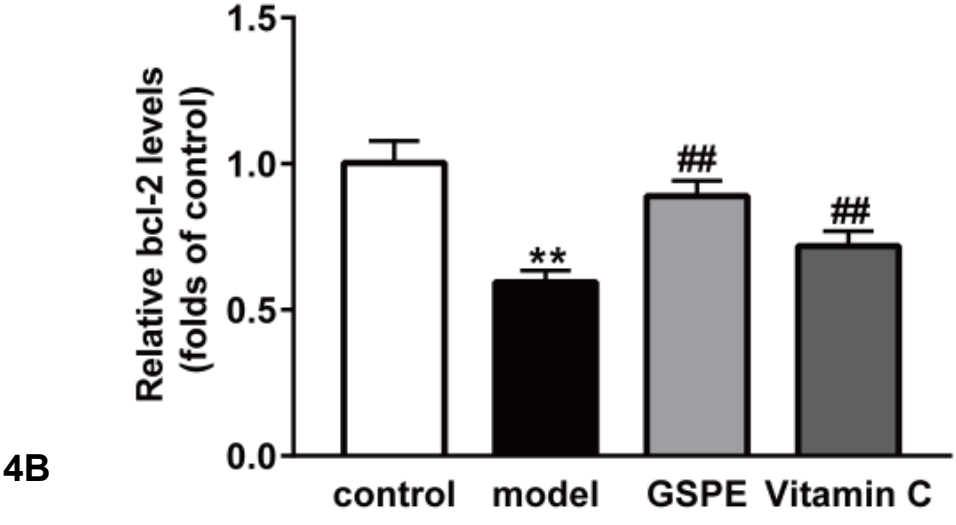

Figure 4B. GSPE increasing antiapoptotic Bcl-2 but decreasing pro-apoptotic Bax in livers of fluoride treated rats. After indicated treatment, the levels of Bcl-2 and Bax expression in hepatic from different groups were detected by western blot assay. 4B:.The levels of Blc-2 normalized to control. The results were presented as means±SD (n=8). **P<0.01 vs. control group, ##P<0.01 vs. model group.

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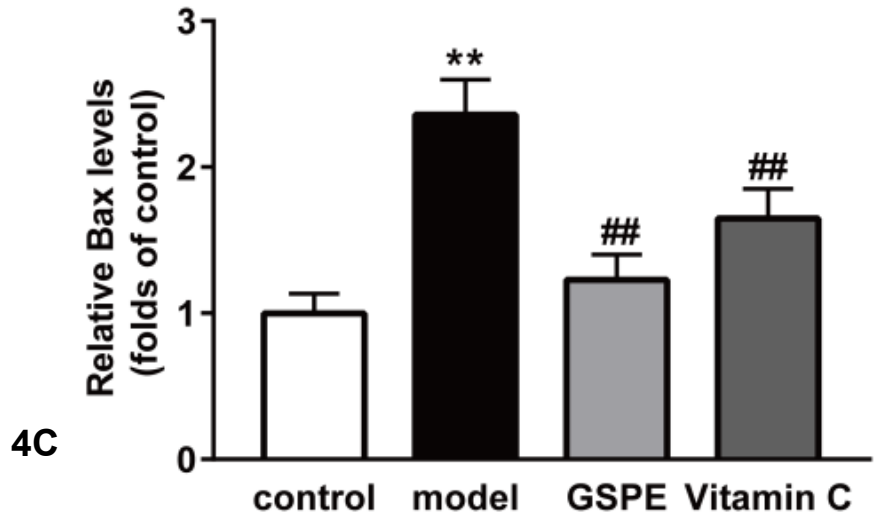

Figure 4C. GSPE increasing antiapoptotic Bcl-2 but decreasing pro-apoptotic Bax in livers of fluoride treated rats. After indicated treatment, the levels of Bcl-2 and Bax expression in hepatic from different groups were detected by western blot assay. 4C:.The levels of Bax normalized to control. The results were presented as means±SD (n=8). **P<0.01 vs. control group, ##P<0.01 vs. model group.

DISCUSSION

It is well-documented that fluoride induces hepatotoxicity in both humans and animals.[5-7] However, the mechanisms governing this hepatotoxicity are not completely clear yet. Numerous previous studies found that fluoride-induced hepatotoxicity is closely related to elevated levels of oxidative stress and apoptosis.[5-11] However, this hepatotoxicity process can be significantly prevented by treatment with antioxidants such as quercetin, gallic acid, and anthocyanins.[2, 5, 6] GSPE has been shown to serve as a robust antioxidant and free radical scavenger,[17] and reportedly has antioxidant and anti-apoptotic properties.[15-19] Therefore, whether these properties could prevent fluoride-induced hepatotoxicity needs to be determined. Herein, we explored the hepatoprotective properties of GSPE in a rat model of NaF-induced oxidative stress and hepatotoxicity.

To explore the protective effects of GSPE in a fluoride-induced hepatic injury rat model, we measured the levels of key oxidative stress-related parameters in liver samples, including MDA content, GSH levels, and SOD/CAT activity. Following a 21-day NaF exposure period, our model rats exhibited significantly impaired SOD and CAT activity together with increased hepatic MDA content and reduced GSH levels. This is consistent with many prior reports which have indicated that fluoride exposure decreased GSH levels, reduced CAT/SOD activity, and increased MDA production, thereby resulting in sustained oxidative stress and associated damage to cell membranes and other macromolecules. Due to its high level of metabolic activity, the liver is particularly susceptible to fluoride-induced toxicity, which can cause the leakage of important liver enzymes (ALT, AST, ALP) and can thereby alter

other serum biochemical parameters (including albumin, bilirubin, glucose, and cholesterol levels).[6,18] Indeed, we found that NaF-exposed rats exhibited increased serum ALP, AST, and ALT levels and abnormal serum biochemical parameters consistent with hepatotoxicity, confirming these prior results.[6,18] However, GSPE pre-treatment reversed these observed changes, as did vitamin C pre-treatment. Pretreating these rats with GSPE (100 mg/kg) was sufficient to normalize these serum parameters (Table 1).

Oxidative stress and associated tissue damage are thought to be key mediators of fluoride-associated toxicity. The liver functions as the primary organ responsible for detoxification and coordinates systemic metabolic activities. Fluoride exposure has previously been linked to pathological and metabolic changes in the liver.[19,20] Consistently, we found that liver tissue samples from NaF-treated model rats exhibited evidence of severe tissue pathology including nuclear and cellular shrinkage, apoptotic bodies, and disrupted cellular boundaries. These findings suggested that oxidative damage may drive fluoride-mediated hepatotoxicity. GSPE treatment alleviated these harmful fluoride-related phenotypes, reversing the observed liver histopathological damage, while also reducing MDA levels and increasing SOD and CAT activities. The antioxidant properties of GSPE may be attributable to its high proanthocyanin content—flavonoid polymers that are known to exhibit more robust antioxidant activity than vitamin C or vitamin E.[21] Proanthocyanins are that exhibit diverse antioxidant properties and other biological activities.[22]

In prior analyses, fluoride-induced oxidative stress was identified as a key driver of apoptosis.[23, 24] Apoptotic signaling is tightly regulated by the pro- and anti-apoptotic proteins Bax and Bcl-2, respectively. The mitochondria serve as a central coordinator of apoptotic signaling, with mitochondrial swelling and rupture leading to the release of many factors that can promote apoptosis. A prior study of fluoride-treated human gingival fibroblasts exhibited Bcl-2 down-regulation in these cells.[25] Furthermore, the fluoride concentrations in water have been positively correlated with Bax expression in the liver of fluoride-exposed fish.[26] We also found that Bcl-2 protein levels were reduced in the liver tissues of NaF-treated rats, whereas the Bax level was increased. GSPE treatment was sufficient to reverse these changes. These results demonstrated that GSPE exerts hepatoprotective activity through up-regulation of the antioxidant and anti-apoptotic signaling pathways.

Nevertheless, there are some limitations to this study. For example, the molecular mechanisms were not fully elucidated in this study which only provides a glimpse into the protective mechanism of GSPE in fluoride-induced hepatic injury. Further detailed studies are necessary to fully elucidate the underlying molecular mechanisms.

CONCLUSIONS

The results of this study indicate that GSPE can protect against fluoride-induced hepatic oxidative damage and may prevent hepatocytes apoptotic by modulating the levels of Bcl-2 and Bax. This hepatoprotective activity appears to be related to the antioxidant and anti-apoptotic properties of GSPE. Overall, our study offers new insights regarding the hepatoprotective properties of GSPE in rats and suggests that

GSPE may be a valuable therapeutic agent in alleviating fluoride-induced liver injury.

ABBREVIATIONS

ALB, albumin

ALP, alkaline phosphatase

ALT, alanine transaminase

AST, aspartate aminotransferase

CAT, catalase

DBIL, direct bilirubin

GSH, reduced glutathione

GSPE, grape seed proanthocyanins extract

H&E, hematoxylin and eosin

HDL, high-density lipoprotein

MDA, malondialdehyde

NaF, sodium fluoride

PMSF, phenylmethanesulfonyl fluoride

SD, standard deviation

SOD, superoxide dismutase

TBIL, total bilirubin

TC, total cholesterol

TG, triacylglycerol

TP, total protein

Vc, vitamin C

CONFLICT OF INTEREST STATEMENT

The authors declared that there was no conflict of interest.

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