Protective Effects of Grape Seed Procyanidin Extract on Fluoride-Induced nephrotoxicity and the possible role of Nrf2 signaling pathway

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Research Article

Keywords: Renal injury, Grape Seed Procyanidin Extract, Oxidative stress, Apoptosis, Nrf2 signaling pathway

Posted Date: March 13th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2663224/v1

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Abstract

To investigate effects of fluoride-induced renal damage and possible role of the Nrf2 signaling pathway and explore the protective activity of Grape Seed Procyanidin Extract (GSPE). A fluoride-exposed rat model was established through drinking water. Oxidative stress parameters and serum markers that reflect renal function were analyzed. Pathological changes were assessed using HE and TUNEL methods. Nrf2 signaling pathway-related and apoptosis-related cleaved Caspase-3 proteins were detected by Western blot. Fluoride exposure caused a significant increase in serum markers of renal function, renal histopathological alterations, oxidative stress, and apoptosis. The protein expression levels of Nrf2, HO-1, and NQO-1 along the Nrf2 signaling pathway were depressed, while the cleaved Caspase-3 protein were upregulated after fluoride exposure. HE and TUNEL examination showed that the renal tissue of fluoride rats was repaired by GSPE. The level of MDA was decreased; while the level of GSH, activities of CAT, and SOD of the kidney were promoted by GSPE. Decreased serum markers such as BUN, UA, and Cr indicate a marked improvement in renal function. GSPE therapy up-regulated the expression levels of Nrf2, HO-1, NQO-1 and down-regulated cleaved Caspase-3 proteins in the kidney. Supplementary GSPE with fluoride significantly alleviated its nephrotoxicity. The results above mentioned indicated that fluoride induced nephrotoxicity possibly through activate the Nrf2 signaling pathway and the presence of GSPE mitigate its nephrotoxicity.

Introduction

Fluoride is an indispensable trace element that is of great importance for the health of living beings. An appropriate amount of fluoride intake is essential for the health, but excessive fluoride exposure will cause harmful effects. Fluoride is widely distributed in the environments and fluorine-containing products are widely used in industry and agriculture. With the rapid development of industry and agriculture in recent decades, fluoride has become one of the important contaminants. Human beings have realized the serious problem of fluoride pollution and made great efforts to solve this severe environmental problem. However, fluorosis still exists [1]. Fluoride enters and accumulates in the body, excessive accumulation of fluoride will lead damage to liver, kidney, and teste and even nervous system [1–3]. Kidneys play an important role in excretion and hematopoiesis [4]. Kidney is the main excretory organ of fluorine. It is reported that the kidney tissue is responsible for the excretion of up to 50–60% of the fluoride ingested each day [5]. This physiological characteristic determined the renal tissue is inevitably vulnerable to toxic effects of excessive fluorine [6].

Previous studies have demonstrated that fluoride exposure increases renal oxidative stress and this fact was evidenced by increased lipid peroxidation levels and reduced the activities of glutathione peroxidase (GSH-Px), CAT, and SOD in the kidney [7, 8]. Some studies further reported fluoride-induced renal cell apoptosis [9, 10]. These results demonstrated that oxidative stress and apoptosis play vital roles in the pathological process of fluoride-related renal dysfunction. However, the exact mechanism of fluoride-induced renal oxidative damage and apoptosis is still unclear at present. The Nrf2 pathway is an important primary transcription factor that affects the oxidative status of the body by regulating
antioxidant enzyme expression [11]. Nrf2 has been proven to play vital roles in a wide range of biological activities including antioxidant, anti-inflammatory, and anti-apoptotic effects [12, 13].

GSPE is a polyphenol bioactive agent that has been reported to exhibit various biochemical activities, such as antioxidant, anti-apoptosis, and anti-inflammatory effects [14, 15]. Hence, natural agents which possess anti-oxidant and anti-apoptotic biological properties could be potential therapeutic against fluoride-induced nephrotoxicity. Recently, many research papers have reported that GSPE exhibit anti-oxidative and anti-apoptosis properties by activating Nrf2 signaling in the protection of various toxic effects caused by exogenous chemicals such as arsenic, lead, and titanium dioxide [16–18]. In our previous research, the administration of GSPE alleviates both oxidative stress and cell apoptosis in the kidney tissue of fluoride rats [10]. The decreased oxidative stress and apoptosis may be a potential molecular mechanism for the treatment of GSPE on fluoride-induced renal injury via activation of the Nrf2 pathway.

However, the exact molecular mechanism by which fluoride induce renal dysfunction was not adequately understood. Therefore, we give the hypothesized that the Nrf2 pathway were inhibited after fluoride exposure and GSPE can alleviate fluoride-induced renal damage by activating the Nrf2 signaling pathway, thereby preventing oxidative stress and apoptosis responses. To test this hypothesis, we explored the protective effect of GSPE against fluoride-induced oxidative stress and apoptosis in rat kidneys, and then the possible involvement of the Nrf2 pathway in the current study.

Materials And Methods

Chemicals

GSPE (≥ 95%) was obtained from Tianjin Peak Natural Product Research Development Co., Ltd. According to the manufacturer, this procyanidin extract contained approximately 56% dimeric, 12% trimeric procyanidins, and 6.6% tetrameric proanthocyanidins. Malondialdehyde (MDA) assay kit (cat. no. S0131) and superoxide dismutase (SOD) assay kit (cat. no. S0101) were obtained from Beyotime Biotechnology (Shanghai, China). Reduced glutathione (GSH) assay kit (cat. no. BC1170) and catalase (CAT) assay kit (cat. no. BC0200) were purchased from Beijing Solar bio Science & Technology Co, (Beijing, China). Total protein assay kit (cat. no. A045-4-1) was acquired from Nanjing Jian Cheng Bioengineering Institute (Nanjing, China). TUNEL assay kit (cat.no. KGA703) was obtained from KeyGen Biotechnology (Nanjing, China). Antibodies against GAPDH (ab181602), Nrf2 (12721), HO-1 antibody (43966), NQO-1 (ab80588), cleaved-caspase-3 (ab184787) and Histone-3 (ab1791) proteins were purchased from Abcam (Abcam, Cambridge, UK).

Animals and treatment

Male SD rats (8 weeks) weighing 240-260g were provided by the animal model center of Nanjing University (license certificate No: sick (Su) 2015-0001). All rats were maintained at a constant temperature of 23 ± 2°C in a 12/12 h light/dark cycle and 60 ± 5% humidity. All experimental procedures
were conducted in conformity with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85 – 23, revised 1985). The animal experimental protocol was approved by the Animal Care and Use Committees of Xinxiang Medical University (XXLL20170108). After adaption for 7 days, 32 rats were randomly divided into four groups: Control, GSPE, NaF (sodium fluoride), and NaF + GSPE. The control group was fed a standard diet for 28 days. The GSPE group was treated the same as the control and supplied with GSPE (100 mg/kg) by gavage daily. NaF and NaF + GSPE groups were intoxicated with NaF (600 ppm through drinking water) for 21 days. The NaF + GSPE group was pretreated daily with GSPE (100 mg/kg) by gavage for a week before intoxication. The dosage of NaF and GSPE selection refers to our previous literature. At the end of the experiment, the blood samples and kidney tissues were collected for further analysis.

**Oxidative Stress And Renal Function Assessment**

All rats fasted overnight. Kidney samples were washed with ice saline and homogenized in phosphate-buffered saline (PBS). After homogenization, the solution was centrifuged at 12000 rpm for 10 min at 4°C. The supernatants were obtained for analysis of activities of GSH, CAT, SOD, and MDA contents. The serum samples were collected by centrifugation at 3000rpm for 10 min. The concentration of serum CR, UA, and BUN were detected using commercially available kits.

**Histological And Apoptosis Examination**

The kidney was quickly removed and washed with ice saline. To observe histopathology changes in kidney tissues, HE was conducted by a trained pathologist. TUNEL staining was applied to evaluate kidney apoptosis. Cells with brown granules in the nucleus are considered as TUNEL positive cells, which reflect apoptotic. Five fields from each tissue (section at magnification×200) were randomly selected to assess and calculate the apoptotic index. The apoptosis index was measured as the percentage of TUNEL-positive cells per total cell.

**Western Blot Analysis**

The kidney tissue homogenates were generated by suspending tissues in total protein extraction lysis buffer containing PMSF. The protein was separated by 10% SDS-PAGE, and then electrophoretically transferred onto the PVDF membrane. After the membranes were blocked in 5% fat-free dry milk in Tween 20 Tris-buffered saline (TBST) for 1.5 h, the membranes were incubated with primary antibodies at a dilution of 1:1000 anti-Nrf2, 1:1000 anti-HO-1, 1:1000 anti-NQO-1 and 1: 2000 Caspase-3 antibodies t at 4°C overnight. Then, the membranes were washed with TBST three times and incubated in horseradish peroxidase (HRP) conjugated secondary antibody (1:5000) for 1 h at room temperature and washed with TBST three times. Finally, blots were developed using the ECL chemiluminescence detection reagent.
Band intensities were quantified by the Tanon 6600 Multi fluorescence image analysis system (Tanon, Shanghai, China).

**Statistical analysis**

The results were analyzed by using Statistical Product and Service Solutions (SPSS, version 11.5). Results are expressed as the mean ± standard deviation (SD). The differences of different groups were analyzed by using a one-way ANOVA test to evaluate the homogeneity of the data followed by a least-squared differences model or Dennett’s multiple comparison test if the homogeneity evaluation indicated significant deviation variances. Differences were considered to be significant at $P<0.05$

**Results**

**GSPE alleviates fluoride-induced Oxidative Stress in the kidneys of rat**

To assess the anti-oxidative effects of GSPE, the levels of MDA and GSH and activities of SOD and CAT were measured in rat kidney tissues. We observed a significant increase in MDA levels and a decrease in activities of SOD, CAT, and the level of GSH in the kidney of the NaF group ($P<0.05$; Table 1). However, after treatment with GSPE a significant decline in MDA content and a remarkable rise in GSH level were observed ($P<0.05$). In addition, a noticeable improvement in the activities of SOD and CAT in the kidney appeared ($P<0.05$; Table 1). These data indicated that GSPE therapy can mitigate fluoride-induced oxidative stress.

<table>
<thead>
<tr>
<th>Oxidative parameters</th>
<th>Control</th>
<th>GSPE</th>
<th>NaF</th>
<th>NaF + GSPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/mg protein)</td>
<td>2.97 ± 0.22</td>
<td>2.76 ± 0.37</td>
<td>9.02 ± 1.26**</td>
<td>4.71 ± 0.40##</td>
</tr>
<tr>
<td>GSH (µg/mg protein)</td>
<td>3.18 ± 0.31</td>
<td>3.33 ± 0.81</td>
<td>0.99 ± 0.07**</td>
<td>2.39 ± 0.31##</td>
</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td>206.74 ± 29.88</td>
<td>187.91 ± 19.56</td>
<td>73.47 ± 5.08**</td>
<td>120.88 ± 16.09##</td>
</tr>
<tr>
<td>CAT (U/mg protein)</td>
<td>3.83 ± 0.39</td>
<td>4.11 ± 0.53</td>
<td>0.97 ± 0.21**</td>
<td>3.40 ± 0.45##</td>
</tr>
</tbody>
</table>

**$P<0.01$, vs. control group, $##P<0.01$, vs. NaF model group.**

**Gspe Protects Against Fluoride-induced Renal Function Injury**

The effects of GSPE in protecting renal function were investigated using a fluoride-induced rat model. The serum levels of renal function markers in all treatment groups were measured (Table 2). After 21 days of intoxication with NaF significant changes were observed in serum markers which indicated renal
function. GSPE therapy mitigates these deleterious changes (Table 2). Hence, our results demonstrated that GSPE treatment can improve renal function in NaF-treated rats.

### Table 2

Indicators of kidney function for each group. (Mean ± SD, n = 8).

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Control</th>
<th>GSPE</th>
<th>NaF</th>
<th>NaF + GSPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr (µmol/L)</td>
<td>59.52 ± 7.85</td>
<td>62.89 ± 8.89</td>
<td>133.49 ± 8.24**</td>
<td>67.08 ± 9.54##</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>6.12 ± 0.69</td>
<td>5.72 ± 0.58</td>
<td>10.67 ± 1.05**</td>
<td>7.32 ± 1.63##</td>
</tr>
<tr>
<td>BUN (mmol/L)</td>
<td>6.57 ± 0.82</td>
<td>6.72 ± 0.82</td>
<td>15.39 ± 1.11**</td>
<td>9.03 ± 1.35##</td>
</tr>
</tbody>
</table>

**P < 0.01, vs. control group ##P < 0.01, vs. NaF model group.

#### Gspe Ameliorates Fluoride-induced Renal Pathological Damage

The protective effects of GSPE against fluoride-induced renal damage were observed in HE staining (Fig. 1) and TUNEL assay (Fig. 2). As shown in Fig. 1, the renal cells in the control group were intact, and in the cells of the NaF group various abnormal changes were observed including: partially empty to varying degrees, vesicular degeneration, and the unclear boundary between cells. However, after the administration of GSPE, the histopathological changes caused by NaF exposure were markedly ameliorated (Fig. 1). TUNEL analysis was used to investigate the apoptotic rate of cells in the kidney (Fig. 2). Furthermore, the renal cell apoptosis rate was observed using the TUNEL assay. GSPE treatment significantly inhibited NaF-induced kidney cell apoptosis (P < 0.05; Fig. 2). Thus, taken together, our data suggested that GSPE has protective effects against NaF-induced histopathology damage and cell apoptosis of the kidney.

#### Gsep Activates Nrf2 Signaling Pathway In The Kidney Of Rat Exposed To Fluoride

To further confirm the positive impact of GSPE on protecting against oxidative stress and renal cell apoptosis the oxidative stress-related Nrf2 signaling and apoptosis-related cleaved Caspase-3 protein were explored. The protein expression levels of Nrf2, NQO-1, HO-1, and cleaved Caspase-3 were all assayed by western blot analysis. As shown in Fig. 3, the expression levels of Nrf2, NQO-1, and HO-1 proteins were significantly up-regulated compared with the control group which indicates that the Nrf2 signaling pathway was activated due to fluoride exposure. The application of GSPE promoted the nuclear translocation of the Nrf2 signaling. Furthermore, GSPE inhibits the caspase-3 apoptosis pathway by inhibiting the expression of cleaved Caspase-3. These data confirm the role of GSPE in regulating the Nrf2 signaling pathway and caspase-3 apoptosis pathway.
Discussion

The purpose of the current study was to investigate the protective effects of GSPE against fluoride-induced renal injury and explore the possible role of the Nrf2 signaling pathway. Although the exact molecular mechanism by which fluoride-induced renal injury is far from completely vitrified, it has been proved that oxidative stress and apoptosis play pivotal roles in the histopathology of this process. Thus, natural agents that possess anti-oxidative stress and anti-apoptosis properties may be potentially good candidates for the therapy of fluoride-induced renal injury. An increasing amount of antioxidants was used for the treatment of such kidney dysfunction [8, 19–21]. To explore the therapeutic potential of GSPE, we assessed the protective effects of GSPE on renal injury induced by fluoride in a rat model [10]. Our results raveled that GSPE is probably a protective agent against fluoride-induced renal oxidative damage and apoptosis.

The kidney is the main excretory organ of fluorine, most of the fluoride ingested each day is excreted through the kidney tissue. Under a high fluoride environment, renal function is inevitably degenerated due to injury in the kidney. Several biochemical parameters are usually considered important indexes that reflect renal function. Therefore, the serum markers (CR, UA, and BUN) were tested in fluoride rats. In the current study, after fluoride exposure, typical renal dysfunction phenomena appeared such as increased CR, UA, and BUN levels, which is consistent with our previous study [10]. However, positive changes were observed that treatment with GSEP reserved these deteriorated alterations in renal function evidenced by the decreasing level of serum markers reflecting kidney function. Furthermore, this favorable change was evidenced by the observation of obviously improved renal pathology results.

Nrf2 is a significant regulator of redox balance that has been shown to improve kidney injury by eliminating ROS [22]. To date, researchers have found that the application of Nrf2-activated agents can effectively reduce ROS generation [23–25]. These results indicated that the Nrf2 pathway was involved in fluoride-induced renal injury and the activation of Nrf2 mitigates renal injury. The Nrf2 pathway has been proven to play vital roles in the anti-oxidant and anti-apoptotic processes [26, 27]. GSPE has been proven to exhibit anti-oxidative and anti-apoptosis properties by activating Nrf2 signaling in the protection of toxic effects caused by exogenous chemicals such as arsenic, lead, and titanium dioxide [16–18]. The above results implied that GSPE may be a promising potential agent to protect against fluoride-induced renal injury by activating the Nrf2 signaling Pathway. To further explore the mechanism of GSPE against fluoride-induced renal injury the protein expression level of the Nrf2 pathway was investigated by western blot. In our results, we found the Nrf2 pathway was down-regulated in the kidney of fluoride rats. However, GSPE treatment can dramatically up-regulate the Nrf2 pathway.

Nrf2 helps protect the kidney against oxidative stress by playing a pivotal role in the cooperative induction of genes that encode antioxidant and detoxifying enzymes [28]. Kidney cell is sensitive to ROS injury, excessive production of ROS can induce an enhancement in MDA content, an indicator of lipid peroxidation. In the present study, we found that rat exposure to fluoride exhibited an increased level of MDA, which was consistent with the results of previous literature [7–11]. However, after treatment with
GSPE significant improvement could be observed. Thus, our results confirmed again that GSPE can reduce the production of ROS and oxidative stress in fluoride rats. In general, there is a balance between the production of ROS and the scavenging of ROS by the antioxidant system. However, the balance is somehow shifted towards the formation of excessive ROS. Fluoride is a strong oxidant, and more and more animal experiments proved the fact that fluoride not only generates reactive oxygen species (ROS) but also interferes with the antioxidant defense system by inhibiting the activities of antioxidant enzymes [29]. Thus, to further assess the renal protective role of GSPE, we explored the activities of several important antioxidant enzymes in the antioxidant defense system in fluoride-induced renal damage. Our results demonstrated that treatment with GSPE significantly alleviates fluoride-induced renal oxidative stress.

It is widely accepted that the generation of ROS not only induces cytotoxicity but also activates the apoptosis pathway through trigger the expression of apoptosis protein, including Bax and cleaved Caspase-3, eventually leading to excessive apoptosis of renal cells and renal injury. Many previous studies have proved that renal cell apoptosis plays a vital role in fluoride-induced renal injury. And our recent article has reported that Bax protein was markedly increased in fluoride rats and treatment with GSPE can significantly inhibit the expression of Bax [10]. To deeply explore the potential protective mechanism of GSPE against renal apoptosis in fluoride rats, the role of the Caspase-3 apoptosis pathway in this process was investigated. Our current study observed an obvious enhancement in TUNEL-positive cells in the kidney compared with the control group. However, a prominently decrease in renal cell apoptosis rate was observed and combined with a significant decrease in the expression of cleaved Caspase-3 protein in fluoride rat kidneys after treatment with GSPE. The above results indicated that GSPE could mitigate fluoride-induced renal injury by inhibiting the apoptotic pathway.

**Conclusion**

In conclusion, the findings of the current study confirmed the protective role of GSPE against fluoride-induced renal injury and found that GSPE inhibited oxidative stress and apoptosis in NaF rats. The Nrf2 signaling pathway was verified to be the key pathway by which GSPE protects against fluoride-induced nephrotoxicity. GSPE could be a promising therapeutic choice for preventing fluoride-induced renal injury.

**Declarations**

**Funding information** The research was supported by National Natural Science Foundation of China (Grant No. 81703230) and the scientific research start-up funds of Shaoxing University (Grant No. 20210036).

**Compliance with Ethical Standards**

**Conflict of Interest** The authors declare that they have no conflict of interest.
References


**Figures**

**Figure 1**

GSPE alleviates fluoride-induced renal histopathology damage (n=8).

**Figure 2**

GSPE Inhibits renal cell apoptosis rate in the kidneys of fluoride rats. Representative photomicrographs of TUNEL-positive cells (A). Quantification of TUNEL-positive cells in each group (B). The results were presented as means ± SD (n=8). **P<0.01, vs. control group #P<0.05, ## P<0.01, vs. NaF model group.
Figure 3

GSPE activates the Nrf2 signaling pathway in the renal tissue of fluoride rats. The levels of Nrf2, Ho-1, NQO1, and cleaved Caspase-3 expressions in the kidney from different groups were detected by western blot assay, and representative bands were shown in (A). The levels of Nrf2 (B, C), Ho-1 (D), NQO1 (E), and cleaved Caspase-3 (F) were normalized to control. The results were presented as mean±SD (n = 8). ** P<0.01, vs. Control group, ## P< 0.01, vs. NaF group.