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Original article

Potential ameliorative effects of grape seed-derived polyphenols against cadmium induced prostatic deficits

Yongfang Lei[a], Qian Chen[a], Jinglou Chen[b,*], Dong Liu[a,*]

[a] Department of Pharmacy, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[b] Department of Pharmacy, Puai Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

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ABSTRACT

Grape (*Vitis vinifera*) is consumed as fruit and wine for people. In this study, rat model of prostatic deficits was induced by orally receiving 60 mg/L cadmium chlorine ($CdCl_2$) through drinking water for 20 weeks. Grape seed-derived polyphenols extract (GSP) was orally given for 20 weeks. Finally, the prostatic levels of E-cadherin, fibronectin, and α-smooth muscle actin were measured by immunohistochemical and qPCR analysis. The oxidative stress was measured by detecting the levels of malondialdehyde, nitric oxide, reduced glutathione/oxidized glutathione and enzymatic antioxidant status. Additionally, the prostatic expressions of transforming growth factor-β1 (TGF-β1), type I TGF-β receptor (TGF-βRI), Smad3, phosphorylation-Smad3 (p-Smad3), Smad7, nuclear related factor-2 (Nrf-2), heme oxygenase-1 (HO-1) and γ-glutamate cysteine ligase catalytic subunit (γ-GCLC) were measured by western blot. The levels of microRNA (miR)-133a/b were measured by qPCR. It was observed that GSP ameliorated the prostatic oxidative stress and fibrosis induced by $CdCl_2$. GSP also inhibited the over-generation of TGF-β1 and p-Smad3, as well as enhanced the levels of Smad7, Nrf-2, HO-1, γ-GCLC and miR-133a/b. These results showed that GSP could attenuate Cd-induced prostatic deficits.

1. Introduction

Heavy metals are naturally present in the environment. However, the generation and accumulation of heavy metals are significantly sped up because of human undertakings. Consequently, the residue of heavy metals has become a serious environment pollution problem accompanied with the development of human society. The use of cadmium (Cd) in the modern industrial manufacturing (including petrochemical operation and batteries generation) is more and more wide. As a result, Cd residue contaminates soil, drinking water and atmosphere [1]. Humans are generally exposed to Cd from crops, aquatic products and tobacco smoke [2]. It has been known that Cd is a non-biodegradable and non-essential metallic element for human beings. Cd can cause acute and chronic toxicities on liver, kidney, lung, pancreas, bones and reproductive organs, as well as on nervous and cardiovascular systems [3]. Studies indicate that a single injection of Cd results in significant decrease in the activities of antioxidant enzymes and in the level of non-enzymatic antioxidant reduced glutathione (GSH) [4,5]. Meanwhile, chronic exposure to Cd through drinking water produces significant damage on the balance of extracellular matrix (ECM) deposition and degradation, as well as on the homeostasis of prostatic cells proliferation and apoptosis [6,7]. Lower urinary tract symptoms (LUTS) are common disorders in elderly men all over the world and seriously harm the patients' quality of life [8]. Chronic prostatic deficits (including fibrosis) are believed to be critical factors for the development of LUTS [9]. So, more and more attentions have been focus on the damage of environmental heavy metals pollution to prostate.

Grape (*Vitis vinifera*) is a member of Vitaceae and consumed as fruit and wine for people. Grape seed contains an abundance of natural polyphenolic substances and has become a functional food resource [10]. Phytotherapy is widely used for the treatment of prostate deficits [11]. It demonstrates that grape seed has benefits of anti-inflammatory, scavenging free radicals, inhibiting lipid peroxidation, anti-carcinogenic and so on [1]. Our previous studies found that grape seed-derived polyphenols extract (GSP) had prostatic protective nature *in vivo* and could modulate prostatic oxidative stress [12,13]. However, it is still unclear whether GSP has potential protective effects against prostatic deficits induced by chronic Cd exposure. So, this study was undertaken to investigate the effects of GSP on Cd-induced prostatic deficits in rats and to explore the possible mechanisms.

* Corresponding authors.
E-mail addresses: jinglouchen@126.com (J. Chen), ld_2069@163.com (D. Liu).

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2. Materials and methods

2.1. Reagents

The antibodies of transforming growth factor-β1 (TGF-β1), type I TGF-β receptor (TGF-βRI), nuclear related factor-2 (Nrf-2), heme oxygenase-1 (HO-1), γ-glutamate cysteine ligase catalytic subunit (γ-GCLC), collagen-I (col-I), fibronectin and α-smooth muscle actin (α-SMA) were obtained from Abcam, UK. The antibodies of Smad3 and phosphorylation-Smad3 (p-Smad3) were got from CST, USA. The antibodies of Smad7 and E-cadherin were purchased from Santa Cruz, USA. The evaluation kits for malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD) and glutathione peroxidase (GPx) and glutathione/oxidized glutathione (GSH/GSSG) were obtained from Nanjing Jiancheng, China. GSP was prepared and analyzed according to our previous study [12]. The levels of (+)-catechin, (−)-epicatechin, procyanidin B2 and total procyanidin B1-4 in GSP was 62.4 ± 1.3, 30.5 ± 0.8, 11.5 ± 0.3 and 39.4 ± 0.8 mg/g, respectively.

2.2. Animals and administration

Male Wistar rats weighing 180 ± 20 g were randomly divided into 5 groups, which include the vehicle control group (Control), $CdCl_2$ exposure model group (Cd exposure), GSP treated control group (GSP control) and $CdCl_2$ exposure + two doses GSP treated groups (Cd + GSP), respectively. There are 8 rats in each group. The rats from Cd exposure and Cd + GSP groups were orally received 60 mg/L $CdCl_2$ through drinking water for 20 weeks [6,7]. The rats from Cd + GSP groups were daily orally given 200 or 400 mg/kg GSP for 20 weeks to be the low or high dose GSP groups, respectively. The rats from GSP control group were orally treated with 400 mg/kg/d GSP without Cd exposure for measuring the possible impacts of GSP itself on prostate. These rat experiments were performed in accordance with the ethical rules in the NIH Guidelines for the Care and Use of Laboratory Animal.

2.3. Histological and immunohistochemical analysis

At the end of the 20th week, the rat prostate samples were collected, weighted and dissected. One part of each prostate sample was made into paraffin-embedded block and serially sectioned for the assessment of prostatic collagen deposition through masson and Picro-Sirius Red (PSR) staining. Additionally, immunohistochemical analysis was performed for evaluation the expressions of col-I, E-cadherin, fibronectin and α-SMA using deparaffinized tissue slides. The negative control was performed with the same operations but omit the primary antibody. Three slides were prepared per rat and ten randomly selected fields of each slide were observed under optical microscope at ×400 and recorded using MOTIC software.

2.4. Prostatic oxidative stress

The prostatic oxidative stress was estimated *via* detecting the levels of prostatic oxidative stress indicators (the contents of MDA, NO and GSH/GSSG) and the enzymatic antioxidant status (the activities of SOD and GPx) using prostate tissue homogenate samples according to the instructions of commercial kits.

2.5. Western blot analysis

Western blot analysis was performed to assess the prostatic expressions of TGF-β1, TGF-βRI, Smad3, p-Smad3, Smad7, Nrf-2, HO-1 and γ-GCLC using fresh prostate tissue. After separated on 10% SDS-polyacrylamide gel electrophoresis, the protein samples were transferred to PVDF membranes *via* electrophoretic transfer, blocked for 1 h at 37 °C with 5% nonfat milk in Tris-buffered saline, and incubated with the primary antibody of TGF-β1 (1:1000), TGF-βRI (1:1000), Smad3 (1:1000), p-Smad3 (1:1000), HO-1 (1:1000), γ-GCLC (1:1000), Smad7 (1:800) or Nrf-2 (1:500) overnight at 4 °C, respectively. Then, these membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies in TBST with 3% nonfat milk for 1 h. Immunoblots were developed using the enhanced chemiluminescence technique and the quantification of bands was determined by integrated optical density analysis using Gel-Pro Analyzer software. LaminB (1:1000) was applied as the internal control for normalizing nuclear Nrf-2. The other data were normalized using β-actin (1:8000) as the internal control.

2.6. qPCR analysis

The qPCR analysis was carried out to test the prostatic mRNA levels of E-cadherin, fibronectin and α-SMA, as well as the microRNA (miR) levels of miR-133a/b using a fast qPCR master mix kit in real-time PCR system (ABI StepOne Plus) according to the manufacturer's protocol. The forward primer of miR-133a-3p was 5′-CCCTTTGGTCCCCTTCAAC-3′, the reverse primer of miR-133a-3p was 5′-CAGTGCAGGGTCCGAGGTAT-3′, the RT primer of miR-133a-3p was 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGCTG-3′. The forward primer of miR-133b-3p was 5′-CCCTTTGGTCCCCTTCAAC-3′, the reverse primer of miR-133b-3p was 5′-CAGTGCAGGGTCCGAGGTAT-3′, the RT primer of miR-133b-3p was 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGCTG-3′. The forward primer of fibronectin was 5′-CCCTACCAAGGCTGGATGAT-3′, the reverse primer of fibronectin was 5′-GAGCAGGTTCCCTCTGTTGTC-3′. The primers of E-cadherin and α-SMA for rat prostate were according to previously study [14]. U6 was used as the internal standard for miRs and GAPDH was used as the internal standard for the others.

2.7. Statistical analysis

The values were expressed as mean ± S.D. Date were analyzed statistically by one-way ANOVA followed by Tukey's multiple comparison using SPSS software. Differences were considered as significant at $p < 0.05$.

3. Results

3.1. Body weight, prostatic collagen deposition and the levels of E-cadherin, fibronectin and α-SMA

Fig. 1 shows that chronic Cd exposure inhibits the body weight gaining of rats at 10th, 15th and 20th weeks when compared to the vehicle control. Co-treated with 400 mg/kg/d GSP increased the body weight from 10th to 20th weeks when compared to the Cd exposure group. And 400 mg/kg/d GSP alone did not significantly change the body weight when compared to the vehicle control. The prostatic collagen deposition was estimated by masson and PSR staining, and by immunohistochemical analysis for expression of col-I. As can be seen from Fig. 2, chronic Cd exposure enhances the prostatic production of collagen in rats when compared to the vehicle control. The prostatic deposition of collagen was suppressed by co-treatment with 200 or 400 mg/kg/d GSP when compared to the untreated Cd exposure group. As indicated in Fig. 3, chronic Cd exposure at the dose of 60 mg/L through drinking water for 20 weeks leads to reduced expression and mRNA level of E-cadherin, as well as enhanced expressions and mRNA levels of fibronectin and α-SMA when compared to the vehicle control. GSP administration contributed to an increased prostatic level of E-