DOP1 Attenuates Cisplatin-induced Reproductive Injury in Male Mice via the Nrf2/ARE Signaling Pathway

Weiwei Yao[1], Zhaoping Hong[1], Xuezhen Chen[1], Biao Liu[2,a] and Chunbin Lu[1,b]

[1]Department of Developmental and Regenerative Biology, Jinan University, Guangzhou 510632, China;

[2]Ministry of Environmental Protection, Nanjing Institute of Environmental Sciences, Nanjing 210042, China.

[a]liubiao@nies.org, [b]tcblu@jnu.edu.cn

Abstract

In the present study, the mechanisms involved in the action of Dendrobium officinale polysaccharide 1 (DOP1) in alleviating cisplatin (CDDP)-induced reproductive injury using a mouse model were investigated. This crude polysaccharide from the stem of D. officinale was obtained by water extraction and ethanol precipitation, and DOP and DOP1 fractions were isolated using diethylaminoethyl (DEAE) cellulose column chromatography. An intraperitoneal injection of CDDP (2.5 mg/kg/d) for 5 days was followed by the oral administration of DOP1 (60, 120, or 240 mg/kg/d) or drinking water (control) for 16 days. This study analyzed sperm parameter, performed pathologic examinations, and assessed changes in biomedical parameters in reproductive organs—including superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GSH-PX), and malondialdehyde (MDA) activities. In addition, mRNA and protein expression of hemeoxygenase-1 (HO-1) and NQO1 (NAD(P)H quinone dehydrogenase 1) in testis were quantified using quantitative real-time polymerase chain reaction analysis (qRT-PCR) and western immunoblotting analysis. DOP1 treatment of reproductively injured mice showed recovery of the aforementioned sperm parameters, increased reproductive organ weight, ameliorated altered histologic indices, and increased antioxidant enzyme activities to differing degrees. Moreover, our data showed a marked increase in nuclear erythroid 2-related factor 2/antioxidant response element (Nrf2/ARE)-related protein expression and an increase in the ratio of Bcl-2/Bax after DOP1 administration when compared to the reproductively injured group. These findings suggest that DOP1 alleviates reproductive injury induced by CDDP in male mice via Nrf2/ARE signaling, thereby implicating a possible mechanism for DOP1 in preventing reproductive injury.

Keywords

DOP1; Cisplatin; Reproductive Injury; Nrf2.

1. Introduction

Cancer is a major public health concern worldwide, and approximately 5% of individuals currently diagnosed with cancer are of reproductive age [1,2]. Although chemotherapeutic agents constitute useful treatments for cancer, their side effects can cause infertility complications in younger patients [3,4]. Cisplatin is widely used to treat reproductively related cancers—for example, testicular [5] and ovarian cancers [6]); however, treatment of testicular cancer with cisplatin regimens leads to temporary azoospermia and oligozoospermia in most men [7], which may cause temporary or permanent infertility [8,9]. As the mechanisms underlying cisplatin-induced reproductive injury are still not fully understood, many reports suggest that reactive oxygen species (ROS) play an important role during the injury process [10-12], and this can be measured by the degree of lipid peroxidation and ability to scavenge oxygen-free radicals.

Dendrobium officinale, or Tie-pi Shih-hu, is a perennial evergreen herbaceous distributed principally in the southern areas of China, which is used because of its excellent efficacy in traditional Chinese

medicine [13]. Many studies suggest that the plant polysaccharides are a primary class of biologic macromolecules that play an important role in the efficacy of plant-source extracts [14-17]. Therefore, we hypothesized that the polysaccharides from *D. officinale* were responsible for its functional capabilities, making investigations into the antioxidative effects of the polysaccharides essential.

Nuclear erythroid 2-related factor 2 (Nrf2), a key transcription factor in the cellular defense system, translocates from cytoplasm to the nucleus under oxidative stress, binds to antioxidant response elements (AREs) in the nucleus, which are cis-acting enhancer sequences located in regulatory regions of antioxidant and detoxifying genes. It then produces corresponding downstream phase-II detoxifying enzymes and antioxidative proteins (e.g., superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), and hemeoxygenase-1 [HO-1]); this results in the activation of cellular antioxidant defense mechanisms via the Nrf2/ARE signaling pathway [18-20]. Therefore, we speculated that *Dendrobium officinale* polysaccharide 1 (DOP1) attenuates reproductive oxidative stress injury by activating the Nrf2/ARE pathway.

The purpose of the present study, then, was to characterize the extracted polysaccharides from *D. officinale*, and to evaluate their antioxidative activity in vivo. We thus evaluated the polysaccharide efficacy via oral administration to Kunming male mice on a reproductive injury model induced by cisplatin (CDDP), including sperm counts and other parameters; content of malondialdehyde (MDA), GSH, GSH-PX, and SOD in the testis; and Nrf2/ARE-related mRNA and protein expression and Hoechst staining.

2. Materials and Methods

2.1 DOP1 Preparation

D. officinale powder was purchased from and authenticated by the Guangdong Academy of Agricultural Sciences Institute of Crops. The powder was extracted 3 times with hot deionized water (1:20 [m/v], 90°C). The filtered extracts were combined and concentrated by lyophilization, and subsequently mixed with ethanol (1:4, v/v) at 4°C for 24 h to precipitate polysaccharides. The sample was then centrifuged at 4000 rpm, and the precipitate was dissolved in a moderate amount of distilled water and deproteinized twice using the Sevag method [21]. DOP was then dissolved in distilled water and applied to a diethylaminoethyl (DEAE)-cellulose column (GE HealthCare DEAE Sepharose Fast Flow), and eluted with distilled water, 0.05 M NaCl and 0.2 M NaCl solutions [22]. Fractions (6.0 ml/tube) were collected and the carbohydrate content was analyzed with the phenol-sulfuric acid assay. The first peak with the highest polysaccharide content was collected, concentrated, and dialyzed against distilled water for 48 h.

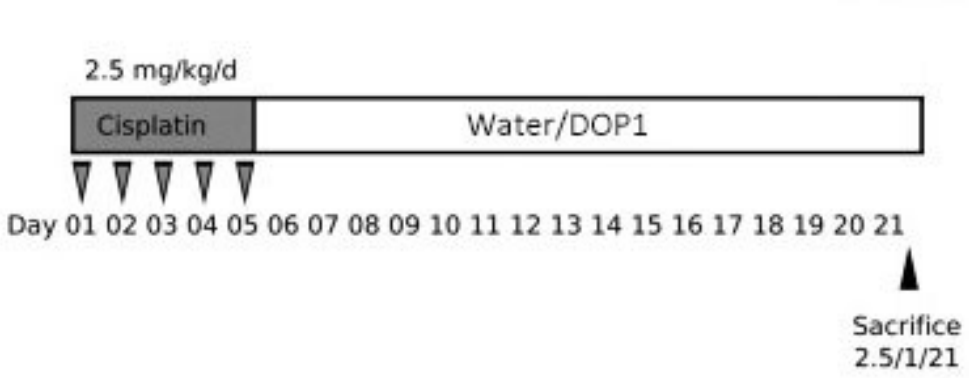

Fig. 1 Timeline and dosage used in the animal experiment

2.2 Animals and Experimental Treatments

Male SPF Kunming mice, 6–7 weeks of age, were obtained from the Jinan University Medical Center Animal Center, Guangzhou, China. Animals were maintained in a temperature-controlled environment (22 ± 2°C) with a 12-h light/12-h dark cycle, and with free access to water and standard

rodent chow. All of the mice were weighed and randomly assigned to the following 6 groups, as shown in Fig. 1: control group (CON), reproductive injury group (CDDP), DOP1 group (240 mg/kg), or reproductive injury model groups treated with DOP1 (60, 120, or 240 mg/kg). Reproductive injury was induced by intraperitoneally administering 2.5 mg/kg of cisplatin (Jiangsu Hausen Pharmaceutical Co., Ltd., with dose selection based upon previous studies [23]) on 5 consecutive days, except for the CON and DOP1 groups. The optimal DOP1 dose was selected based on our pre-experimental results.

2.3 Body Weight and Reproductive Organ Weight

The body weights of all of the experimental mice were measured at the end of the experiment and mice were sacrificed by cervical dislocation, and testes and epididymides were immediately dissected and weighed. The organ coefficients were calculated as (organ weight / body weight) × 100%.

2.4 Evaluation of Sperm Characteristics

The epididymides were cut and incubated in saline for 5 min at 37°C in 5% CO_2 in air. Total sperm numbers were counted with a hemocytometer, and sperm viability and morphologic normality were observed using an inverted microscope (C-DS, Nikon, Japan) after eosin staining of cells. Sperm nuclear maturity was measured by aniline blue staining.

2.5 Pathologic Sampling of Mice and Detection of Apoptosis

On day 21, mice in each group were selected randomly and weighed after they were sacrificed via cervical dislocation. Testicular and epididymal tissues were quickly removed, washed with a precooled saline solution, blotted of excess moisture, and weighed. Each testis was divided into 2 portions: 1 was used for real-time polymerase chain reaction analysis (RT-PCR), western blotting (WB), and biochemical assays to detect the contents of MDA, GSH, SOD, and GSH-PX; and the other 1 was fixed in Bouin's fluid solution for future H&E and Hoechst staining. The epididymides provided the sperm used for analysis of sperm counts and other parameters.

2.6 Biochemical Analysis of Antioxidant Status

Frozen testicular tissue was homogenized in ice-cold saline (testicular tissue weight: saline weight = 1:9) for 5 min to prepare a 10% (w/v) homogenate. The homogenates were centrifuged at 3000 *g* and 4°C for 15 min using a refrigerated centrifuge, and the activities of SOD and GSH-PX; and MDA and GSH content were measured using a microplate reader (BioTek Synergy4, USA) according to the instructions provided with the assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Protein content of the supernatant was also determined according to the instructions in the assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Assay results were normalized to the protein concentration in each sample and were expressed as U/mg prot, nmol/mg prot, or μmol/mg prot.

2.7 RT-PCR Analysis

Total ribonucleic acid (RNA) extraction from testes was performed using RNAiso plus (TaKaRa, China), and the total RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, China). Total RNA from each sample was reverse-transcribed into cDNA and amplified. Forward and reverse primer sequences (5′-3′) were designed as follows: β-actin (F, GGCACCACACCTTCTACAATGAG; R, AGAGGCATACAGGGACAGCAC), Nrf2 (F, AACCACCCTGAAAGCACGC; R, TGAAATGCCGGAGTCAGAATC), HO-1 (F, ATGGCCTCCCTGTACCACATC; R, TGTTGCGCTCAATCTCCTCCT), NQO1 (F, CGCAGACCTTGTGATATTCCAG; R, CGTTTCTTCCATCCTTCCAGG), Bax (F, GGCCCACCAGCTCTGAAC; R, TTCTTCCAGATGGTGAGCGA), and Bcl-2 (F, GGGGAAACACCAGAATCAAG; R, TCCCTTTGGCAGTAAATAGCT). We performed RT-PCR in triplicate on a real-time PCR detection system (CFD-3120, Bio-Rad, USA), and the cycle conditions consisted of an initial denaturation of 3 min at 95°C; followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 15 s. We normalized all of the target genes to β-actin in duplicate, and all of the data were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.8 Western Blotting Analysis

The supernatants were collected after centrifugation at 12,000 *g* for 10 min, and the amount of extracted proteins in each sample was quantified using a BCA Protein Assay Kit (Solarbio, China). Total protein (60 μg) was loaded onto gels and electrophoresed, followed by transferring proteins onto PVDF membranes (Absin, China). Membranes were blocked with 5% BSA for 2 h; and primary antibodies against HO-1, NQO1, and GAPDH were incubated overnight for 2 h at 4°C, followed by washing 3 times with PBST buffer. Secondary HRP-labeled antibodies (anti-mouse or anti-rabbit IgG; Cell Signaling Technology Inc., USA) were then incubated at room temperature for 2 h. After washing the membranes 3 times, 1 ml of ECL was added and the gels were scanned using a chemiluminescence gel-imaging system (Synoptics Ltd., USA).

2.9 Statistical Analyses

Data are expressed as means ± 1 standard deviation (SD), and all of the experiments were repeated at least 3 times. Differences between groups were calculated using Tukey's test by using GraphPad Prism software. A value of $P < 0.05$ was considered to be significant.

3. Results and Discussion

3.1 DOP Elution Results

After ion-exchange chromatography of DOP on a DEAE-cellulose column, we obtained 2 peaks from the distilled water and NaCl elution, and named them DOP1 (eluted with distilled water) and DOP2 (eluted with 0.05 M NaCl); the remaining small peaks were eluted with 0.2 M NaCl (Fig. 2). The primary fractions of DOP1 accounted for 58.7% of the total polysaccharides by weight.

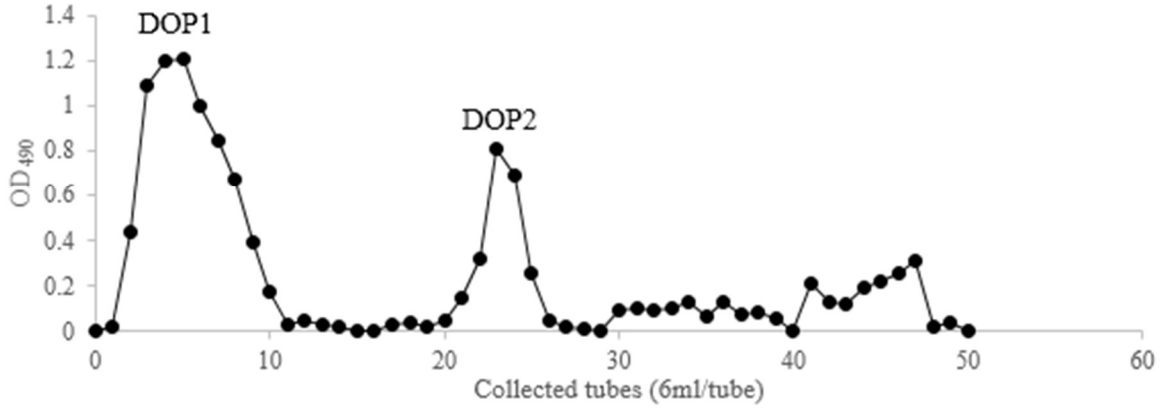

Fig. 2 DOP elution curve. Isolation of D. officinale polysaccharides. Ion-exchange chromatogram of the crude polysaccharide (DOP) on a DEAE-cellulose column (200 × 26 mm), and elution with distilled water, and NaCl solution (0.05 M and 0.2 M)

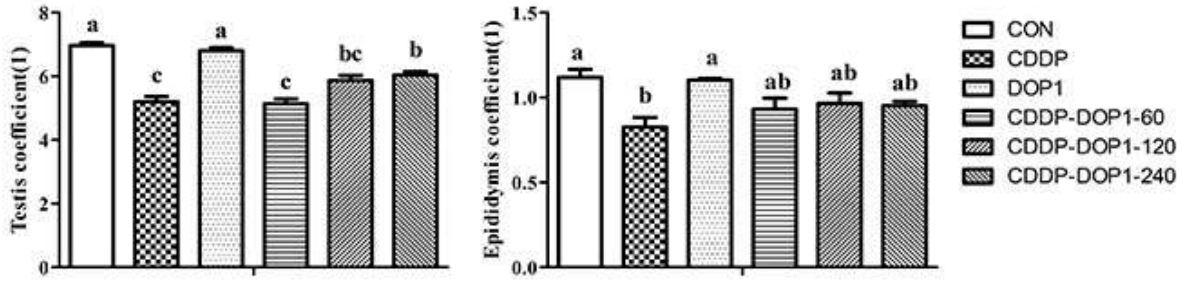

Fig. 3 The effect of DOP1 on reproductive organ weight. Significant differences among the groups are marked with different superscript letters ($\alpha < 0.05$). CON is the control group, CDDP is the CDDP-induced group, the CDDP-DOP1-60 group was gavaged with 60 mg/kg/d and induced by CDDP, the CDDP-DOP1-120 group was gavaged by 120 mg/kg/d and induced by CDDP, and the CDDP-DOP1-240 group was gavaged by 240 mg/kg/d and induced by CDDP

3.2 Effect of DOP1 on Reproductive Organ Weight

The testis and epididymis constitute important reproductive organs of the male body that are capable of producing a large number of spermatozoa [24]. Compared with the CDDP group, the testicular coefficient of the CDDP-DOP1 groups was significantly ($P < 0.05$) increased with a polysaccharide concentration of 240 mg/kg (Fig. 3), whereas the epididymal coefficient showed no significant changes. This may suggest that DOP1 exerted differential effects on different components of the reproductive axis.

3.3 Effect of DOP1 on Sperm Parameters

We observed various sperm indices to investigate the biologic effects of DOP1 on oxidative stress injury to the reproductive system in CDDP-induced mice (Fig. 4). The mouse model induced by CDDP led to physical malformations that included a tapered (Fig. 4A) or pyriform-shaped head (Fig. 4B), and reduced spermatozoal vitality (Fig. 4C) and maturity (Fig. 4D) compared with the normal control group. In contrast, DOP1 administration effectively reduced CDDP-induced abnormalities dose-dependently; and augmented sperm number, viability, and maturity (Fig. 5). Although sperm indices with DOP1 treatment were worsened relative to normal controls, DOP1 still relieved some sperm pathologies and protected sperm from oxidative stress.

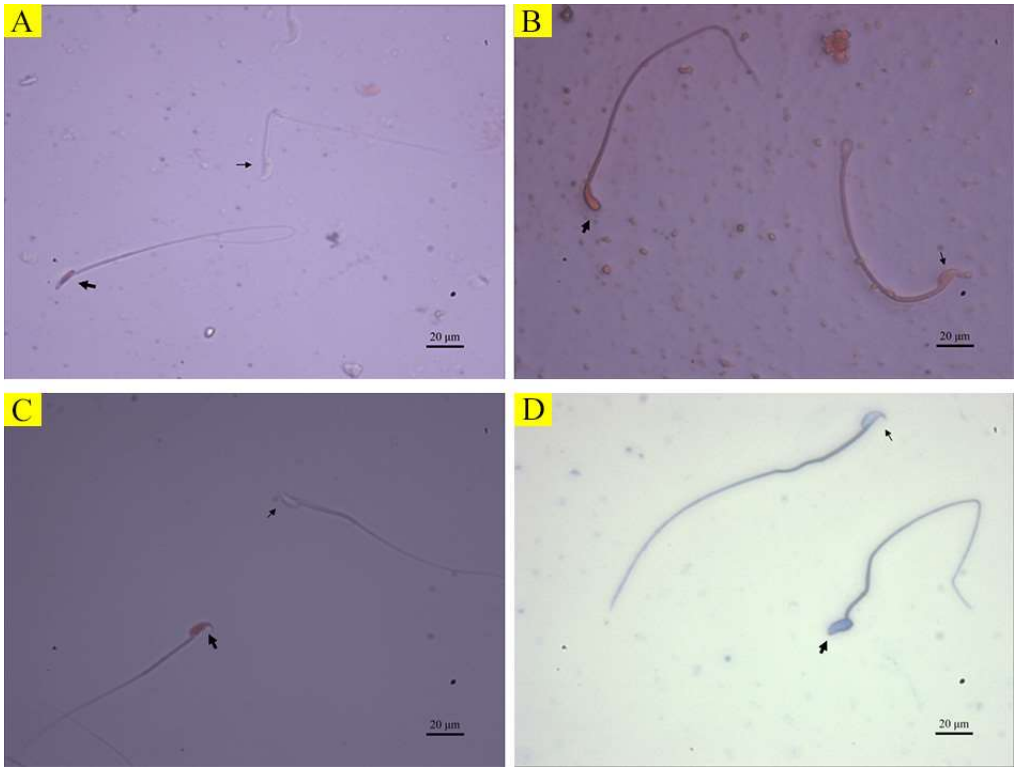

Fig. 4 The effect of DOP1 on sperm characteristics. Micrographs of spermatozoa, with arrows pointed to the sperm head. (A) The thick arrow points to a tapered head, and the thin arrow points to a normal sperm head (eosin staining). (B) The thick arrow points to a pyriform head, and the thin arrow points to a normal head (eosin staining). (C) The thick arrow points to dead sperm, and the thin arrow points to normal sperm (eosin staining). (D) The thick arrow points to an immature sperm nucleus, and the thin arrow points to mature spermatozoa (aniline blue staining)

3.4 Effects of DOP1 on Testicular Oxidative Stress

GSH occupies an important role in biologic processes, and its use in infertile men resulted in improvement of fertility [25]. Increasing the levels of antioxidants such as SOD and GSH-PX increased sperm vitality; and, conversely, increasing ROS in sperm preparation medium resulted in DNA damage, deleterious changes in the acrosome reaction, and failure of sperm attachment to the zona pellucida [26].

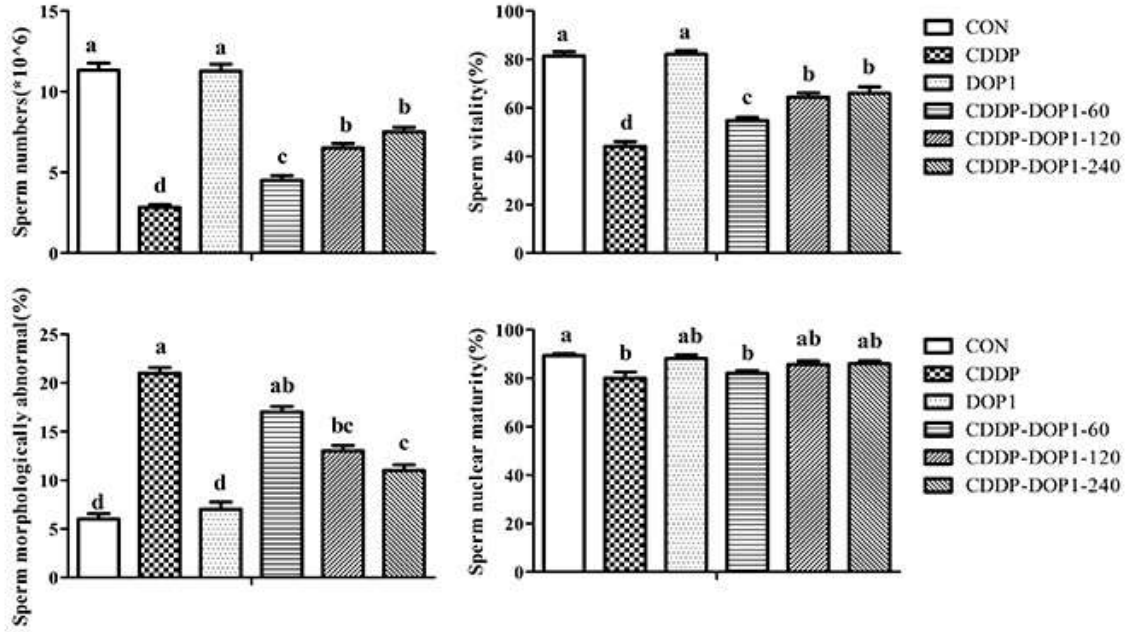

Fig. 5 Changes in sperm numbers, vitality, morphologically abnormal sperm, and mature sperm after administration of DOP1. CON is the control group, CDDP is the CDDP-induced group, the CDDP-DOP1-60 group was gavaged with 60 mg/kg/d and induced by CDDP, the CDDP-DOP1-120 group was gavaged by 120 mg/kg/d and induced by CDDP, and the CDDP-DOP1-240 group was gavaged by 240 mg/kg/d and induced by CDDP

As shown in Fig. 6, GSH-PX and SOD levels of the DOP1-treatment groups at all 3 concentrations were significantly different from the model group ($P < 0.05$). Furthermore, the level of MDA was significantly lower ($P < 0.05$) and the concentration of GSH was significantly higher ($P < 0.05$) when the DOP1 concentration was increased to 240 mg/kg.

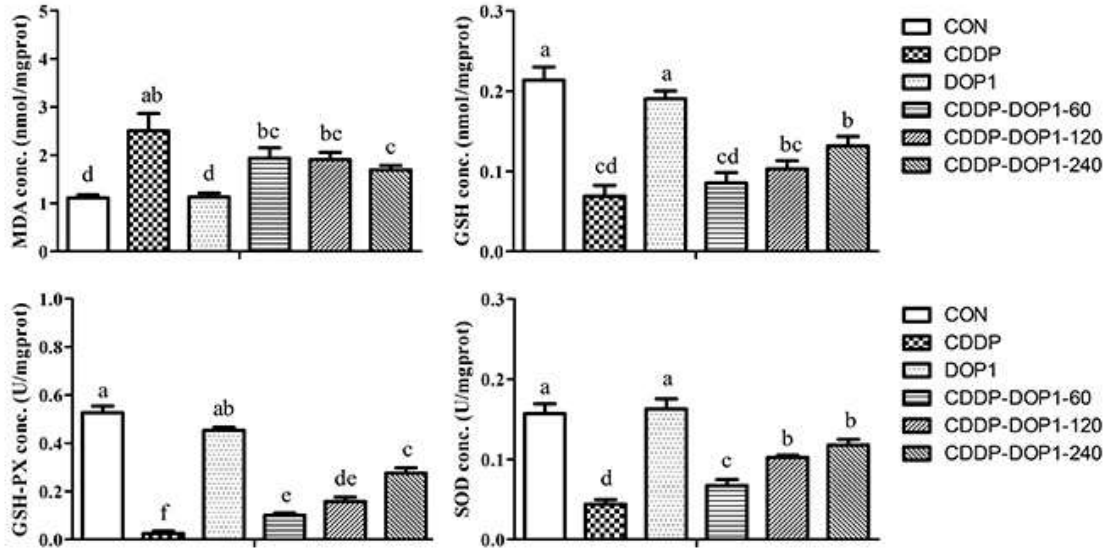

Fig. 6 The effect of DOP1 on oxidative stress in mouse testis (n = 5/group). CON is the control group, CDDP is the CDDP-induced group, the CDDP-DOP1-60 group was gavaged with 60 mg/kg/d and induced by CDDP, the CDDP-DOP1-120 group was gavaged by 120 mg/kg/d and induced by CDDP, and the CDDP-DOP1-240 group was gavaged by 240 mg/kg/d and induced by CDDP

3.5 Effects of DOP1 on Testicular Pathology

Pathologic sections of testes were prepared to investigate the effects of DOP1 on oxidative stress injury in CDDP-induced mice. As shown in Fig. 7A, spermatogenic cells were characteristically hierarchically arranged in the convoluted seminiferous tubules. Compared with the normal group, spermatogenic cells were significantly shrunken and diminished in number in the CDDP model group. These results indicated that we successfully created a reproductively impaired mouse model, showed that CDDP administration caused notable pathologic changes in the testes, and showed that, to an extent, DOP1 administration alleviated CDDP-induced reproductive injury in a dose-dependent fashion.

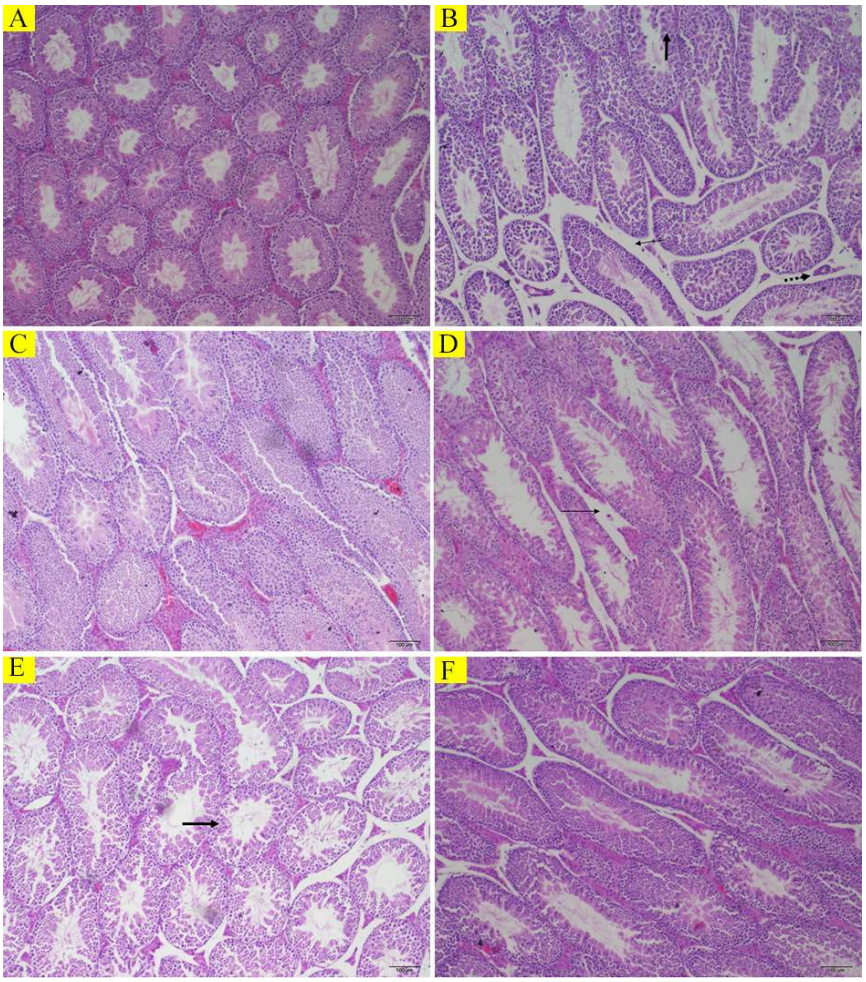

Fig. 7 H&E staining of testis after intragastric administration of different doses of DOP1 (100×). (A) CON group. (B) CDDP 21-d group: thick arrow points to residual nuclear contour, thick dashed arrow points to a congested blood vessel. (C) DOP1 21-d group. (D) CDDP-DOP1-60 group: thin arrow points to interstitial loose; (E) CDDP-DOP1-120 group. (F). CDDP-DOP1-240 group

3.6 Effect of DOP1 on Expression of Nrf2/ARE and Apoptosis-related Genes

To determine roles for Nrf2 in the mouse testis after treatment with DOP1 and/or CDDP, we assessed the expression levels of Nrf2 RNA and protein by quantitative RT-PCR (Fig. 8) and western blotting analysis (Fig. 9), respectively. Pretreatment with CDDP significantly inhibited the expression of Nrf2, HO-1, and NQO1 compared with the normal group, and also upregulated the expression of Bax/Bcl-2 compared with normals. Compared to the model group, CDDP treatment significantly increased the expression of activated Nrf2, HO-1, and NQO1; and these genes were gradually upregulated commensurately with increasing DOP1 dosage. In addition, Bax/Bcl-2 expression were also downregulated compared with the model group. These data showed that treatment with DOP1 significantly activated Nrf-2 protein expression in CDDP-induced testicular tissues.

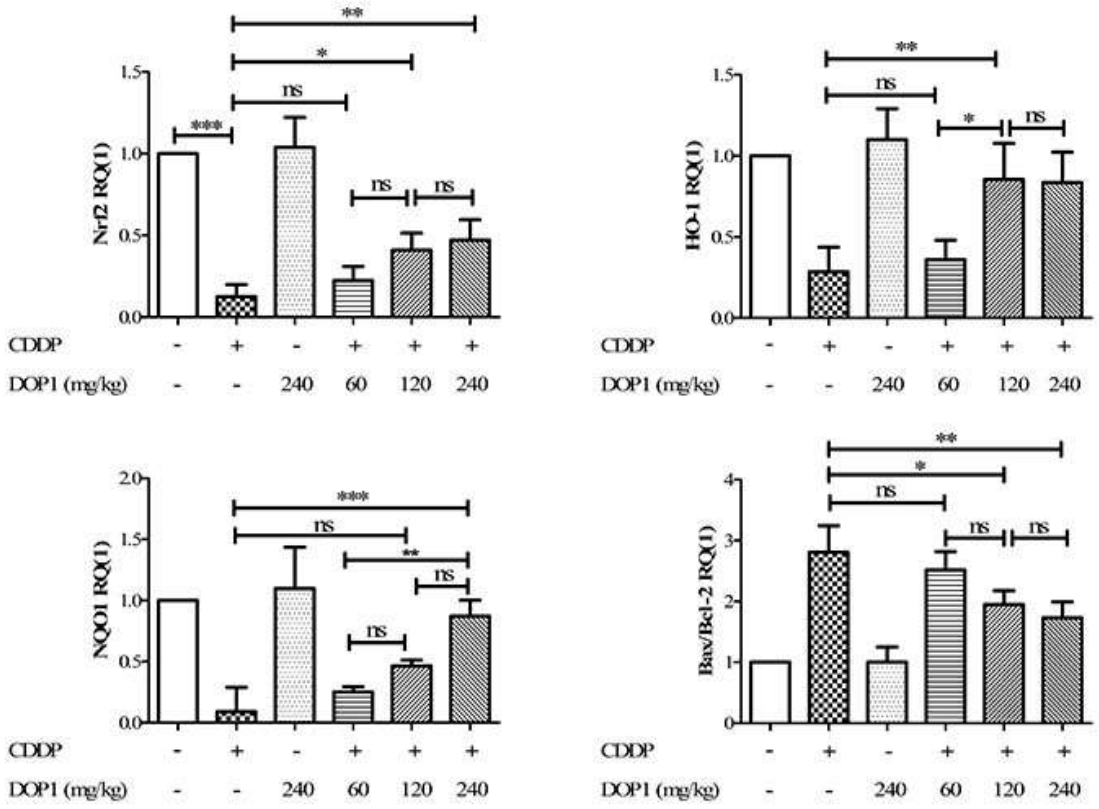

Fig. 8 Expression levels of Nrf2/ARE-related mRNA in testis after intragastric administration of different doses of DOP1

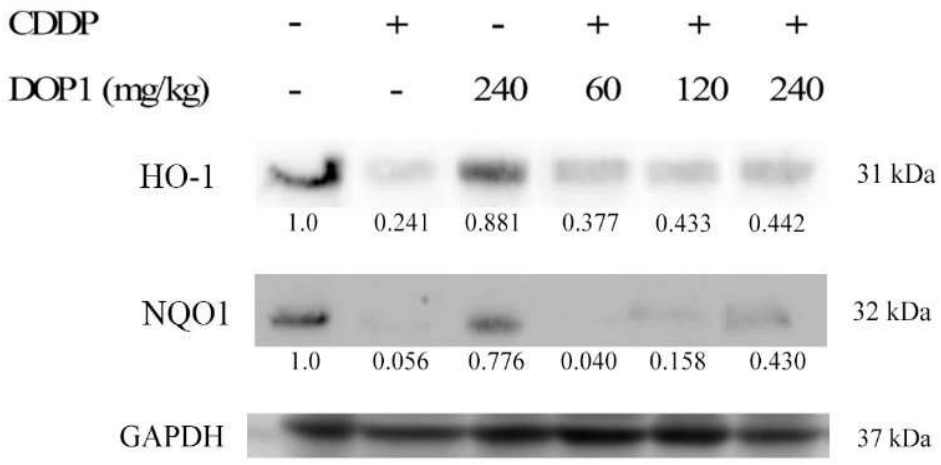

Fig. 9 Expression levels of ARE-related protein in testis after intragastric administration of different doses of DOP1

3.7 Effects of DOP1 on Apoptosis in the Testis

We performed Hoechst staining of the testicular tissue from each group of mice and made observations under a fluorescence microscope (Fig. 10). In the normal group, Sertoli, Leydig, and peritubular myoid cells showed normal blue stain, whereas the model group showed cells as more deeply stained. After DOP1 treatment, the brightness of the fluorescence gradually weakened. These results suggested that DOP1 can attenuate the apoptotic status of testicular somatic cells.

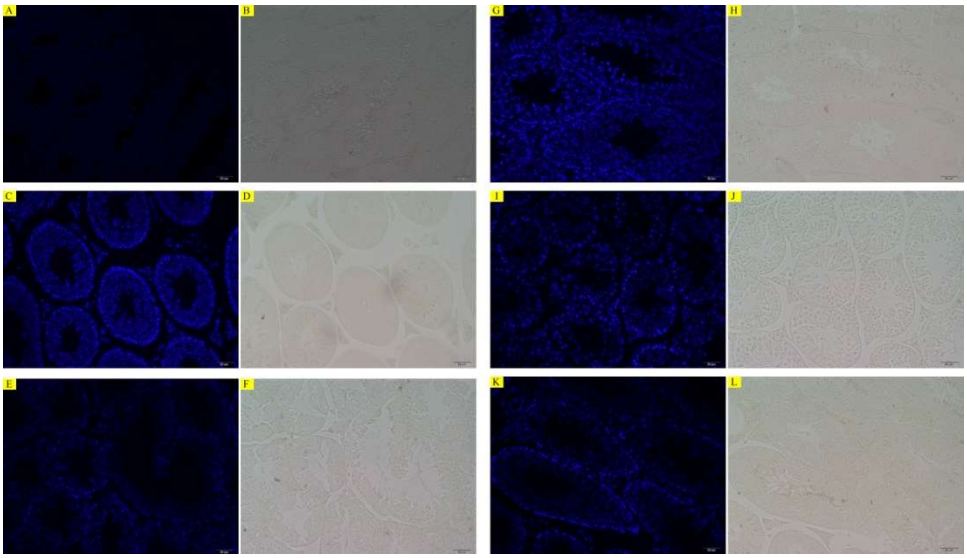

Fig. 10 Hoechst staining results are shown in A, C, E, G, I, and K; and DIC results are shown in B, D, F, H, J, and L. A, B–CON group. C, D–CDDP group. E, F–DOP1 group. G, H–CDDP-DOP1-60 group. I, J–CDDP-DOP1-120 group. K, L–CDDP-DOP1-240 group

4. Conclusions

Administration of DOP1 exerted a therapeutic effect on the induction by CDDP of oxidative stress damage to the reproductive system of male mice, its therapeutic effects were manifested as improvement in general symptoms, reduction in antioxidant injury, modulation of sperm characteristics, and attenuation of reproductive oxidative stress. But for the further study, each chemical form of DOP1 that goes through the process of absorption, distribution, metabolism, and excretion needs to be clarified. Based on these results, we hypothesize that DOP1 will become a potential treatment of sperm defects caused by oxidative stress damage to the reproductive system.

References

[1] G. Bedoschi, P. A. Navarro and K. Oktay: Chemotherapy-induced damage to ovary: mechanisms and clinical impact, Future Oncology, Vol. 12 (2016) No. 20, p. 2333–2344.

[2] R. Siegel, D. Naishadham and A. Jemal: Cancer statistics, 2013, CA: A Cancer Journal for Clinicians, Vol. 63 (2013) No. 1, p. 11–30.

[3] S. W. Hansen, J. G. Berthelsen and H. von der Maase: Long-term fertility and Leydig cell function in patients treated for germ cell cancer with cisplatin, vinblastine, and bleomycin versus surveillance, Journal of Clinical Oncology, Vol. 8 (1990) No. 10, p. 1695–1698.

[4] T. Afsar, S. Razak, M. R. Khan and A. Almajwal: Acacia hydaspica ethyl acetate extract protects against cisplatin-induced DNA damage, oxidative stress and testicular injuries in adult male rats, BMC Cancer, Vol. 17 (2017) No. 1, p. 883.

[5] R. E. Drasga, L. H. Einhorn, S. D. Williams, D. N. Patel and E. E. Stevens: Fertility after chemotherapy for testicular cancer, Journal of Clinical Oncology, Vol. 1 (1983) No. 3, p. 179–183.

[6] D. K. Armstrong, B. Bundy, L. Wenzel, H. Q. Huang, R. Baergen, S. Lele, L. J. Copeland, J. L. Walker and R. A. Burger: Intraperitoneal cisplatin and paclitaxel in ovarian cancer, New England Journal of Medicine, Vol. 354 (2006) No. 1, p. 34–43.

[7] S. J. Howell and S. M. Shalet: Testicular function following chemotherapy, Human Reproduction Update, Vol. 7 (2001) No. 4, p. 363–369.

[8] M. Schrader, M. Müller, B. Straub and K. Miller: The impact of chemotherapy on male fertility: a survey of the biologic basis and clinical aspects, Reproductive Toxicology, Vol. 15 (2001) No. 6, p. 611–617.

[9] M. L. Meistrich: Effects of chemotherapy and radiotherapy on spermatogenesis, European Urology, Vol. 23 (1993) No. 1, p. 136–142.

[10] P. a. Ma, H. Xiao, C. Yu, J. Liu, Z. Cheng, H. Song, X. Zhang, C. Li, J. Wang, Z. Gu and J. Lin: Enhanced cisplatin chemotherapy by iron oxide nanocarrier-mediated generation of highly toxic reactive oxygen species, Nano Letters, Vol. 17 (2017) No. 2, p. 928–937.

[11] S. Dasari and P. B. Tchounwou: Cisplatin in cancer therapy: molecular mechanisms of action, European Journal of Pharmacology, Vol. 740 (2014), p. 364–378.

[12] J. C. Dabrowiak, J. Goodisman and A.-K. Souid: Kinetic study of the reaction of cisplatin with thiols, Drug Metabolism and Disposition, Vol. 30 (2002) No. 12, p. 1378–1384.

[13] L. Xia, X. Liu, H. Guo, H. Zhang, J. Zhu and F. Ren: Partial characterization and immunomodulatory activity of polysaccharides from the stem of Dendrobium officinale (Tiepishihu) in vitro, Journal of Functional Foods, Vol. 4 (2012) No. 1, p. 294–301.

[14] Y.-F. Hua, M. Zhang, C.-X. Fu, Z.-H. Chen and G. Y. S. Chan: Structural characterization of a 2-O-acetylglucomannan from Dendrobium officinale stem, Carbohydrate Research, Vol. 339 (2004) No. 13, p. 2219–2224.

[15] T.-B. He, Y.-P. Huang, L. Yang, T.-T. Liu, W.-Y. Gong, X.-J. Wang, J. Sheng and J.-M. Hu: Structural characterization and immunomodulating activity of polysaccharide from Dendrobium officinale, International Journal of Biological Macromolecules, Vol. 83 (2016), p. 34–41.

[16] X. Huang, S. Nie, H. Cai, G. Zhang, S. W. Cui, M. Xie and G. O. Phillips: Study on Dendrobium officinale O-acetyl-glucomannan (Dendronan): Part IV. Immunomodulatory activity in vivo, Journal of Functional Foods, Vol. 15 (2015), p. 525–532.

[17] Q. Zeng, C.-H. Ko, W.-S. Siu, L.-F. Li, X.-Q. Han, L. Yang, C. Bik-San Lau, J.-M. Hu and P.-C. Leung: Polysaccharides of Dendrobium officinale Kimura & Migo protect gastric mucosal cell against oxidative damage-induced apoptosis in vitro and in vivo, Journal of Ethnopharmacology, Vol. 208 (2017), p. 214–224.

[18] S. Li, Z. Song, T. Liu, J. Liang, J. Yuan, Z. Xu, Z. Sun, X. Lai, Q. Xiong and D. Zhang: Polysaccharide from Ostrea rivularis attenuates reproductive oxidative stress damage via activating Keap1-Nrf2/ARE pathway, Carbohydrate Polymers, Vol. 186 (2018), p. 321–331.

[19] H. Motohashi and M. Yamamoto: Nrf2–Keap1 defines a physiologically important stress response mechanism, Trends in Molecular Medicine, Vol. 10 (2004) No. 11, p. 549–557.

[20] M. He, H. Pan, R. C.-C. Chang, K.-F. So, N. C. Brecha and M. Pu: Activation of the Nrf2/HO-1 antioxidant pathway contributes to the protective effects of Lycium barbarum polysaccharides in the rodent retina after ischemia-reperfusion-induced damage, PLoS One, Vol. 9 (2014) No. 1, p. e84800.

[21] L. Shi, K. Chen, Q. Dong, J. Fang and K. Ding: Separation, purification and structure elucidation of a polysaccharide from root of Cudrania tricuspidata, Frontiers of Chemistry in China, Vol. 3 (2008) No. 2, p. 209–212.

[22] M. Li, H. Yue, Y. Wang, C. Guo, Z. Du, C. Jin and K. Ding: Intestinal microbes derived butyrate is related to the immunomodulatory activities of Dendrobium officinale polysaccharide, International Journal of Biological Macromolecules, Vol. 149 (2020), p. 717–723.

[23] J. G. Harman and J. H. Richburg: Cisplatin-induced alterations in the functional spermatogonial stem cell pool and niche in C57/BL/6J mice following a clinically relevant multi-cycle exposure, Toxicology Letters, Vol. 227 (2014) No. 2, p. 99–112.

[24] R. L. Oliveira, A. Parent, D. G. Cyr, M. Gregory, C. A. Mandato, C. E. Smith and L. Hermo: Implications of caveolae in testicular and epididymal myoid cells to sperm motility, Molecular Reproduction and Development, Vol. 83 (2016) No. 6, p. 526–540.

[25] M. E. Anderson: Glutathione: an overview of biosynthesis and modulation, Chemico-Biological Interactions, Vol. 111–112 (1998), p. 1–14.

[26] H. Fingerova, I. Oborna, J. Novotny, M. Svobodova, J. Brezinova and L. Radova: The measurement of reactive oxygen species in human neat semen and in suspended spermatozoa: a comparison, Reproductive Biology and Endocrinology, Vol. 7 (2009), p. 118.