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Two resveratrol analogs, pinosylvin and 4,4′-dihydroxystilbene, improve oligoasthenospermia in a mouse model by attenuating oxidative stress via the Nrf2-ARE pathway

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ABSTRACT

Two synthesized resveratrol analogs from our laboratory, namely pinosylvin (3,5-dihydroxy-*trans*-stilbene, PIN) and 4,4′-dihydroxystilbene (DHS), have been carefully evaluated for treatment of oligoasthenospermia. Recent studies have demonstrated that PIN and DHS improved sperm quality in the mouse. However, the mechanism of action of PIN and DHS on oligoasthenospermia remains unknown. Herein, we investigated the mechanistic basis for improvements in sperm parameters by PIN and DHS in a mouse model of oligoasthenospermia induced by treatment with busulfan (BUS) at 6 mg/kg b.w.. Two weeks following busulfan treatment, mice were administered different concentrations of PIN or DHS daily for 2 consecutive weeks. Thereafter, epididymal sperm concentration and motility were determined, and histopathology of the testes was performed. Serum hormone levels including testosterone (T), luteinizing hormone (LH), and follicle stimulating hormone (FSH) were measured using corresponding specific enzyme-linked immunosorbent assay (ELISA) kits. Testicular mRNA expression profiles were determined by RNA sequencing analysis. These findings were validated by quantitative real-time PCR, western blotting and ELISA. Both PIN and DHS improved the epididymal sperm concentration and motility, enhanced testosterone levels, and promoted testicular morphological recovery following BUS treatment. PIN treatment was found to significantly reduce oxidative stress via the nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE)-dependent antioxidant, glutathione peroxidase 3. DHS treatment significantly reduced oxidative stress via the Nrf2-ARE-dependent antioxidants glutathione S-transferase theta 2 and glutathione S-transferase omega 2. In summary, PIN and DHS ameliorated oligoasthenospermia in this mouse model by attenuating oxidative stress via the Nrf2-ARE pathway.

1. Introduction

About 13% of couples worldwide suffer from infertility [1], and men contribute to about 50% of infertility [2]. Oligoasthenospermia is one of the most notable causes of male-factor infertility. While there are many reasons, but the molecular mechanism is still unclear. Oxidative stress is considered to be an important contributing factor, caused by the overproduction of reactive oxygen species (ROS) in the male reproductive tissues [3], leading to sperm cell damage, reduced sperm motility, and germ cell apoptosis, as well as DNA damage in testicular tissues [4–6]. The nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) signaling pathway is considered to be one of the most important pathways of defense against oxidative stress [7–8]. Ubiquitination is inhibited after Nrf2 is stimulated by noxious chemicals, when more Nrf2 is generated and accumulated. Thereafter, Nrf2 enters into the cell nucleus to bind to ARE gene sequences and activates downstream genes encoding antioxidative enzymes and the glutathione redox system, inducing ROS degradation,

Abbreviations: PIN, pinosylvin; DHS, 4,4′-dihydroxystilbene; RES, resveratrol; BUS, busulfan; ELISA, enzyme-linked immunosorbent assay; Nrf2, nuclear factor erythroid 2-related factor 2; ARE, antioxidant response element; ROS, reactive oxygen species; MDA, malondialdehyde; Gpx3, glutathione peroxidase 3; GSTT2, glutathione S-transferase theta 2; GSTO2, glutathione S-transferase omega 2; GAPDH, glyceraldehyde-3- phosphate dehydrogenase; HE, hematoxylin and eosin; T, testosterone; FSH, follicle stimulating hormone; LH, luteinizing hormone; GO, gene Ontology; PVDF, polyvinylidene difluoride

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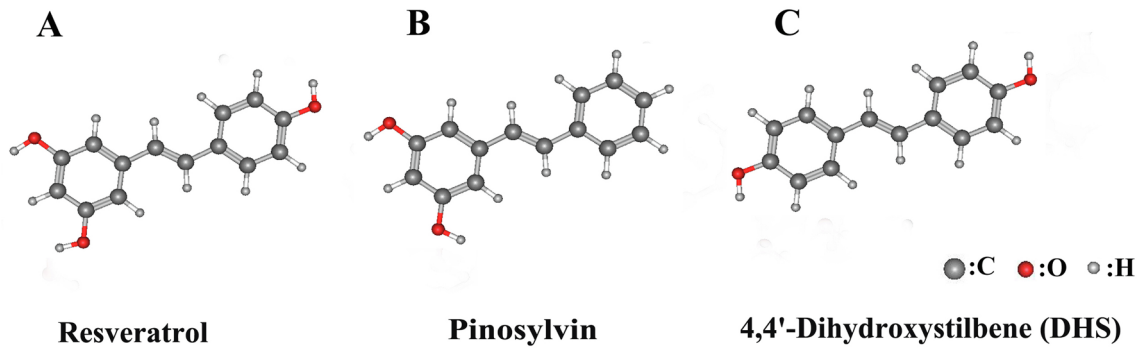


Fig. 1. Chemical structure of Resveratrol, Pinosylvin and DHS. (A): Resveratrol (RES), (B): Pinosylvin (PIN), (C): 4,4′-dihydroxystilbene (DHS).

which in turn protects cells and tissues against ROS-mediated damage [9–11]. Current therapeutic approch for the treatment of oligoasthenospermia is ineffective [12]. At present, Traditional Chinese Medicine is receiving increasing attention as it often offers more effective treatment options [13].

Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene, RES; Fig. 1A), a polyphenolic stilbene compound, has been used to treat oligoasthenospermia and attenuate testicular damage of mouse, mainly because of its strong antioxidant activity [14–17]. However, the clinical application of resveratrol has limitations because this drug is relatively unstable, it has poor bioavailability and is cleared rapidly from the systemic circulation [18–20]. Therefore, it is necessary to find more active antioxidants based on RES to overcome these disadvantages. We removed the 4′-hydroxyl group from RES to increase its lipophilicity. Also, we altered the position of the hydroxyl group to make the compound more symmetrical and stable. Based on these principles, two RES analogs, pinosylvin (3,5-dihydroxy-*trans*-stilbene, PIN, Fig. 1B) and 4,4′-dihydroxy-stilbene (DHS, Fig. 1C), have been synthesized in our laboratory [21].

Busulfan (BUS), an anticancer drug, can cause male reproductive system damage because of its toxic side effects [22]. Different concentrations of BUS can cause different degrees of testicular tissue damage. For instance, high concentrations of BUS can cause aspermia, and lower concentrations can cause oligoasthenospermia [23–24]. Our pilot study demonstrated that PIN and DHS improved sperm quality of mouse in a concentration-dependent manner (Supplementary Fig. S1; *note*: Supplemental data can be found at: https://figshare.com/articles/figure/Supplemental_Data_BIOORG-D-20–00181-R1/12930947). To further understand the roles and mechanisms of PIN and DHS in treating oligoasthenospermia, we investigated the mechanistic basis for improving epididymal sperm parameters by treatment with high concentrations of PIN (100 mg/kg b.w.) and DHS (100 mg/kg b.w.) in a mouse model of oligoasthenospermia induced by treatment of adult mice with BUS. We found that PIN and DHS ameliorated BUS-induced oligoasthenospermia by attenuating oxidative stress via the Nrf2-ARE pathway.

2. Results

2.1. Synthesis of PIN and DHS

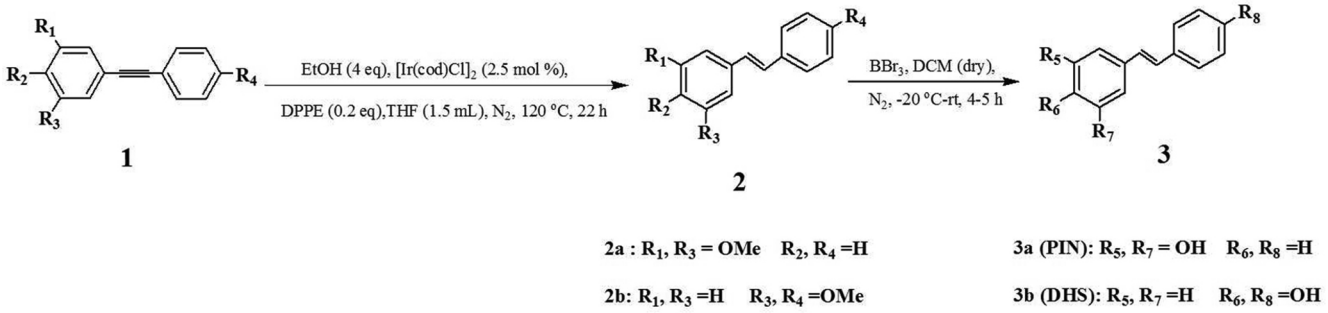
The synthesis of PIN and DHS is noted in Scheme 1. To a 15 mL pressure tube were added diarylacetylene **1** (0.20 mmol), $[Ir(cod)Cl]_2$ (5 μmol, 3.6 mg), DPPE (0.04 mmol, 15.9 mg) under N_2, and then EtOH (4 mmol, 232 μL) and THF (1.5 mL) were also added. The resulting solution was stirred at 120 °C for 22 h. Then, the solution was cooled to room temperature, and diluted with ethyl acetate (10 mL). The combined organic phases were washed with saturated saline, and the aqueous phase was extracted with ethyl acetate and dried over anhydrous Na_2SO_4. After filtration, the solvent was evaporated and the crude product was purified by column chromatography (*n*-Hexane/EtOAc = 40:1) to obtain the desired product **2** (**2a**, 80% yield; **2b**, 73% yield). **3a (PIN) and 3b (DHS):** To a solution of product **2** (0.2 mmol) in anhydrous DCM (6 mL) maintained under nitrogen at −20 °C with stirring was added BBr_3 dropwise (0.34 g, 1.34 mmol, 30% in DCM). The mixture, maintained under nitrogen with stirring, was allowed to warm up to room temperature for 4–5 h, and then it was poured into water (10 mL) and extracted with DCM (3 × 10 mL). The collected organic phases were washed with brine (10 mL) and then dried over Na_2SO_4, filtered and concentrated in vacuo. The residue purified by column chromatography (*n*-Hexane/Acetone = 6:4) to afford the desired product **3**. **PIN:** 83% yield, white solid, ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 7.57 (d, J = 7.2 Hz, 2H), 7.35 (t, J = 7.2 Hz, 2H), 7.25 (t, J = 7.2 Hz, 1H), 7.10 (s, 2H), 6.59 (d, J = 2.0 Hz, 2H), 6.31 (t, J = 2.4 Hz, 1H); ^{13}C NMR (100 MHz, $(CD_3)_2CO$): δ 159.56, 140.37, 138.36, 129.77, 129.50, 129.17, 128.35, 127.34, 105.94, 103.06. **DHS:** 77.8% yield, white solid, ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 8.40 (s, 2H), 7.40 (d, J = 8.8 Hz, 4H), 6.96 (s, 2H), 6.83 (d, J = 8.8 Hz, 4H); ^{13}C NMR (100 MHz, $(CD_3)_2CO$): δ 157.72, 130.47, 128.29, 126.46, 116.35.

2.2. PIN and DHS treatments improved epididymal sperm quality

In this report, we elected to use RES, PIN and DHS at a concentration of 100 mg/kg b.w. because results of pilot experiments as noted in Fig. S1 had indicated that this is the most effective dose to improve oligospermia. Herein, it was shown that at the end of the experiment, there were no significant differences in body weight among the five groups of mice (Fig. 2A). However, BUS treatment considerably reduced testis weight compared with the normal control group. There was a significant (P = 0.011) increase in testicular weight in the PIN treatment group after BUS administration, while there were no significant increases in testicular weights after the RES and DHS treatments (Fig. 2B). The epididymal sperm concentration and motility in the BUS group were lower than in the normal group, demonstrating that our mouse model for oligoasthenospermia had been established successfully. Treatment with RES, PIN, and DHS significantly improved epididymal sperm concentration (P = 0.036, 0.001, 0.001) and motility (P = 0.001, 0.001, 0.007). PIN and DHS treatments improved sperm concentrations better than RES, while the DHS treatment group had a lower improvement in sperm motility compared with the RES and PIN groups (Fig. 2C, D).

2.3. PIN and DHS treatments reversed testicular damage

Histological analysis by HE staining of testis sections from the normal group indicated normal spermatogenesis, including regular arrangements of primary/secondary spermatocytes, spermatids, and spermatogenic epithelial cells across the epithelium of seminiferous



Scheme 1. Synthesis of resveratrol analogs, pinosylvin and 4,4′-dihydroxystilbene. Reagents and conditions: (i) EtOH (4.0 eq), $[Ir(cod)Cl]_2$ (2.5 mol %), DPPE (0.2 eq), THF, N_2, 120 °C, 22 h; (ii) BBr_3, DCM (dry), N_2, −20 °C-rt, 4–5 h.

tubules (Fig. 3A, A′), whereas BUS induced significant tissue damage including disorganization and loss of spermatogenic cells (Fig. 3B, B′). Administration of RES, PIN, and DHS all reversed the BUS-induced histopathology damage, but PIN and DHS treatments were more effective in this than RES (Fig. 3C, C′, D, D′, E, E′).

2.4. Effects of PIN and DHS treatments on serum levels of T, FSH, and LH

Serum hormone assays showed that the levels of T and FSH were significantly (P = 0.001, 0.001) reduced by BUS administration compared with the normal group, the levels of LH were also reduced by BUS administration (P = 0.049). The results showed that PIN treatments markedly increased the levels of T, FSH, and LH (P = 0.001, 0.001, 0.001) after BUS administration, which was similar to RES. The serum T levels significantly (P = 0.0025) increased with DHS treatment but the levels of FSH and LH levels decreased (Fig. 4A–C).

2.5. Differentially expressed mRNAs between the BUS, PIN, and DHS treatment groups

To investigate the mechanism of action of PIN and DHS on oligoasthenospermia, expression profiling studies were performed on the mRNA from two independent samples in the normal, BUS, PIN, and DHS groups for RNA-Seq and transcriptome profiling. GO classification (Fig. 5A, B) analyses demonstrated the expression patterns of mRNAs. As PIN and DHS are both polyphenolic compounds acting as antioxidants, we also studied antioxidant-related genes. GO analysis indicated that the differentially expressed mRNAs were enriched in terms of peroxidase activity, oxidoreductase activity (acting on peroxide as an acceptor), and antioxidant activity (Fig. 5A, B; yellow background). Table S1 shows the differentially expressed mRNAs of antioxidant-related genes. Most of these were enriched in the glutathione redox system including Gpx3, GSTT2, and GSTO2. RNA-seq analysis showed that, compared with the BUS group, the expression of Gpx3 was significantly upregulated in the PIN group with a > 1.5-fold change

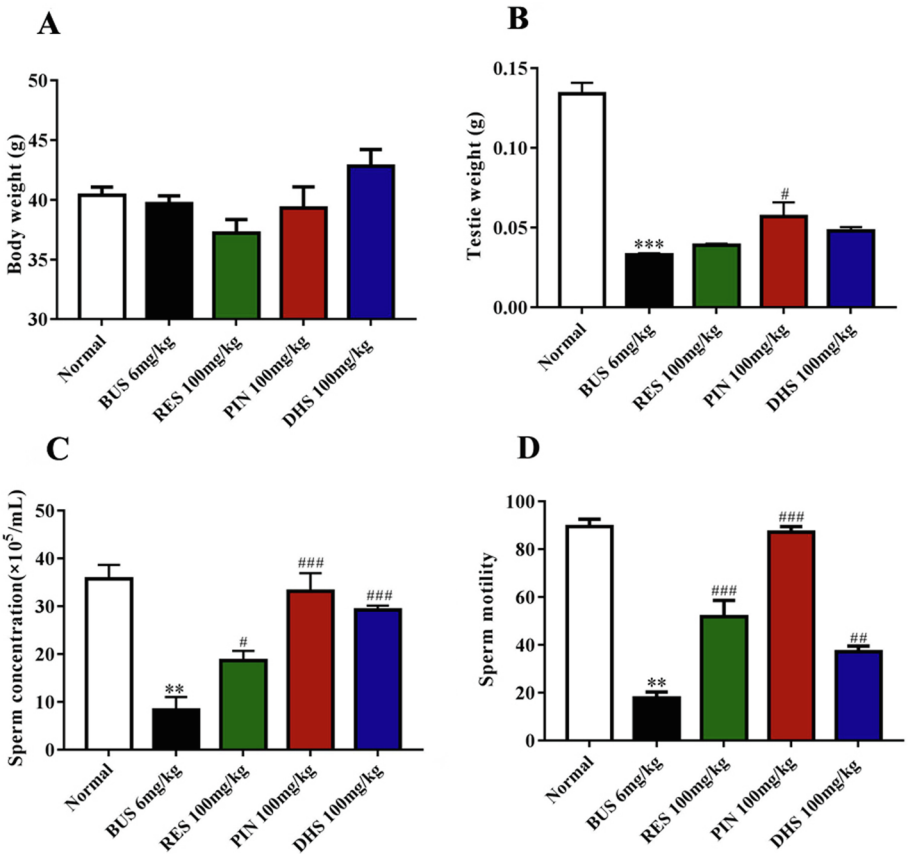


Fig. 2. Effects on body, testicular organ index and sperm parameters. (A): Body weight, (B): Testicular weight, (C): Sperm concentration, (D): Sperm motility. **P < 0.01 vs. Normal group, ***P < 0.001 vs. Normal group, #P < 0.05 vs. BUS group, ##P < 0.01 vs. BUS group, ###P < 0.001 vs. BUS group. Each column represents the mean ± SEM, n = 6.

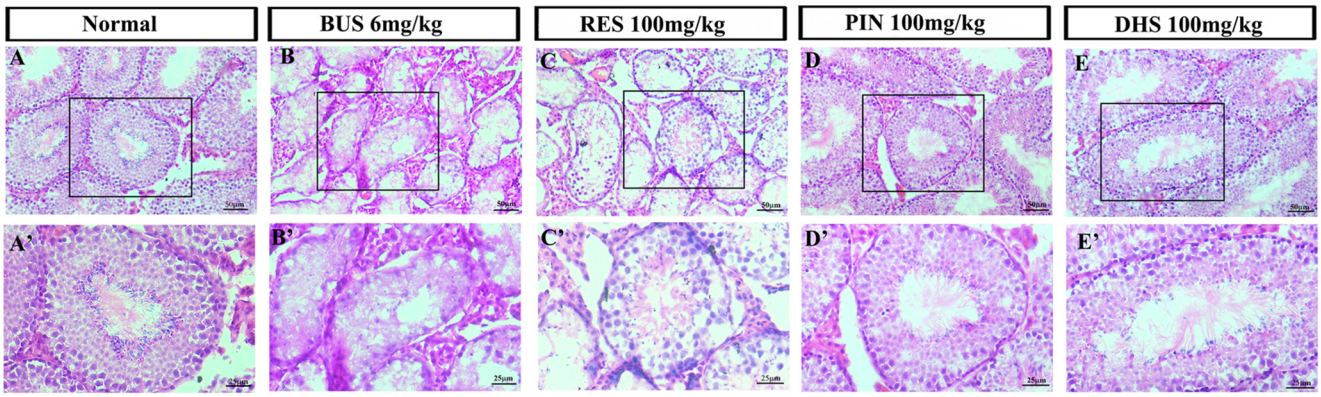


Fig. 3. Histological examination of mice testes. (A–E): Scale bar = 50 μm, (A′–E′): Scale bar = 25 μm.

($P < 0.05$), whereas it was downregulated in the DHS group with a > 1.5-fold change ($P < 0.05$). Compared with the BUS treatment group, the expressions of both GSTT2 and GSTO2 were significantly upregulated in the DHS group with a > 1.5-fold change ($P < 0.05$).

2.6. Validation of the expression of genes in the glutathione redox system by RT-qPCR

The relative Gpx3 gene expression in the PIN group was higher than in the BUS group, while it was lower in the DHS group. The relative gene expression levels of GSTT2 and GSTO2 in the DHS group were higher than in the BUS group (Fig. 6). These results of the qRT-PCR analysis were consistent with the RNA-seq data, indicating that the expressions of Gpx3, GSTT2, and GSTO2 might be related to the antioxidative actions of PIN and DHS.

2.7. Validation of the Nrf2-ARE pathway

RT-qPCR was also used to detect the expression of Nfe2l2, an upstream gene in the glutathione redox system. The relative Nfe2l2 expression in the BUS group was obviously less than in the normal group, but was induced in both the PIN and DHS groups, even higher than the control group (Fig. 7A). The protein levels of Nrf2, Gpx3, GSTT2, and GSTO2 were quantified by western blotting. This showed that the Nrf2 protein level was lower in the BUS group than in the normal group, and higher in both the PIN and DHS groups than in the BUS group and also the control group (Fig. 7B, C). The Gpx3 protein level in the BUS group was significantly lower than in the normal group, and was significantly increased after PIN treatment (Fig. 7D, G). The GSTT2 and GSTO2 protein levels in the BUS group were obviously lower than in the normal group, and were significantly increased after DHS treatment (Fig. 7E, F, H, I).

2.8. Effects of PIN and DHS treatments on the levels of ROS and MDA

The levels of ROS and MDA in testicular tissue were measured using corresponding specific ELISA kits. The concentrations of ROS and MDA in the BUS group were markedly ($P = 0.001, 0.026$) higher than in the normal group, and were significantly decreased by PIN ($P = 0.005, 0.001$) and DHS ($P = 0.009, 0.001$) treatments (Fig. 7J, K). These findings thus support that notion that PIN and DHS treatments appeared to improve experimental oligoasthenospermia by attenuating oxidative stress via the Nrf2-ARE pathway.

2.9. Interactions of PIN and DHS with Nrf2

Molecular docking studies demonstrated that PIN and DHS could bind effectively with Nrf2 (Fig. 8A, D). The docking grid score for PIN combined with Nrf2 was −22.248619, and for DHS with Nrf2 it was −34.274078. Furthermore, we used MOE software to analyze the interactions of PIN and DHS with the Nrf2 protein. This showed that PIN might have hydrogen bonds that interacted with Nrf2 specifically at Cys513 and Tyr520 (Fig. 8B, C), and that DHS might also have a hydrogen bond to interact with Nrf2 specifically at Val465 (Fig. 8E, F). Based on these docking results, PIN and DHS are highly likely to be potent activators of Nrf2. Further work is needed to test this hypothesis.

3. Discussion

In Asia, and especially in China, Traditional Chinese Medicine

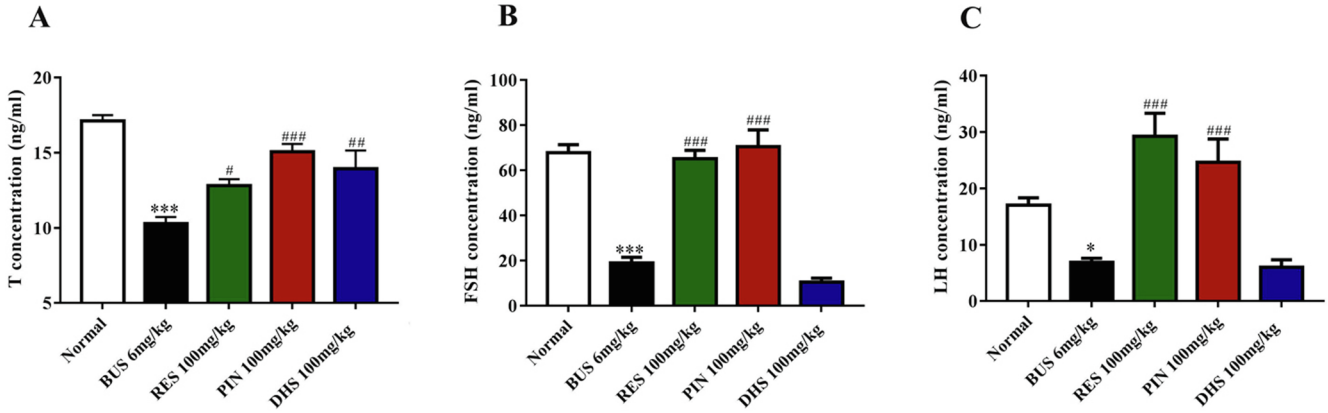


Fig. 4. Effect of PIN and DHS on hormone levels. (A): T concentration, (B): FSH concentration, (C): LH concentration. *P < 0.05 vs. Normal group, ***P < 0.001 vs. Normal group, #P < 0.05 vs. BUS group, ##P < 0.01 vs. BUS group, ###P < 0.001 vs. BUS group. Each column represents the mean ± SEM, n = 6.

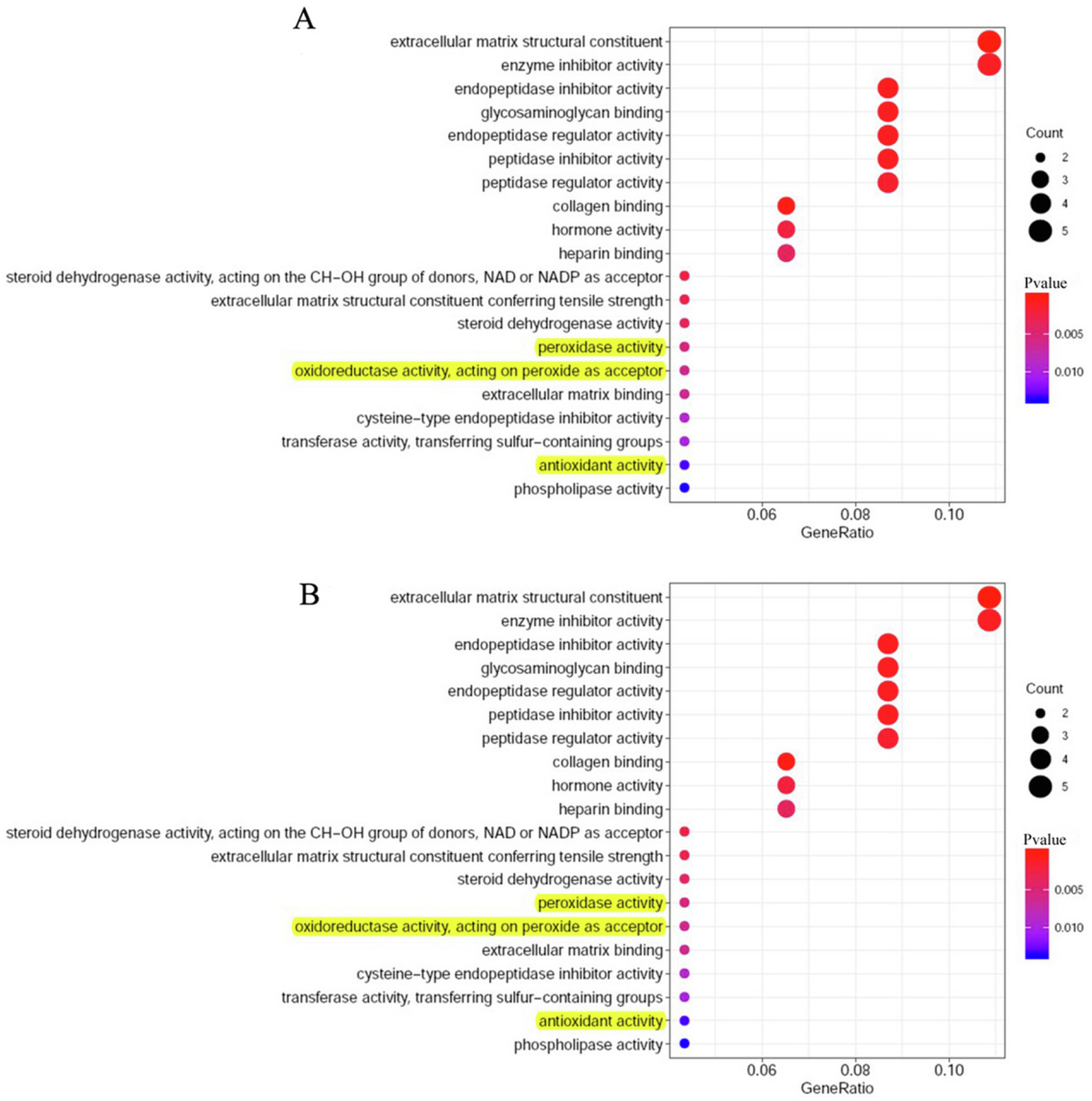


Fig. 5. The data analysis of RNA sequencing. (A): GO classification of differentially expressed mRNAs in the between the BUS and PIN treatment mouse testis. The x-axis shows counts of genes enriched in GO and the y-axis shows the GO classification. (B): GO classification of differentially expressed mRNAs in the between the BUS and DHS treatment mouse testis. The x-axis shows counts of genes enriched in GO and the y-axis shows the GO classification.

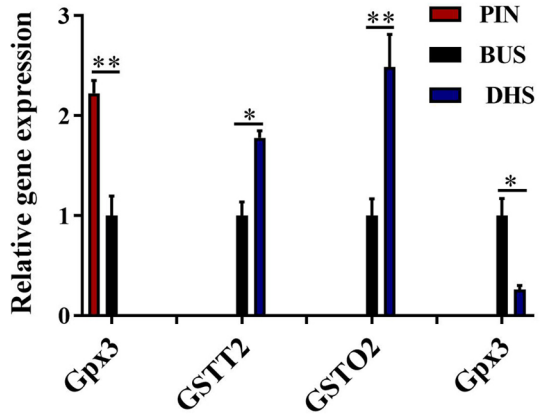


Fig. 6. qRT-PCR validated the expression of antioxidative-related glutathione redox system. *p < 0.05 and **p < 0.01. Each column represents the mean ± SEM, n = 3.

(TCM) has been used to treat human oligoasthenospermia for decades with limited success [25]. The efficacy of RES for treating men with oligoasthenospermia is also limited because of its chemical instability and low bioavailability. The two RES analogs we have synthesized PIN and DHS are both naturally occurring bisphenol-based compounds. PIN is found in the wood and leaves of various *Pinus* species [26]. It possesses various biological activities and has been suggested as a potential therapeutic agent against cancers and inflammatory diseases [27–28]. DHS is found in the bark of *Yucca periculosa* [29] and exhibits promising antitumor, antioxidant, and anti-inflammatory activities [30–31]. To our knowledge, this is the first study to investigate the effects of PIN and DHS on oligoasthenospermia in a mouse model. Intratesticular injections of BUS in male ICR mice resulted in tissue damage and induced oligoasthenospermia in epididymal sperm suspensions. Treatments with PIN and DHS could attenuate this damage to the testes and ameliorate the impaired epididymal sperm parameters. We conclude that PIN and DHS improved this oligoasthenospermia by attenuating oxidative stress via the Nrf2-ARE pathway.

It is known that RES and its analogs can cross biological membranes [32]. We hypothesize that reduction of the hydroxyl group at the 4′-

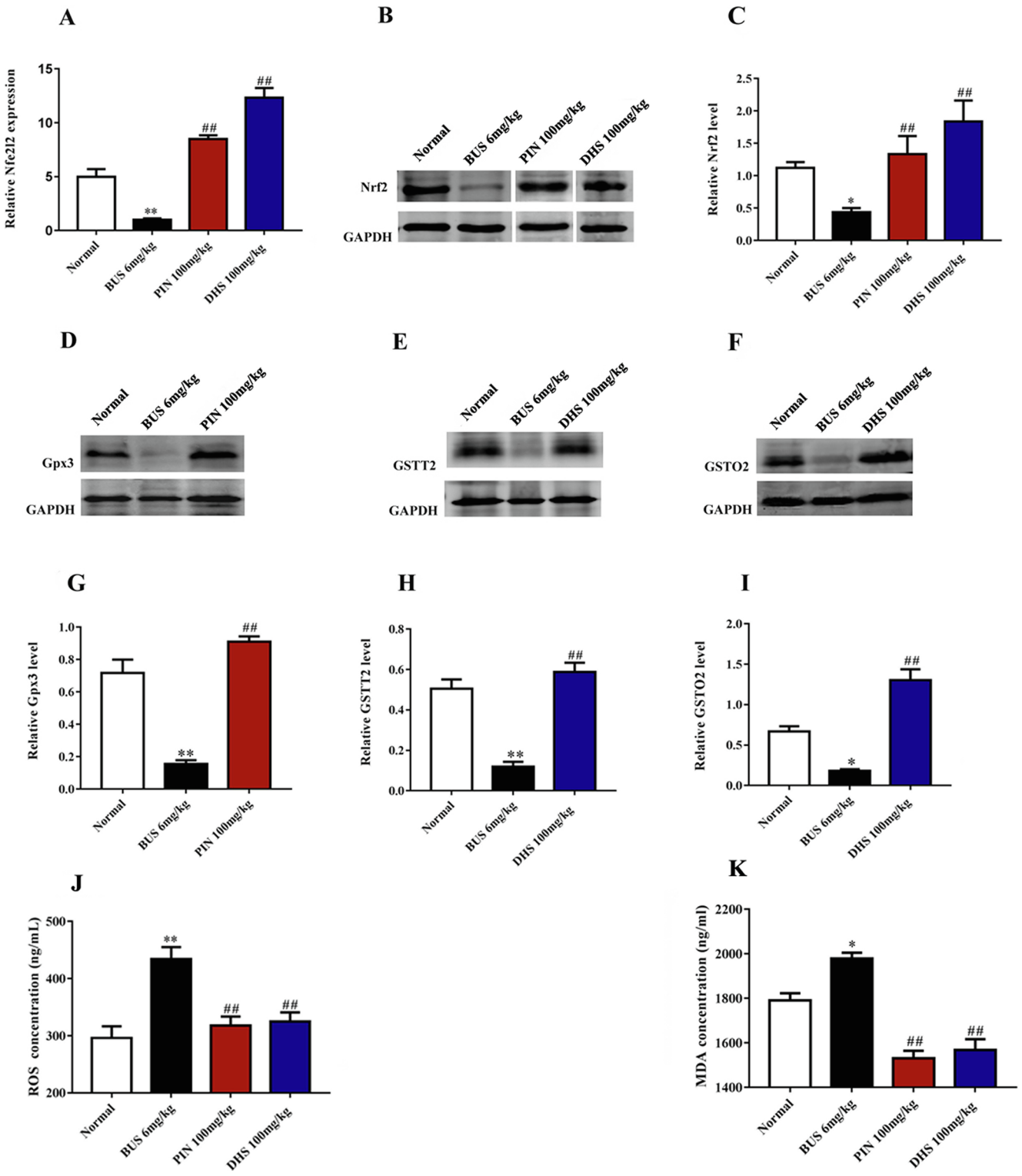


Fig. 7. The effects of of PIN and DHS on the expression of Nrf2-AREs pathway. (A): The relative Nfe2l2 expression in testes. (B and C): The expression of Nrf2 was analyzed by western blot. Relative integrated density value analysis of Nrf2. (D and G):The expression of Gpx3 was analyzed by western blot. Relative integrated density value analysis of Gpx3. (E and H):The expression of GSTT2 was analyzed by western blot. Relative integrated density value analysis of GSTT2. (F and I): The expression of GSTO2 was analyzed by western blot. Relative integrated density value analysis of GSTO2. (J): ROS concentration, (K): MDA concentration. *P $<$ 0.05 vs. Normal group, **P $<$ 0.01 vs. Normal group, $^{\#\#}$P $<$ 0.01 vs. BUS group. Each column represents the mean $\pm$ SEM, n = 6.

position of PIN might make it more lipophilic than RES, which would help PIN to enter damaged cells more easily and improve its efficacy [33]. We found that PIN treatment produced better improvements in mouse epididymal sperm concentrations and motility compared with RES treatment (Fig. 2C, D), and that PIN treatment also improves the recovery from BUS-induced histopathological damage to the testes more than RES treatment (Fig. 3). These results confirmed that the lipophilicity of PIN might be more helpful than RES in improving oligoasthenospermia. We also proposed that both PIN and DHS are more stable and have better lipophilicity. The log P of RES, PIN and DHS is

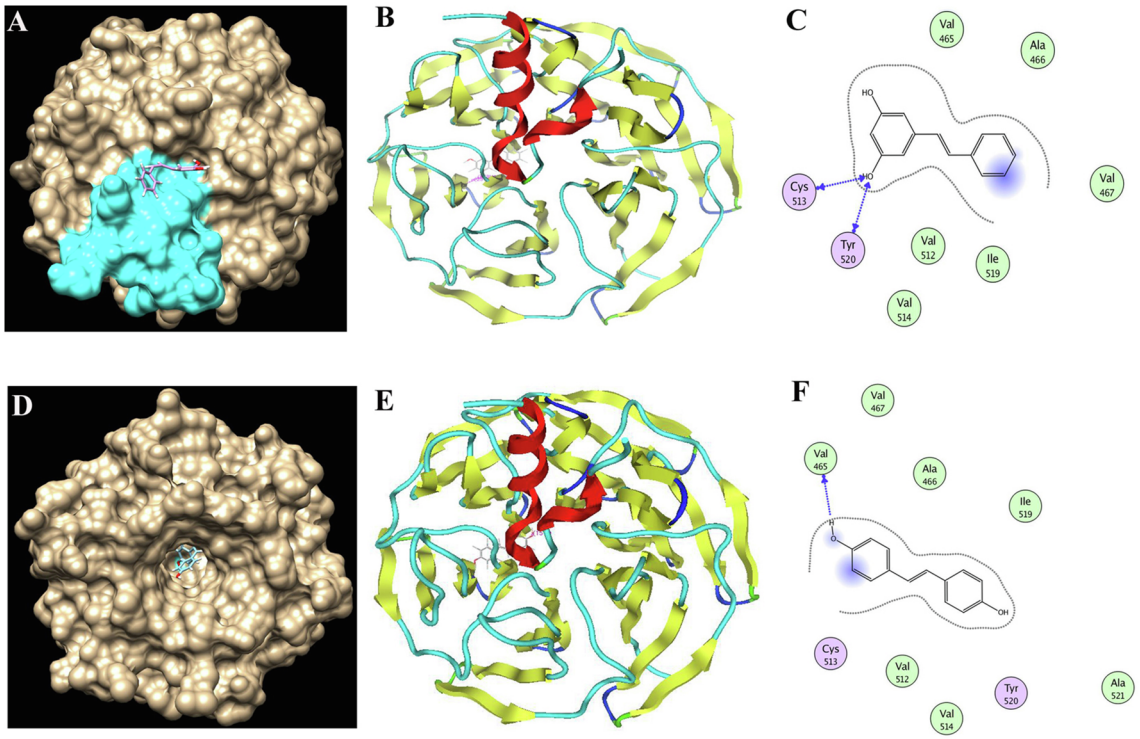


Fig. 8. The interaction of PIN and DHS with Nrf2 protein. (A): Molecular docking of PIN with Nrf2 protein. Grid Score is −22.248619. (B): Diagrams of the interactions between PIN with Nrf2 protein. (C): Interactive visualisation of PIN binding to Cys513 and Tyr520 with Nrf2 protein through hydrogen bonding. (D): Molecular docking of DHS with Nrf2 protein. Grid Score is −34.274078. (E): Diagrams of the interactions between DHS with Nrf2 protein. (F): Interactive visualisation of DHS binding to Val465 with Nrf2 protein through hydrogen bonding.

3.06, 3.45, and 3.45, respectively. We have also examined these analogs for the drug-likeness on the Lipinski rule of 5 at https://chemicalize.com/app/calculation, indicating that both PIN and DHS fulfill the Lipinski Rule of 5. We speculate that the symmetrical structure of the hydroxyl group at the 4,4′ position of DHS makes it more stable with more potent antioxidant activity, which would help DHS improve oligoasthenospermia. It has been shown that DHS exhibited more antioxidant activity and cytotoxicity than RES [34]. Here, DHS treatment had a better improvement in epididymal sperm concentration compared with RES treatment, while the lower improvement in sperm motility might be related to DHS being more toxic than RES (Fig. 2C, D). These findings suggest that the symmetrical structure of DHS might help improve oligoasthenospermia and increase sperm output.

Oxidative stress is known to be involved in the pathophysiology of oligoasthenospermia [35]. Oxidative stress is caused by an excessive production of ROS. In the function of the male reproductive system, seminal sperm parameters, including concentration and motility are particularly vulnerable to ROS [36]. ROS cause sperm damage, and ultimately damage nuclear DNA; factors associated with human male infertility [35]. It has been suggested that Nrf2 plays a critical role in the defense against oxidative stress by activating the expressions of genes encoding antioxidative enzymes downstream of AREs to reduce the production of ROS [10]. BUS testicular injection induces oligoasthenospermia with tissue damage and oxidative stress [22]. Our results indicated that BUS caused a considerable reduction in Nrf2 protein expression in the mouse testis, and both PIN and DHS treatments were capable of reversing this (Fig. 7A, C, D). RNA-seq, RT-qPCR, and western blot analysis showed that PIN treatment enhanced the expression of the gene encoding Gpx3, and DHS treatment enhanced the expressions of the genes for GSTT2 and GSTO2 (Figs. 6, 7). We have also shown that BUS increased the ROS and MDA levels in mouse testes, and that either PIN or DHS treatment was able to reverse these induction (Fig. 8). It has been shown that RES activates the transcription factor Nrf2 [37], and, based on this, we identified PIN and DHS as novel Nrf2 activators. Our molecular docking studies have shown that PIN has a hydrogen bond to the Nrf2 protein specifically at Cys513 and Tyr520, and that DHS has a hydrogen bond specifically at Val465 (Fig. 8). Different binding sites with Nrf2 might cause the differences in response between the PIN and DHS treatments, including epididymal sperm concentration and motility, hormone levels, and AREs. These findings indicate that PIN might improve oligoasthenospermia by attenuating oxidative stress via the Nrf2-ARE-dependent antioxidant Gpx3, and that DHS might act similarly by attenuating oxidative stress via the Nrf2-ARE-dependent antioxidants GSTT2 and GSTO2 (Fig. 9).

The development of novel potential candidates for the treatment of oligoasthenospermia has very important practical significance. Even though the protective effects of RES on testicular damage and sperm function are known [14–17], its clinical application is limited due to the relative unstability of RES, and its poor bioavailabaility, as well as its rapid metabolic clearance from the systemic circulation [18–20]. The two new analogs of RES, namely PIN and DHS, were obtained through structural modification of resveratrol. Most importantly, PIN and DHS were found to improve oligoasthenospermia in a mouse model by attenuating oxidative stress via the Nrf2-ARE pathway. PIN and DHS may be promising candidates for clinical treatment of oligoasthenospermia.

4. Experimental procedures

4.1. Chemicals

PIN and DHS were synthesized as described [21]. Their chemical structures were identified fully using ^{1}H NMR and ^{13}C NMR spectral analyses (Fig. S2 A-D). The purity (> 99%) of PIN and DHS was checked using gas chromatography-mass spectroscopy (Supplementary Fig. S3; Table S2, S3). RES, BUS, and Proteoprep® total extraction sample kits were purchased from Sigma-Aldrich (Darmstadt, Germany). Reverse transcription (RT) kits, BCA protein assay kits, and TB Green Premix Ex Taq II (Tli RNaseH Plus) were purchased from Takara

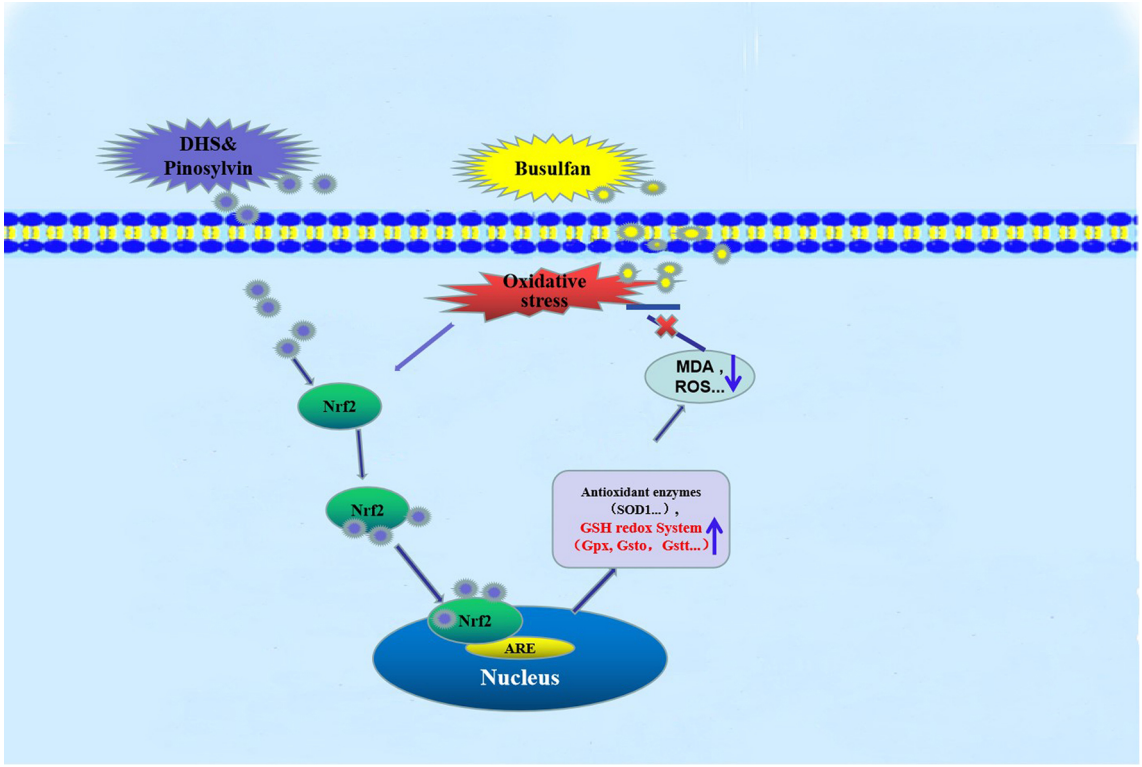


Fig. 9. Schematic representation of proposed mechanism of PIN and DHS in improving oligoasthenospermia. Our findings indicated PIN may improve oligoasthenospermia by attenuating oxidative stress via the Nrf2-ARE-dependent antioxidants Gpx3 and DHS may improve oligoasthenospermia by attenuating oxidative stress via the Nrf2-ARE-dependent antioxidants GSTT2 and GSTO2.

Biotechnology (Dalian, China). Testosterone (T), follicle stimulating hormone (FSH), luteinizing hormone (LH), ROS and malondialdehyde (MDA) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Elabscience Biotechnology Inc. (Houston, USA). TRIzol reagent was purchased from Life Science Products (ThermoFisher Scientific, Waltham, MA, USA). Primary rabbit anti-mouse antibodies against Nrf2, glutathione peroxidase 3 (Gpx3), glutathione S-transferase theta 2 (GSTT2) and glutathione S-transferase omega 2 (GSTO2), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Proteintech (Rosemont, IL, USA). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

4.2. Animals

Male Institute of Cancer Research (ICR) strain mice (n = 50, 8 weeks old, 40 ± 2 g), were purchased from the Experimental Animal Center of Nantong University (Nantong, P. R. China). Mice were housed under standard laboratory environmental conditions (room temperature 22–24 °C on a 12/12-h light/dark cycle) with free access to food and water. Our project was submitted to the ethics committee supervising animal experimentation in Nantong University, and all procedures were approved according to the Jiangsu Province Animal Care Ethics Committee with Approval ID: SYXK(SU)2007-0021.

4.3. Experimental groups, treatment, and sample preparation

Fifty mice were divided randomly into five groups of 10 each as follows: (1) a normal control group fed with normal diet and water; (2) a BUS group with oligoasthenospermia induced by the intratesticular injection of BUS at 6 mg/kg (3 mg/kg/side, all drugs were dissolved in 5% DMSO aqueous solution); (3) a RES group receiving 100 mg/kg via intragastric administration daily for 2 consecutive weeks, 2 weeks after testes had been injected with BUS as above; (4) a PIN group receiving 100 mg/kg via intragastric administration daily as in group 2; and (5) a DHS group receiving 100 mg/kg via intragastric administration daily as in group 2. After the final administration, all mice were weighed, then euthanized under anesthesia; testes and epididymides were then removed and weighed separately. Six testes of each group were fixed in 4% paraformaldehyde solution, and the rest were snap-frozen in liquid nitrogen and stored at −80 °C; the following experiments were performed immediately.

4.4. Analysis of epididymal sperm concentration and motility

The whole epididymides were removed immediately after euthanasia, then the cauda epididymidis cut into small pieces and incubated in a tube containing 2 mL phosphate buffered saline at 37 °C for 15 min and gently shaken to allow the spermatozoa to swim out. Aliquots of 200 μL of the suspension were diluted with 2 mL Tyrode's solution. Sperm concentration and motility parameters were measured using a computer-assisted sperm analysis system: the Hamilton Thorne CEROS II for humans and animals.

4.5. Histology

Testes were fixed overnight in 4% paraformaldehyde and soaked consecutively in 10% and 30% sucrose overnight. Frozen sections of 8-μm thickness were performed using a cryotome. The sections were stained with hematoxylin and eosin (HE) using standard procedures and observed by light microscopy.

4.6. Serum hormone levels

Collection of blood and preparation of serum were performed as described [38]. Briefly, blood was collected by cardiac puncture with a heparinized syringe and then serum collected immediately by centrifugation at 3000*g*, 4 °C for 10 min. Samples were stored at −80 °C for

further analysis. Serum levels of T, FSH, and LH were measured by ELISA kits according to the manufacturer's instructions. The sensitivities of the mouse T, FSH, and LH assays were 0.10 ng/mL, 0.94 ng/mL, and 0.28 ng/mL, respectively. The intra- and inter-assay coefficient of variation were < 10% for all assays.

4.7. RNA sequencing and data analysis

Total RNA was extracted from testes using Trizol reagent according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Samples of ~60 mg were ground into powder with liquid nitrogen and transferred into 2-mL tubes containing 1.5 mL Trizol. The mix was centrifuged at 12,000*g* for 5 min at 4 °C. The supernatant was transferred to a new 2.0-mL tube to which was added 0.3 mL of chloroform/isoamyl alcohol (24:1) per 1.5 mL of Trizol reagent. After the mix was centrifuged at 12,000*g* for 10 min at 4 °C, the aqueous supernatant was transferred to a new 1.5-mL tube to which was added an equal volume of isopropyl alcohol. The mix was centrifuged at 12,000*g* for 20 min at 4 °C, and the supernatant was removed. After washing with 1 mL 75% ethanol, the RNA pellet was air-dried in a biosafety cabinet and then dissolved by adding 25–100 μL of diethyl pyrocarbonate treated water. Subsequently, total RNA was qualified and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and 2100 Bioanalyzer (Agilent Technologies, San Diego, CA, USA). The products were enriched by PCR to create the final cDNA library. The libraries were assessed for quality and quantity using two methods: first by checking the distribution of fragment sizes using an Agilent 2100 bioanalyzer, and second by quantifying the library using real-time qPCR (see below) and TaqMan probes. The libraries were sequenced pair end-on using the BGISEQ-500/MGISEQ-2000 System (BGI-Shenzhen, P. R. China). Fastp software (https://github.com/OpenGene/fastp) was used to perform all-round quality control and to clean up raw data, including linker processing, global trimming by directly cutting off low-quality bases at the beginnings and ends of sequences, sliding-window quality trimming, filtering short sequences, base correcting for double-end sequences, and quality filtering. STAR software was used to perform sequence alignment on the clean data, with reference *Mus musculus* genome GRCm38, and each read was aligned to the genome to obtain the mapping rate of each sample. FeatureCounts software was used to count each gene in all samples. Finally, DEseq2 software was used to analyze the differentially expressed genes of different samples, and the differential genes were analyzed using Gene Ontology (GO) annotations.

4.8. Quantitative real-time PCR

The expression of antioxidative-related genes involving the Nrf2-ARE signal pathway were further evaluated by quantitative real-time PCR. Total RNA was extracted from frozen mouse testes as above. The RNA was used to synthesize cDNA using reverse transcription kits. cDNA was estimated using NanoDrop 2000/2000C spectrophotometer. Gene-specific primers were synthesized by Genewiz (USA) for the corresponding mRNA sequences: Nfe2l2 (Nrf2) (Forward) 5′-TCCTATGC GTGAATCCCAAT-3′, (Reverse) 5′-GCGGCTTGAATGTTTGTCTT-3′; Gpx3 (F) 5′-CCGGGGACAAGAGAAGTCTA- A-3′, (R) 5′-AGGCAAGCAG ACCTGAGTTT-3′, GSTT2 (F) 5′-TGGAGCTCTACCTG- GACCTG-3′, (R) 5′-GCTGTGCTTTCGGTCAACAC-3′; GSTO2 (F) 5′-TGTACGGAT- CTGA AGGTCGC-3′, (R) 5′-GGGCCAGACGAGGTAATCAA-3′; and β-actin (F) 5′-CTGTCCCTGTATGCCTCTG-3′, (R) 5′-TTGATGTCACGCACGATT-3′. The β-actin gene served as an internal control. PCR reactions were run in triplicate using TB Green Premix Ex Taq II (Tli RNaseH Plus) on a Lightcycler 480 system (Roche, Mannheim, Germany). The reaction conditions were as follows: 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s. Melting curves were generated with a three-segment cycle of 95 °C for 5 s, 60 °C for 60 s, and 95 °C for 1 s in the continuous acquisition mode. The quantitative real-time PCR data analysis used the $2^{-\triangle\triangle CT}$ method.

4.9. Western blotting

The protein levels of Nrf2, Gpx3, GSTT2, and GSTO2 were measured by western blot analysis to validate the qPCR data. Total testicular protein was extracted using Proteoprep® total extraction sample kits (Merk-Shanghai, P. R. China). BCA protein assay kits were used to estimate protein concentrations. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes, then blocked with 5% skimmed milk for 1 h at room temperature. PVDF membranes were incubated with primary antibodies against Nrf2, Gpx3, GSTT2, GSTO2, or GAPDH (served as a protein loading control) at 4 °C overnight. Thereafter, they were incubated with the corresponding secondary antibodies (Goat anti-rabbit IgG H&L (Alexa Fluor 790) and Goat anti-mouse IgG H&L (Alexa Fluor 791)) for 2 h at room temperature. Densitometry analysis was performed using an Odyssey infrared imaging system. Image J software (https://imagej.nih.gov/ij/download.html) was used for the analysis of relative signal intensity.

4.10. Testicular levels of ROS and MDA

Testicular levels of ROS and MDA were measured using ELISA kits according to the manufacturer's instructions. The sensitivities of the mouse ROS and MDA assays were 0.039 U/mL and 18.75 ng/mL, respectively. The intraassay and interassay coefficient of variation were < 10% for both assays.

4.11. Molecular docking

Molecular docking studies of PIN and DHS were carried out using DOCK v. 6.7 (http://dock.compbio.ucsf.edu/DOCK_6/index.htm). The grid score is defined as the total docking score of the DOCK 6 scheme. Negative values indicate molecular combination, and positive values indicate no binding. Molecular Operating Environment (MOE) software (https://www.chemcomp.com/Products.htm) was used to determine interactions between ligand and target proteins. The crystal structure of Nrf2 (Protein Data Bank [PDB] entry 3WN7) in complex with Kelch-like ECH-associated protein 1 was derived from the PDB (http://www.rcsb.org/structure/3WN7) [39].

4.12. Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA, USA). Student's *t*-tests were used to analyze differences between two experimental groups, and one-way analysis of variance were used to analyze the differences between multiple experimental groups Data are expressed as the mean ± standard error of mean, of at least three independent experiments, and $P < 0.05$ was considered to be statistically significant.

Author contributions

F. S. and C.Y. C. designed this study. C. W. wrote the manuscript. C. W. and S. G. performed the experiments. M. S. and J. Y. helped collecting and analyzing the data. All the authors read and approved this manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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