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Rg1 alleviates oxidative stress and spermatogonium apoptosis in D-gal-induced testicular toxicity by activating Akt

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ABSTRACT

Objectives: High reactive oxygen species (ROS) levels lead to cell death, and the testes are among the most vulnerable organs to oxidative damage. Rg1, an active ingredient extracted from the natural medicine ginseng, has potential anti-inflammatory, antioxidant and antiapoptotic properties. Our previous studies showed that Rg1 can effectively improve spermatogenic function in mice, but the specific mechanism remains unclear. The purpose of this study was to investigate the effect of Rg1 on oxidative stress and spermatogonium apoptosis in D-gal-induced testicular toxicity and elucidate the associated mechanism.

Methods: Male C57BL/6 mice at 6–8 weeks of age were intraperitoneally injected with D-gal (200 mg/kg) for 42 days to establish a testicular injury model, and on day 16, 40 mg/kg Rg1-rich saline was injected intraperitoneally. Concurrently, we established an in vitro model of D-gal-damaged spermatogonia, which was treated with Rg1.

Results: We found that treatment with the ginsenoside Rg1 reduced D-gal-induced oxidative stress and spermatogonium apoptosis in vivo and in vitro. Mechanistically, we found that Rg1 activated Akt/bad signaling and reduced D-gal-induced spermatogonium apoptosis.

Discussion: We provide evidence showing that the antioxidant effect of Rg1 is mediated by the Akt/GSK-3β/NRF2 axis. Based on these findings, we consider Rg1 a potential treatment for testicular oxidative damage.

KEYWORDS
Ginsenoside Rg1; testis; oxidative damage; apoptosis

Introduction

Sperm abnormalities, including reduced sperm count and poor sperm quality, are a direct cause of male infertility. Spermatogenesis occurs in testicular spermatogenic tubules, and testicular function is particularly susceptible to oxidative stress [1]. A variety of diseases, such as diabetes and obesity, can cause loss of male fertility by triggering mitochondrial stress and significantly increasing testicular apoptotic cell death, leading to loss of germ cells [2,3]. In addition, excessive ROS accumulation can lead to testicular damage and male infertility by disrupting the blood-testicular barrier (BTB) [4,5]. Therefore, there is an urgent need to understand the pathogenesis of testicular oxidative stress injury and spermatogonium apoptosis in order to find new therapeutic targets.

Protein kinase B (PKB/Akt) is a serine/threonine protein kinase involved in the regulation of cell survival, proliferation and metabolism [6]. An increasing number of studies have shown that Akt activation prevents apoptosis in a variety of cells, while Akt inhibition augments apoptosis and cellular dysfunction [7–9]. Activated Akt inactivates Bcl-2-associated death promoter (Bad), which promotes cell death by inhibiting Bcl-2 protein expression [10,11]. The available data suggest that in addition to its regulatory role in cell survival, Akt also inactivates glycogen synthase kinase 3β (GSK-3β), which reduces the nuclear translocation of FYN, and in turn, NF-E2-related factor 2 (NRF2) nuclear output and degradation [12,13]. Considering the role of Akt in D-gal-induced testicular toxicity, it is important to identify a novel positive regulator of Akt.

The ginsenoside Rg1 is a natural ginseng extract monomer with a known chemical structure and anti-inflammatory, antioxidant and anti-aging pharmacological effects. Previous studies have shown that Rg1 can antagonize testicular aging induced by D-gal in mice, but the mechanism is not clear [14]. In addition, numerous studies have demonstrated that Rg1 treatment can protect stem cell function and reduce oxidative damage and senescence [15]. Based on these findings, we hypothesized that Rg1 might be a natural therapeutic agent for testicular oxidative damage and further explored the mechanism.

Methods and materials

Cell culture and treatments

GC-2spd (ts) cells were purchased from Fenghui Biotechnology Co., Ltd. (Hunan, China), and were cultured in Dulbecco's Modified Eagle medium (DMEM) with 10% fetal bovine serum and 1% streptomycin/penicillin (Beyotime, China). After synchronization for 24 h, the cells were treated with Rg1

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(50 μM) in the presence or absence of D-gal (40 mg/ml) for 24 h (Additional file 1: Figure S1). For Akt inhibition, GC-2spd (ts) cells were pretreated with Akti (1 μmol/L) for 30 min. To knock down the expression of NRF2, cells were transfected with siNRF2 (100 nmol/L) for 4 h using the Lipofectamine™ 2000 transfection reagent according to the manufacturer's protocol (Thermo Fisher, USA) and then cultured in normal medium for 24 h before the next treatment.

Animals and treatments

All animal care and experimental procedures followed the Guidelines for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication, revised 2011) and were approved by the Animal Ethics Committee of Chongqing Medical University (IACUC-CQMU-2022-0026, 2022). C57BL/6 male mice (6–8 weeks of age) were purchased from the Animal Experiment Center of Chongqing Medical University (Chongqing, China) and were subjected to adaptive feeding for 1 week before the study commenced. All mice were maintained under specific pathogen-free, environmentally controlled (temperature: 20–25°C; humidity: 50 ± 5%) barrier conditions in individual ventilated cages and were given sterile food and water ad libitum. Sixteen days after D-gal (200 mg/kg) or control normal saline (NS) injection (according to our previous study), the mice were treated with an intraperitoneal injection of Rg1 (40 mg/kg). To inhibit Akt activity, mice were treated daily with Akti (20 mg/kg/day) for 14 days via intraperitoneal injection according to previous studies [7,16].

Immunohistochemistry and TUNEL staining

Immunohistochemical staining was performed according to our previous studies [17,18]. Briefly, 3% hydrogen peroxide or 10% goat serum was used to block endogenous peroxidase and nonspecific binding of the antibody, respectively. Sections were examined by light microscopy (OLYMPUS, Tokyo, Japan). TUNEL staining was performed using a commercially available kit (Beyotime) according to the manufacturer's instructions, and the images were captured with a LEICA fluorescence microscope (Germany).

Proteome profiler array analysis

Protein profiles were analyzed using the Proteome Profiler™ Array Mouse Apoptosis Array Kit (R&D Inc. Minneapolis, MN, USA) according to the manufacturer's protocol. The testes were lysed with lysis buffer mixed with protease cocktail inhibitors (Roche, Branford, CT, USA). Cell lysates were resuspended on ice for 30 min and then centrifuged at 14,000 × g for 5 min at 4°C. Protein lysates were collected, and their concentrations were determined by the BCA assay. Lysates were then incubated overnight with nitrocellulose membranes containing 20 soluble receptors (200–400 μg/membrane). The membranes were subsequently incubated first with a specific mixture of biotinylated detection antibodies and then with streptavidin-horseradish peroxidase solution. The signal was detected using a chemical fluorescence detection system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol.

siRNA experiments

Transfection was performed using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's protocol. The target siRNA sequences used are listed in Additional file 2: Table S1. siRNA was used at a concentration of 100 nmol/L. The efficiency of transfection was verified by Western blotting (Additional file 2: Figure S2).

Western blotting

Western blotting was performed according to previous articles [17–19]. GAPDH or β-actin was used to normalize the expression level of each transcript. The antibody information is shown in Additional file 2: Table S1.

Enzyme-linked immunosorbent assay (ELISA)

Serum was collected, and testosterone (T) was measured with an ELISA kit (MEIMIAN, China).

Oxidative stress detection and cell viability

ROS production was evaluated by DCFH-DA staining in vitro [20–22]. Briefly, coverslips were stained with DCFH-DA (10 μmol/L) in the dark at 37°C for 20 min, and then the cells were washed three times with serum-free cell culture medium to fully remove the DCFH-DA that had not entered the cells. After the probe was loaded in situ, the sample was directly observed with a fluorescence microscope (Leica). To further assess the oxidative stress level, we measured the contents of MDA,, T-AOC, and SOD (Beyotime) and the GSH/GSSG ratio in the testis or GC-2spd (ts) cells according to our previous study by using commercially available kits [23]. Cell viability was determined using the CCK-8 assay kit according to the manufacturer's protocol, as described previously [24].

Statistical analysis

All the data are expressed as the means ± standard deviations and were analyzed using SPSS v20.0 (IBM Corporation, Armonk, NY, USA). The data normality and homogeneity of variance were tested by the Kolmogorov–Smirnov test and Levene's test. One-way ANOVA analysis was used for multiple-group comparisons. Asterisks indicate statistical significance (*$P < 0.05$).

Results

Rg1 attenuated D-gal-induced testis injury in mice

To explore the role of Rg1 in D-gal-induced testicular toxicity, we treated D-gal-injured mice with an intraperitoneal injection of Rg1. As shown in Figure 1(C), Rg1 treatment prevented D-gal-induced testicular dysfunction, as indicated by preserved serum testosterone levels. In addition, we observed that D-gal injection reduced testicular volume and the ratio of testicular weight to body weight (the testis index), and these changes were significantly attenuated by Rg1 treatment (Figure 1(E,F)). The morphology of spermatogenic tubules is closely related to the spermatogenic function of the testis [25]. The epithelium of spermatogenic tubules

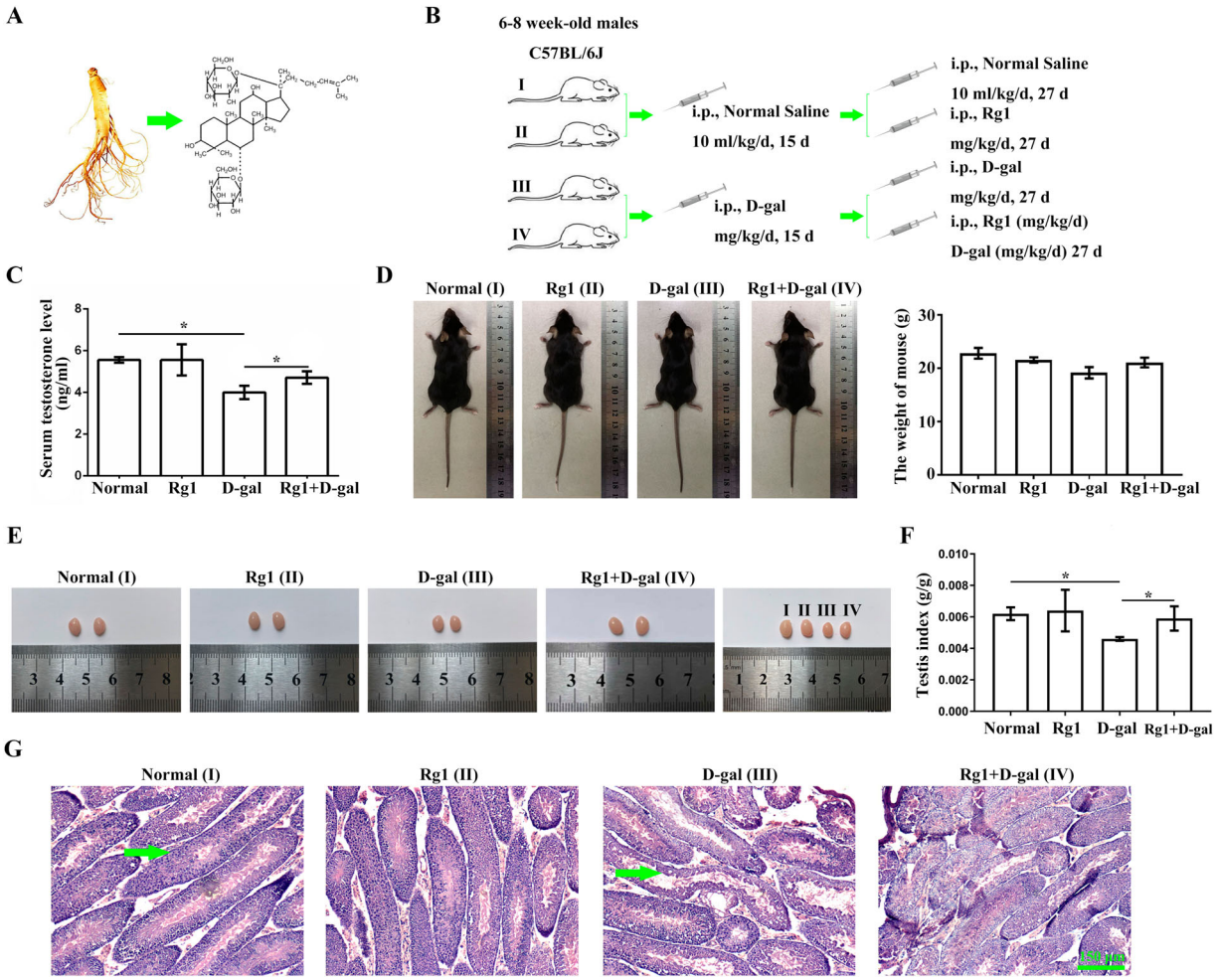

Figure 1. Rg1 attenuated D-gal-induced testis injury in mice. A Schematic diagram of the structure of the ginsenoside Rg1. B Schematic diagram of the animal experiment. C Serum testosterone levels were detected by ELISA. D Representative pictures of mouse gross morphology. E, F Mouse testis morphologic representation and testicular index. G H&E staining showed the microstructure of the mouse testis. Data are represented as the mean ± S.D. in C, D, F. (*$P < 0.05$; one-way ANOVA).

atrophies after toxic injury of the testis, and spermatogenic cells in the middle levels are sparse and disordered. Intriguingly, we found that D-gal damage blocked spermatozoa at the secondary spermatocyte stage, and Rg1 treatment attenuated D-gal damage-induced blockage. Given these results, we concluded that Rg1 attenuates D-gal-induced testicular toxicity in mice.

Rg1 protected the testis from oxidative damage and apoptosis in response to D-gal insult

The testis has been suggested to be one of the most vulnerable organs to oxidative stress; thus, we detected oxidative stress levels in the testis via 8-OHdG staining. D-gal injection resulted in increased oxidative stress in the testis, while Rg1 treatment largely inhibited 8-OHdG production (Figure 2(E)). Consistent with these molecular alterations, we found that Rg1 treatment reduced the abnormal MDA level (Figure 2(C)), and preserved the GSH/GSSG ratio (Figure 2(D)) and total SOD activity (Figure 2(B)). Excessive ROS production induced by D-gal can induce spermatogonium apoptosis and promote the progression of testicular dysfunction; therefore, we next assessed the role of Rg1 in spermatogonium apoptosis. As shown in Figure 2(F), D-gal injection resulted in clear spermatogonium apoptosis in vivo, and treatment with Rg1 mitigated this pathological alteration. The inhibitory effects of Rg1 on spermatogonium apoptosis were further confirmed by Western blots, which showed that Rg1 decreased the expression of C-PARP and C-Caspase3 and increased BCL-2 levels (Figure 2(G,H)).

Rg1 alleviated D-gal-induced oxidative stress and spermatogonium apoptosis in vitro

To further verify the beneficial role of Rg1 on D-gal-induced oxidative stress and spermatogonium apoptosis, we treated GC-2spd (ts) cells with Rg1 in the presence or absence of D-gal in vitro. To further verify the beneficial effect of Rg1 on D-gal-induced oxidative stress and spermatogonia, we treated GC-2spd (ts) cells with Rg1 in the presence or absence of D-gal in vitro. TUNEL staining showed that incubation with D-gal induced a substantial increase in the positive TUNEL staining of GC-2spd cells, and notably, treatment with Rg1 reversed this effect (Figure 3(C)). Moreover, DCFH-DA staining confirmed that Rg1 treatment attenuated the D-gal-induced oxidative damage to spermatogonia (Figure 3(C)). We further examined apoptosis and antioxidant-related proteins and found that in vitro Rg1 treatment had no effect on the baseline levels but significantly attenuated D-gal-induced C-Caspase3 upregulation, reversed Bcl-2 and

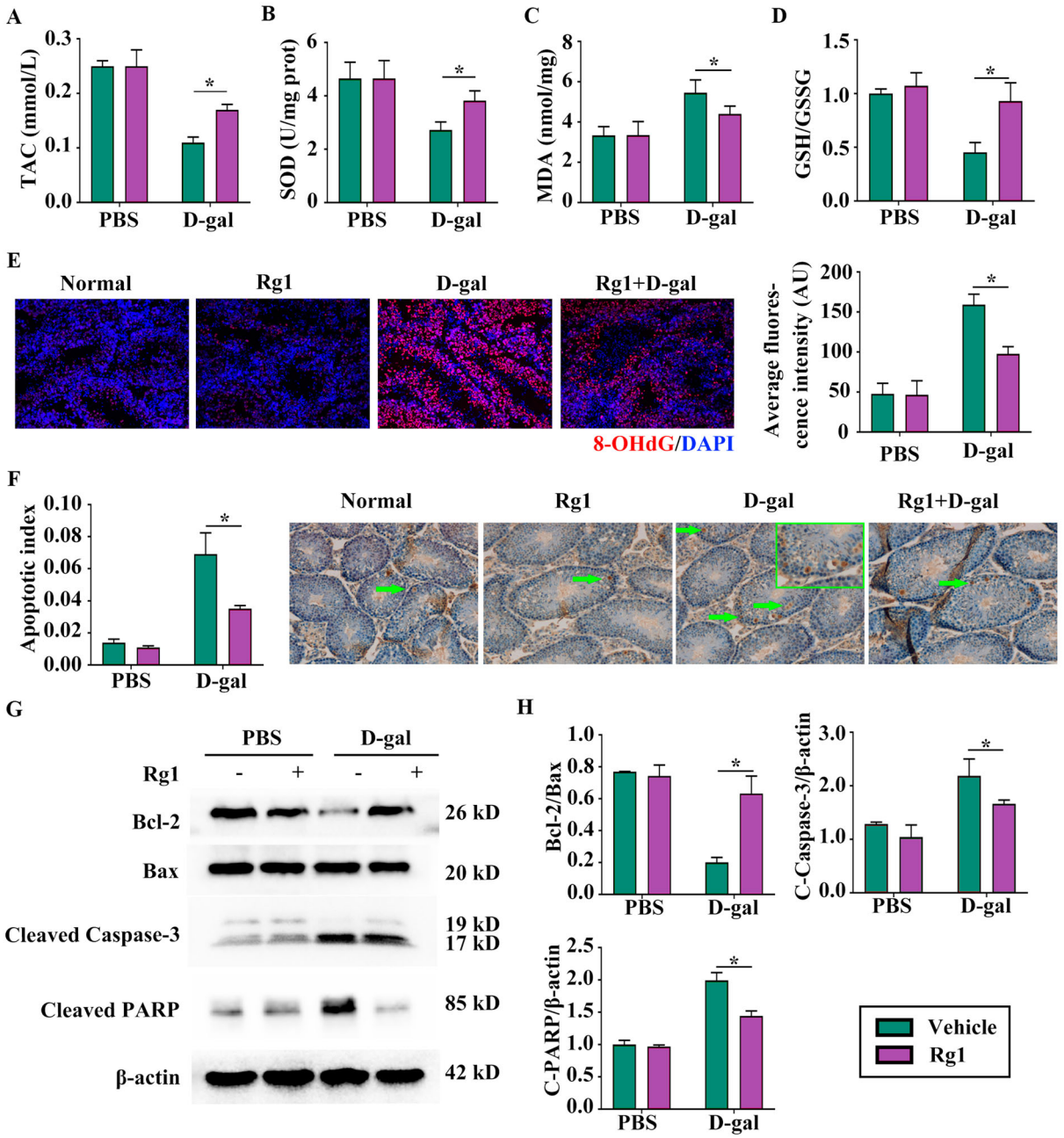

Figure 2. Rg1 protected the testis from oxidative damage and apoptosis. A, B, C, D Quantitative results for testicular TAC levels, SOD activities, and MDA and GSH levels, respectively. E Representative immunofluorescence staining images of 8-OHdG and quantitative results. F Representative TUNEL staining images and quantitative results. G, H Western blots and quantitative results in murine testes ($n = 3$). Data are represented as the means ± S.D. (*$P < 0.05$; one-way ANOVA).

SOD2 downregulation, and exerted a protective effect against oxidative stress and apoptosis (Figure 3(A, B)). These results suggest that Rg1 attenuates D-gal-induced oxidative stress and spermatogonia apoptosis in vitro.

Akt/Bad signaling is responsible for Rg1-mediated protective effects against spermatogonium apoptosis

To further determine the mechanism by which Rg1 ameliorates D-gal-induced apoptosis, we used the Mouse Apoptosis Signaling Pathway Array Kit. The results show the changes in apoptotic markers in Rg1-treated mice and D-gal-injured mice. Based on a semiquantitative analysis comparing the differential expression of apoptosis signaling pathway components between Rg1-treated mice and D-gal-injured mice, we identified Bad as the most significant marker. (Additional file 3: Figure S3).

To determine whether Rg1-mediated amelioration of D-gal-induced testicular damage was associated with inhibition of Bad signaling, we performed Western blotting to examine the expression of Bad signaling pathway-related proteins in the testes after Rg1 treatment. Total Bad expression levels were reduced after Rg1 treatment, and Bad expression was significantly higher in testes from untreated mice (Figure 4 (A)). We also examined changes in the expression of components of the Akt signaling pathway, which regulates signaling upstream of Bad.

Strikingly, p-Akt levels in the testes of mice were significantly higher after Rg1 treatment than in the testes of untreated mice, suggesting that Rg1 treatment enhanced the activation of Akt signaling (Figure 4(B)).

To obtain evidence that the protective effect of Rg1 is mediated by Akt activation, we pretreated mouse spermatogonia with Akt inhibitors. As shown in Figure 4(C and D), Akt

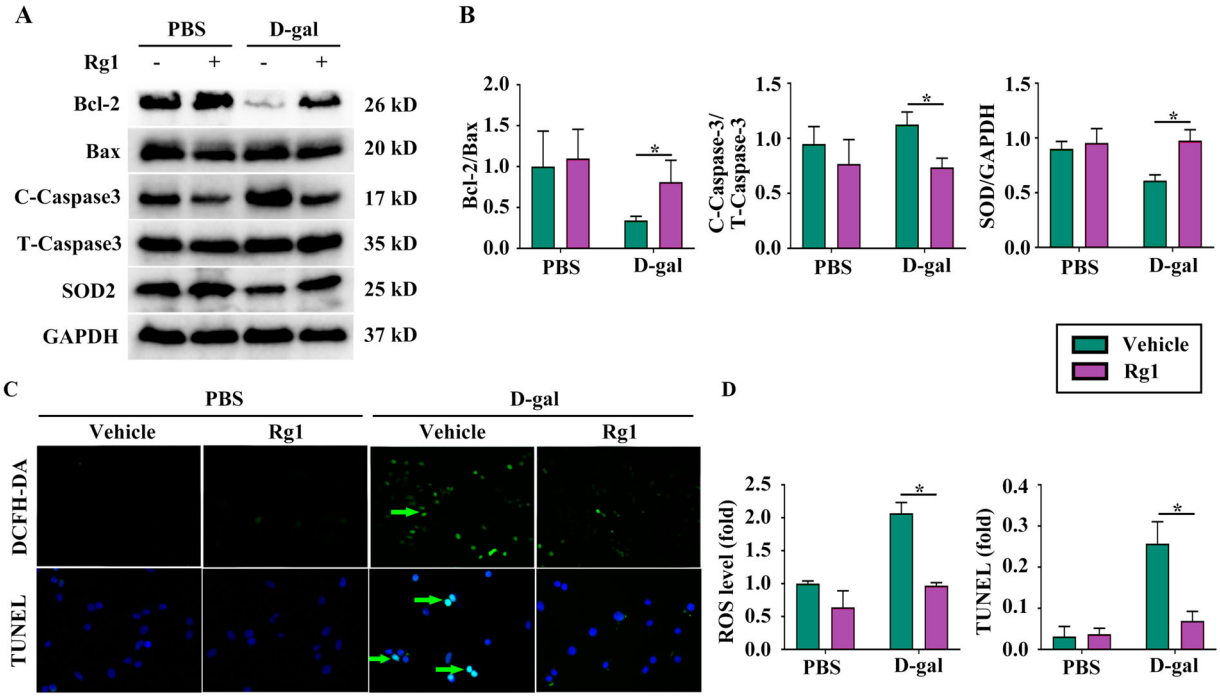

Figure 3. Rg1 alleviated D-gal-induced oxidative stress and spermatogonium apoptosis in vitro. A, B Western blots and quantitative results in GC-2spd cells ($n = 3$). C, D Representative images of DCFH-DA detection, TUNEL staining and quantitative results. Data are represented as the mean ± S.D. (*$P < 0.05$; one-way ANOVA).

inhibition eliminated the beneficial effects of Rg1 on testicular spermatogonium apoptosis both in vivo and in vitro. Collectively, these data suggest that Akt/Bad signaling is responsible for Rg1-mediated protective effects against testicular spermatogonium apoptosis.

Akt/GSK3β/NRF2 signaling is responsible for Rg1-mediated protective effects on oxidative damage

NRF2 is a redox-sensitive transcription factor that plays a key role in redox homeostasis during oxidative stress. Previous studies have shown that NRF2 deficiency seriously affects

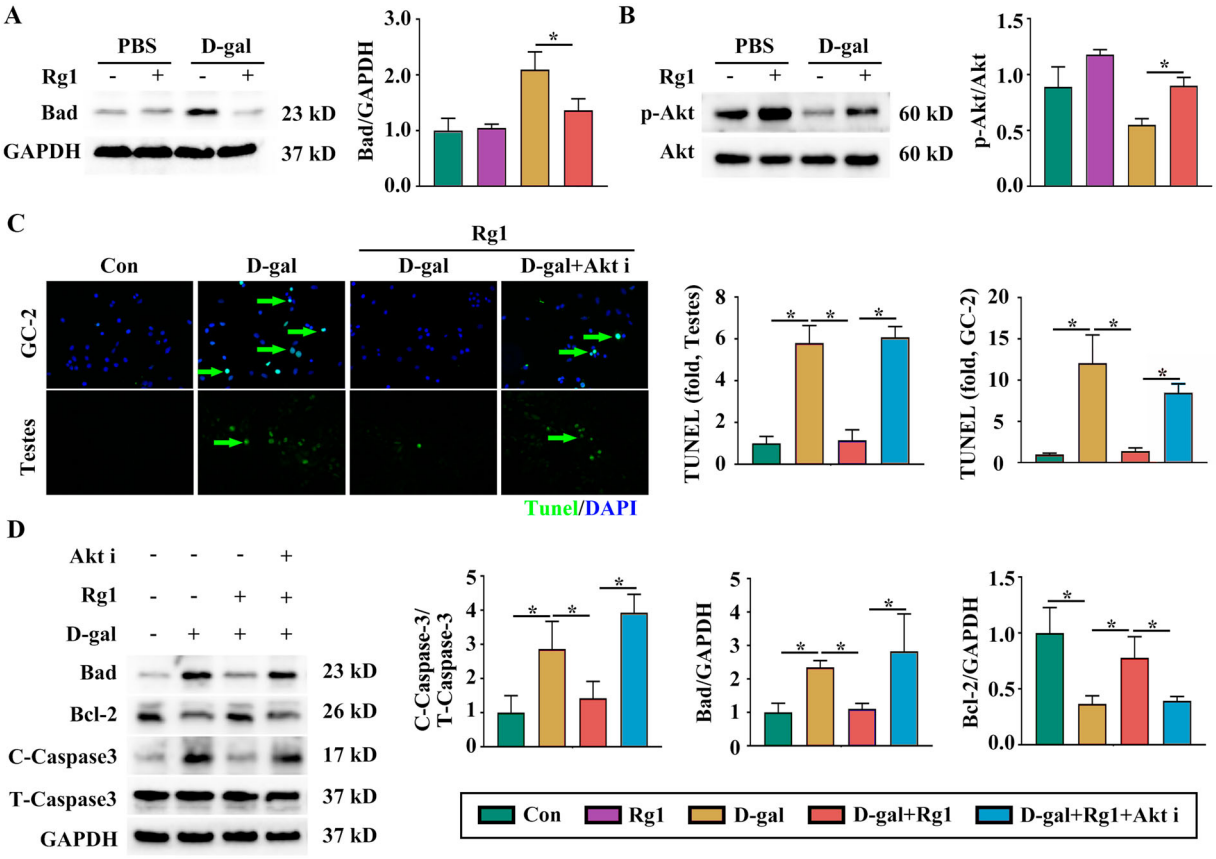

Figure 4. Akt/Bad signaling is responsible for Rg1-mediated protective effects against spermatogonium apoptosis. A Western blots and quantitative results ($n = 3$). B Representative Western blots and quantitative results ($n = 3$). C Representative images of TUNEL staining and quantitative results. D Western blots and quantitative results ($n = 3$). Data are represented as the mean ± S.D. (*$P < 0.05$; one-way ANOVA).

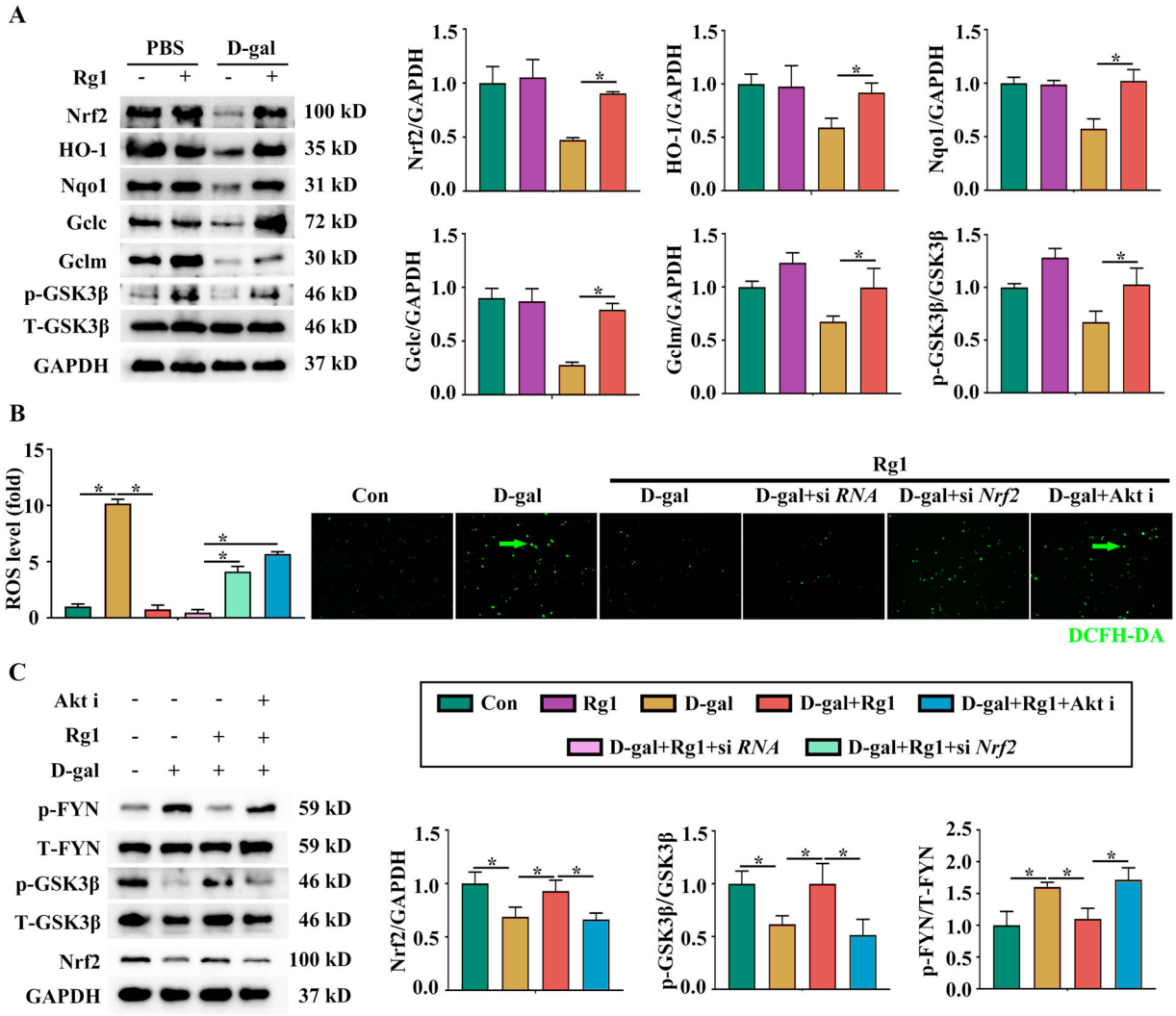

Figure 5. Akt/GSK3β/NRF2 signaling is responsible for Rg1-mediated protective effects on oxidative damage. A Western blots and quantitative results (n =3). B Representative images of DCFH-DA detection staining and quantitative results. C Representative Western blots and quantitative results (n = 3). Data are represented as the mean ± S.D. (*P < 0.05; one-way ANOVA).

the oxidative stress tolerance of mice and that NRF2 activation has a protective effect against oxidative stress injury in mice [26,27]. Here, we found that Rg1 treatment blocked D-gal-induced NRF2 downregulation, maintained NRF2 transcriptional activity, and increased HO-1, Nqo1, Gclc and Gclm protein levels (Figure 5(A)). Many studies have identified the role of the Akt/GSK 3β/FYN axis in the regulation of NRF2 [28,29]. We investigated this axis in GC-2spd (ts) cells considering to the role of Akt in Rg1-mediated antioxidant effects. In addition, NRF2 deficiency blocked the beneficial effect of Rg1 on oxidative stress, which was similar to of the outcome observed for Akt inhibitors (Figure 5(B)). Consistent with the in vitro data, we also observed that Rg1 lost its antioxidant effect in NRF2-deficient mice (Additional file 4: Figure S4). To further verify the role of Akt in NRF2 regulation, we treated GC-2spd (ts) cells with Akt inhibitors and found that Akt inhibition can activate GSK 3β and eliminate the beneficial effect of Rg1 treatment (Figure 5(C)). These data confirm that the Akt/GSK-3β/NRF2 signaling pathway is involved in Rg1-mediated oxidative damage protection.

Discussion

D-gal-treated animals have been reported to exhibit aging-related characteristics, such as elevated levels of advanced glycation end products (AGEs) [30], reduced antioxidant enzyme [31] activity, and mitochondrial [32] damage. Therefore, the D-gal-induced aging model has been widely used in the study of anti-aging drugs. In the present study, we observed senescence and oxidative stress damage in mouse testicular spermatogonia after D-gal injury. The ginsenoside Rg1 is a natural ginseng extract monomer with a well-defined chemical structure and anti-inflammatory, antioxidant, and anti-aging pharmacological effects. Our previous study showed that Rg1 antagonizes D-gal-induced testicular senescence in mice, but the mechanism remained unclear [14]. In the present study, we show that Rg1 attenuated D-gal-induced oxidative stress and spermatogonium apoptosis in testicular injury in vitro and in vivo. Based on these findings, we hypothesized that Rg1 may be a natural therapeutic agent for oxidative testicular injury and further explored its mechanism.

Previous studies have shown that Akt inactivation is the cause of testicular injury, and that enhanced Akt phosphorylation promotes the survival of testicular spermatogonia and prevents oxidative damage and apoptosis [33]. Our study showed that Rg1 could activate Akt/Bad in the mouse testis and protect spermatogonia from apoptosis, suggesting that Akt activation is an important factor in the protection of the testis by Rg1. Akt/mTOR is an important signal transduction

pathway responsible for cell survival and apoptosis [34]. Enhanced activation of the PI3K/Akt/mTOR signaling pathway can enhance immunity to apoptosis in DEHP-injured mouse testicular cells [35]. In this study, we observed that Rg1 attenuated D-gal-induced inactivation of the Akt/mTOR pathway both in vivo and in vitro, which was further confirmed by enhanced phosphorylation of P70/S6 and 4EBP1 (Additional file 5: Figure S5). Considering that mTOR is one of the downstream effectors of Akt and mainly mediates survival signaling pathways, we believe that Rg1 may play an antiapoptotic role through multiple Akt-dependent downstream pathways. Taken together, these data suggest that Rg1-mediated protection against testicular spermatogonium apoptosis is mainly regulated by multiple downstream signaling pathways following Akt activation.

The NRF2 antioxidant system is considered an important therapeutic target against oxidative stress and can exert antioxidant effects through the production of cytoprotective enzymes and the expression of related proteins [27]. Studies have shown that NRF2 plays an important role in preventing the development of oxidative stress in mirror genesis [36]. HO-1, a major enzyme in the heme metabolism catabolic pathway, plays a crucial role in cryoprotection through its antioxidant and anti-inflammatory properties [37]. The NRF2/HO-1 pathway has been reported to show a key antioxidant role in the treatment of multiple organs, including the testis [38]. In contrast, both GCLC and GCLM are NRF2-dependent genes, and GCLC is the rate-limiting enzyme for cellular glutathione biosynthesis [26]. This study showed that Rg1 treatment increased the expression of GCLC and GCLM. It has been reported that NQO1, a component of the NRF2 complex, is activated under oxidative stress [39]. Consistent with these data, we found that the addition of Rg1 significantly increased HO-1 oxidase activity and Nqo1 expression. In addition, studies have shown that Akt dephosphorylation leads to GSK-3β activation, promotes NRF2 degradation, and increases oxidative damage in cells [28]. In this study, we found that Rg1 attenuates oxidative damage via the Akt/GSK-3β/FYN/NRF2 axis. In conclusion, our results define Rg1 as an antioxidant that protects against oxidative damage in spermatogonia.

Previous studies have shown that Akt inactivation is the cause of testicular injury and that enhanced Akt phosphorylation promotes testicular spermatogonium survival and prevents oxidative damage and apoptosis. Our study showed that Rg1 could activate Akt/Bad in the mouse testis and protect spermatogonia from apoptosis, suggesting that Akt inhibition is an important factor in D-gal-induced damage to the testis.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

Data sharing is not applicable to this article because no datasets were generated or analyzed during the current study.

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