

# Role of peroxiredoxin 2 in H<sub>2</sub>O<sub>2</sub>-induced oxidative stress of primary Leydig cells

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**Abstract.** Late-onset hypogonadism is defined as a condition caused by a decline in the levels of testosterone with aging. One of the major factors contributing to the low levels of testosterone is the accumulation of reactive oxygen species (ROS) in Leydig cells during the ageing process. Peroxiredoxin 2 (Prdx2), a member of the peroxiredoxin family, is an antioxidant protein, the predominant function of which is to neutralize ROS. However, its role in Leydig cells remains to be elucidated. In the present study, primary Leydig cells were exposed to low concentrations of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to induce oxidative stress. Cell apoptosis was measured using an Annexin V fluorescein isothiocyanate/propidium iodide apoptosis detection kit and flow cytometry. The level of testosterone was determined by radioimmunoassay, and the mRNA and protein expression levels of Prdx2 were detected by reverse transcription-polymerase chain reaction and western blotting, respectively. The results revealed a significant increase in cell apoptosis and decrease in testosterone production. In addition, the expression of Prdx2 was decreased by H<sub>2</sub>O<sub>2</sub> in a dose- and time-dependent manner, and this decrease may have been caused by the induction of its molecular structure transformation due to H<sub>2</sub>O<sub>2</sub> elimination.

The above findings indicated that Prdx2 may prevent H<sub>2</sub>O<sub>2</sub> accumulation in Leydig cells, and may be important in oxidative stress-induced apoptosis and decreased testosterone production.

## Introduction

Late-onset hypogonadism (LOH) has been defined as a syndrome in middle-aged and elderly men, who report symptoms in the presence of low levels of testosterone (1). Testosterone deficiency is associated with alterations in reproductive function, muscle strength, bone density and other physiological parameters (2).

Testosterone is predominantly produced by Leydig cells. One of the major reasons for the reduced production of testosterone is oxidative stress, which usually results from an imbalance between the production of reactive oxygen species (ROS) and the scavenging ability of cellular antioxidant defense systems. ROS, including H<sub>2</sub>O<sub>2</sub> and superoxides, are produced by cells as by-products of normal cellular metabolism. Elevated levels of ROS have been shown to be associated with several diseases, including neurodegenerative disease and cancer (3,4). Multiple studies have indicated that ROS inhibits testosterone production in Leydig cells by dissipating mitochondrial membrane potential, and reducing the expression and activity of testicular steroidogenic enzymes (5-7). Thus, the accumulation of ROS during the ageing process results in reduced levels of testosterone (8).

Peroxiredoxins (Prdxs) are a family of antioxidant enzymes, which are capable of metabolizing H<sub>2</sub>O<sub>2</sub>. Prdxs are thioredoxin-specific antioxidants, which were first identified in yeast, and are found in archaea, prokaryotes and eukaryotes (9). Prdx2, a member of the peroxiredoxin family, is considered to regulate multiple cellular functions, including cell proliferation, differentiation and intracellular signaling. Of note, through the clearance of excessive H<sub>2</sub>O<sub>2</sub>, Prdx2 is critical in the modulation of cell survival. For example, previous studies have indicated that Prdx2 is upregulated in colorectal cancer and protects cells from oxidative stress (10), whereas Prdx2 knockdown by RNA interference inhibits the growth of colorectal cancer cells (11), and attenuation of Prdx2 inhibits proliferation and induces apoptosis in granulosa cells (12).

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**Abbreviations:** LOH, late-onset hypogonadism; ROS, reactive oxygen species; Prdx2, peroxiredoxin 2; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; Prdxs, peroxiredoxins; HBSS, Hank's balanced salt solution; MDA, malondialdehyde; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; LSD, least significance difference; Trx, thioredoxin

**Key words:** late-onset hypogonadism, Leydig cells, testosterone, H<sub>2</sub>O<sub>2</sub>, peroxiredoxin 2

Despite the protective effect of Prdx2 in several cell types, its biological function in Leydig cell remains to be elucidated. In the present study, primary Leydig cells were treated with H<sub>2</sub>O<sub>2</sub> to induce oxidative stress, following which cell apoptosis, testosterone production and changes in the expression of Prdx2 were investigated. These investigations were performed to determine whether Prdx2 is involved in the modulation of cell survival and testosterone production in Leydig cells.

## Materials and methods

**Materials.** Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F12 (DMEM/F12) was purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Percoll, HEPES and collagenase type I were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hanks' balanced salt solution (HBSS) without Ca<sup>2+</sup> or Mg<sup>2+</sup>, and penicillin-streptomycin were purchased from Life Technologies, Inc. (Paisley, UK). H<sub>2</sub>O<sub>2</sub> was purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). The Annexin V Apoptosis Detection kit APC was purchased from eBioScience, Inc. (San Diego, CA, USA). Mouse monoclonal antibody against 3 $\beta$ -hydroxysteroid dehydrogenase (HSD) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA; cat. no. 515120; dilution, 1:200). Rabbit monoclonal antibody against Prdx2 was purchased from Abcam (Cambridge, MA, USA; cat. no. ab133481; dilution, 1:50,000). Monoclonal rabbit antibodies against  $\beta$ -actin (cat. no. AC-40; dilution, 1:10,000), secondary horseradish peroxidase-conjugated goat anti-mouse (cat. no. BA1050; dilution, 1:10,000) and goat anti-rabbit antibodies (cat. no. BA1054; dilution, 1:10,000) were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China).

**Animals.** Male Sprague-Dawley rats (n=20; age, 9-10 weeks; weight, 200 $\pm$ 20 g) were obtained from the Center of Comparative Medicine, Nanjing Jinling Hospital (Nanjing, China) and bred in the laboratory of the Center of Reproductive Medicine, Nanjing Jinling Hospital, Nanjing University School of Medicine (Nanjing, China). The animal room was maintained at 22-24°C under a constant 12 h light: 12 h dark cycle. The animals were fed with standard pellet diet and water *ad libitum*. The procedures involving the animals were performed following the guidelines for animal treatment of Nanjing Jinling Hospital and approved by the ethics committee of Nanjing Jinling Hospital, in accordance with the principles and procedure of the National Institute of Health guidelines for the care and use of laboratory animals (13).

**Leydig cell isolation and culture.** The Leydig cells were prepared from the immature rat testes by collagenase treatment, as described previously (14). Briefly, the rats were sacrificed by cervical dislocation and immersed in 75% ethanol for 5 min. The sterile testes were dissected and washed three times in phosphate-buffered saline (PBS) chilled to 4°C. The epididymis, visible vessels, adipose and connective tissues were removed from the testes using microscissors. The tunica albuginea was dissected and the decapsulated testes were incubated with collagenase (0.25 mg/ml) for 20 min at 37°C. The crude interstitial cells were collected by centrifugation

at 1,000 g for 10 min at 4°C, and then washed twice in HBSS containing 0.1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich). To purify the Leydig cells, the crude cell suspension was loaded onto a discontinuous Percoll gradient (20, 40, 60 and 90% Percoll in HBSS) and subsequently centrifuged at 800 g for 20 min at 4°C. The fractions enriched in the Leydig cells were obtained and further centrifuged in a continuous, self-generating density gradient (starting at 60% Percoll), at 20,000 g for 30 min at 4°C.

The cells were then resuspended at a density of 10<sup>5</sup> cells/cm<sup>2</sup> in 24-well plates (Costar; Corning, NY, USA) at 0.5 ml/well. A total of 1.0 $\times$ 10<sup>6</sup> The total numbers of purified cells were analyzed for the expression of 3 $\beta$ -HSD to determine the purity of the Leydig cells (15). The purity was found to be ~85-90%, and >90% of these cells were viable, as determined using Trypan blue exclusion dye (Sigma-Aldrich). The purified Leydig cells were then washed twice with DMEM-F/12 and resuspended in DMEM-F/12 supplemented with 15 mmol/l HEPES (pH 7.4), 1 mg/ml BSA, 365 mg/l glutamine (Invitrogen; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin.

For subsequent culturing, 2 $\times$ 10<sup>6</sup> cells were plated into each well of a 6-well plate (Costar; Corning, NY, USA) and incubated at 34°C in a humidified atmosphere of 5% CO<sub>2</sub>.

**R2C cell line and granulosa cell culture.** The R2C cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM/F12 with 15% horse serum (Gibco; Thermo Fisher Scientific, Inc.), 2.5% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 100 U/l penicillin-100  $\mu$ g/l streptomycin, at 34°C in a humidified atmosphere of 5% CO<sub>2</sub>. The granulosa cells were obtained from the Center of Comparative Medicine, Nanjing Jinling Hospital and cultured in 6-well plates in DMEM/F12 medium, 10% FBS, at 34°C in a humidified atmosphere of 5% CO<sub>2</sub>. The cells were used for reverse transcription-polymerase chain reaction (RT-PCR) and western blotting, as described below.

**Measurement of malondialdehyde (MDA) levels.** Following 24 h of Leydig cell separation and culturing, the cells were treated with different concentrations of H<sub>2</sub>O<sub>2</sub> (50, 100 and 200  $\mu$ M) for 4 and 6 h. At the end of the treatment, the cells were harvested and sonicated with phosphate buffer (pH 6.8) containing 1.0 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich), to obtain cell homogenates. The homogenates were centrifuged at 3,000  $\times$  g at 4°C for 10 min and the supernatants were used for measuring cellular levels of MDA using an MDA assay kit (Jiancheng Biochemical, Inc., Nanjing, China). The MDA levels were calculated by evaluating the thiobarbituric acid reacting substance at a wavelength of 532 nm using an Infinite M200 microplate reader (Tecan Group, Ltd., Mannedorf, Switzerland). All values were normalized against the total protein concentration of the corresponding samples. The units of MDA measurements were  $\mu$ mol/g.

**Analyses of Leydig cell viability and apoptosis.** The Leydig cells were cultured and subsequently treated with different doses of H<sub>2</sub>O<sub>2</sub> (50, 100 and 200  $\mu$ M) for 4 and 6 h. This was followed by assessment of their viability and apoptosis using an Annexin V-Propidium iodide (PI) Apoptosis Detection kit

(cat. no. 88-8007) according to the manufacturer's protocol (eBioScience, Inc.). Briefly, the cells pellets were resuspended in 100  $\mu$ l of 1X binding buffer (eBioscience, Inc.) and incubated with 5  $\mu$ l of fluorochrome-conjugated Annexin V for 10-15 min at room temperature. The cells were then washed with 1X binding buffer and resuspended in 400  $\mu$ l of the same binding buffer. Following this, 5  $\mu$ l of PI staining solution was added to the cells, and each sample was analyzed immediately using a Cell Lab BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Data analysis was performed using FlowJo version 7.6.1 software (Tree Star, Inc., Ashland, OR, USA).

**Radioimmunoassay of testosterone.** To determine the levels of testosterone, the Leydig cells were incubated with fresh medium containing increasing concentrations of H<sub>2</sub>O<sub>2</sub> (50, 100 and 200  $\mu$ mol/l) for 4 and 6 h in the presence of human chorionic gonadotropin (hCG; 2 ng/ml; Sigma-Aldrich). The levels of testosterone were determined using a chemiluminescence assay with an Access Testosterone assay kit, according to the manufacturer's protocol (Beckman Coulter, Brea, CA, USA).

**RT-PCR analysis.** Total RNA was extracted from the rat Leydig cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The quality and concentration of the RNA was determined using an Eppendorf BioPhotometer® D30 (Eppendorf, Germany). Total RNA was reverse-transcribed using the PrimeScript™ RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) in a total volume of 25  $\mu$ l, comprising 5xAMV buffer, 2.5 mmol/l dNTPs, OligdT and 10 U/ $\mu$ l AMV, together with RNA as the template. The reaction was performed at 42°C for 60 min, following which the samples were incubated at 95°C for 5 min to terminate the reaction.

The RT-PCR was performed using an AffinityScript One-Step RT-PCR kit (Stratagene, Mississauga, ON, Canada). The primer sequences (synthesized by Shanghai Sangong Pharmaceutical Co., Ltd., Shanghai, China) were as follows: Sense 3'-ATGATGAGGGCATCGCTTAC-5' and antisense 3'-CATTGGGTTTGTATGGTGTCA-5' for Prdx2; and sense, 5'-GACATGCCGCCTGGAGAAAC-3' and anti-sense, 5'-AGCCCAGGATGCCCTTTAGT-3'. The DNA was first denatured at 95°C for 30 sec, followed by annealing at 60°C for 30 sec and extension at 72°C for 30 sec. This was repeated for 30 cycles, prior to a final extension step at 72°C for 10 min. The PCR product was finally run on a 1.5% agarose gel (Invitrogen; Thermo Fisher Scientific, Inc.) and visualized using ethidium bromide (Sigma-Aldrich).

Similarly, for quantitative (q)PCR, the RNA samples from the Leydig cells treated with the various concentrations of H<sub>2</sub>O<sub>2</sub> for different durations were prepared and processed, as described above. To determine the expression levels of Prdx2 RT-qPCR was performed using an ABI Prism 7000 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The GAPDH gene was used as an internal control for Prdx2 template normalization. Fluorescent signals were normalized to that of the internal reference, and the quantification cycle (C<sub>q</sub>) was set within the exponential phase of the PCR. The relative mRNA expression levels were calculated using the  $2^{-\Delta\Delta C_t}$  method (16).

**Western blot analysis.** The Leydig cells were serum-starved for 4 h following washing once with fresh medium. Subsequently, the cells were stimulated by increasing concentrations of H<sub>2</sub>O<sub>2</sub> (50, 100 and 200  $\mu$ M) for 2, 4 and 6 h). Following treatment, the cells were washed twice with ice-cold PBS and lysed in 120  $\mu$ l of ice-cold radioimmunoprecipitation assay buffer (Sangon Biotech Co., Ltd., Shanghai, China), containing 150 mmol/l NaCl, 1% Nonidet P-40, 0.25% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mmol/l Tris (pH 7.4), 1 mmol/l PMSF, 1 mmol/l Na<sub>3</sub>VO<sub>4</sub> and 1 mmol/l NaF. The cell lysates were harvested and centrifuged at 10,000 g for 20 min at 4°C. The supernatants were transferred to new tubes, and the protein concentrations were determined using the Bradford method (17). The total protein (30  $\mu$ g) was then mixed with loading buffer (Sangon Biotech Co., Ltd.) and boiled for 5 min. These protein samples were subsequently separated by running them on 15% SDS-PAGE gels (Sangon Biotech Co., Ltd.) in 1X running buffer (Sangon Biotech Co., Ltd.) at 25 mA for 2 h. The proteins were transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA) at 100 V for 1 h in transfer buffer at 4°C (Sangon Biotech Co., Ltd.) at 4°C. Subsequently, the membrane was blocked with 5% non-fat milk powder in Tris-buffered saline with 0.5% Tween 20 (TBST) for 1.5 h at 37°C, and washed three times with TBST for 30 min. The membrane was then incubated with Prdx2 primary antibody for 16-18 h at 4°C. Following washing in TBST, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody, and the bands were visualized with enhanced chemiluminescence (Promega), according to the manufacturer's protocol. The intensities of the bands were quantitated using Quantity-One version 4.6.2 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Statistical analysis.** All data are expressed as the mean  $\pm$  standard error of the mean, with standard deviation shown as bars in the figures. The differences between means were analyzed using one-way analysis of variance and the least significance difference method using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

**H<sub>2</sub>O<sub>2</sub> induces lipid peroxidation in primary Leydig cells.** The levels of lipid peroxidation in the Leydig cells following H<sub>2</sub>O<sub>2</sub> treatment were assessed by measuring the levels of MDA, which is the most frequently used biomarker to detect oxidative changes. As shown in Fig. 1A, treatment of these cells with different concentrations of H<sub>2</sub>O<sub>2</sub> for 4 h caused significant, concentration-dependent increases in MDA levels, compared with the control group. However, increasing the duration of H<sub>2</sub>O<sub>2</sub> treatment to 6 h (Fig. 1B) had no additional significant effect on the levels of MDA, compared with the cells treated for 4 h.

**Effects of H<sub>2</sub>O<sub>2</sub> treatment on primary Leydig cell viability and apoptosis.** Following exposure of the Leydig cells with varying concentrations of H<sub>2</sub>O<sub>2</sub> (50, 100 and 200  $\mu$ M) for 4 and 6 h, the cell viability and rates of apoptosis were

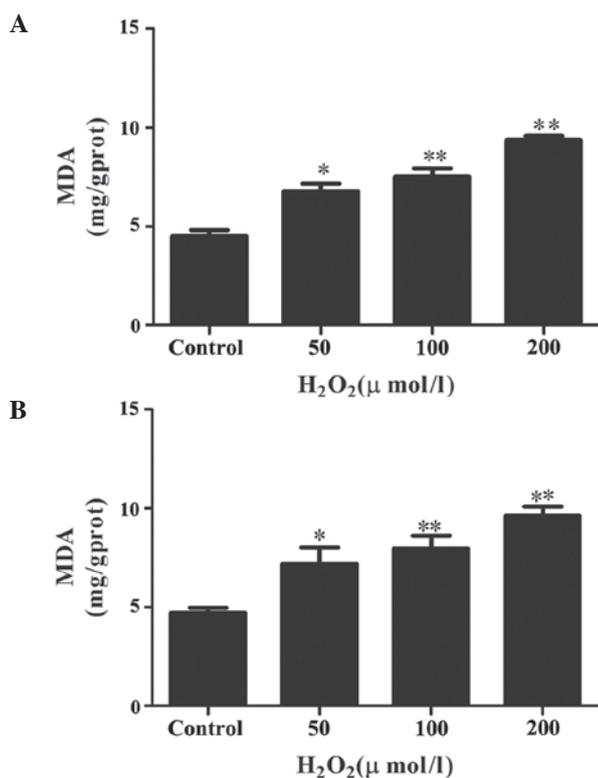


Figure 1. Evaluation of oxidative stress in Leydig cells treated with different concentrations of H<sub>2</sub>O<sub>2</sub>. (A) Levels of MDA in Leydig cells exposed to different concentrations of H<sub>2</sub>O<sub>2</sub> for 4 h. (B) Levels of MDA following 6 h of H<sub>2</sub>O<sub>2</sub> treatment. Data are presented as the mean ± standard error of the mean of three individual experiments. \*P<0.05 and \*\*P<0.01, compared with the untreated control. MDA, malondialdehyde; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide.

determined using PI and Annexin-V staining, respectively (Fig. 2). It was observed that H<sub>2</sub>O<sub>2</sub> treatment for 4 h led to a decrease in cell viability, whereas the apoptotic rate increased in a dose-dependent manner, as seen in Fig. 2A. Following 4 h treatment, the apoptotic rate was highest (13.65±2.44%) in the group treated with 200 μM H<sub>2</sub>O<sub>2</sub>, compared with the control group (Fig. 2C). This was simultaneously associated with decreased cell viability (77%; Fig. 2D). Prolonged treatment for 6 h with the same concentrations of H<sub>2</sub>O<sub>2</sub> led to no significant enhancement of apoptosis or cell viability, compared with the 4 h treatment group (Fig. 2B), and the two time points had relatively similar effects. The apoptotic rate of the cells treated with 200 μM H<sub>2</sub>O<sub>2</sub> was 14.1±0.02% (Fig. 2C) following 6 h treatment, and cell viability decreased to a similar extent as that observed following 4 h treatment (Fig. 2D).

#### H<sub>2</sub>O<sub>2</sub> inhibits testosterone production by primary Leydig cells.

To determine whether H<sub>2</sub>O<sub>2</sub> has any effect on the hCG-stimulated production of testosterone in Leydig cells, the cells were treated with varying concentration of H<sub>2</sub>O<sub>2</sub> for 4 and 6 h in the presence of hCG. A dose-dependant reduction in the levels of testosterone produced by the Leydig cells was observed (Fig. 3). At 4 h, 200 μM of H<sub>2</sub>O<sub>2</sub> inhibited the production of testosterone to the lowest level (2.36±0.29 ng), compared with the control group. At the two time points, significant decreases in testosterone production were observed at all concentrations, with the exception of the cells treated with 50 μM H<sub>2</sub>O<sub>2</sub> for 4 h.

*Detection of the mRNA and protein expression levels of Prdx2 in Leydig cells.* It has been previously reported that Prdx2 is expressed in a variety of cells and tissues (9). To determine whether Prdx2 is expressed in Leydig cells, the present study performed RT-PCR and Western blot analyses. The Prdx2 transcripts and proteins were detected in the R2C Leydig tumor cell line and in the primary Leydig cells (Fig. 4).

*H<sub>2</sub>O<sub>2</sub> treatment has no effect on the mRNA expression of Prdx2 in primary Leydig cells.* The Leydig cells were treated with different concentrations of H<sub>2</sub>O<sub>2</sub> (50, 100 and 200 μM) for 2, 4 and 6 h. Subsequently, the mRNA levels of Prdx2 were measured using RT-qPCR. It was observed that, compared with the control group, none of the concentrations or durations of H<sub>2</sub>O<sub>2</sub> treatment had any significant effect on the mRNA levels of Prdx2, as shown in Fig. 5.

*H<sub>2</sub>O<sub>2</sub> treatment decreases the protein expression of Prdx2 in primary Leydig cells.* The Leydig cells were treated, as above, with different concentrations of H<sub>2</sub>O<sub>2</sub> for different durations, and were analyzed for the protein expression of Prdx2. As shown in Fig. 6A, compared with the control group, no significant change in the protein expression of Prdx2 was observed following 2 h treatment with H<sub>2</sub>O<sub>2</sub>. However, the protein expression levels of Prdx2 decreased following 4 and 6 h of H<sub>2</sub>O<sub>2</sub> treatment. Furthermore, the expression of Prdx2 decreased significantly with increased H<sub>2</sub>O<sub>2</sub> concentration, with the lowest level observed following 200 μM H<sub>2</sub>O<sub>2</sub> treatment for 6 h, as shown in Fig. 6B.

## Discussion

LOH has been considered the most common form of male hypogonadism, with a prevalence rate of ~1 in 100 men (18). Leydig cell dysfunction in older men leads to lower serum testosterone levels, which may be the predominant cause of LOH (19-21).

ROS are produced during steroidogenesis, particularly during steroid hydroxylation by cytochrome P450 enzymes, which are localized in the mitochondria and endoplasmic reticulum (22,23). In aged animals, the generation of mitochondrial superoxide is increased in Leydig cells (24). It has been reported that ROS inhibits the synthesis of testosterone, and that this may be due to the accumulation of ROS in Leydig cells, resulting in lower levels of testosterone and enhanced cell death (6,8).

In the present study, the effect of H<sub>2</sub>O<sub>2</sub> on testosterone production in Leydig cells at low concentrations was investigated. The levels of MDA, the most frequently used biomarker to detect oxidative changes, increased following H<sub>2</sub>O<sub>2</sub> treatment. This result confirmed that H<sub>2</sub>O<sub>2</sub> induced oxidative stress. In addition, the present study demonstrated that H<sub>2</sub>O<sub>2</sub> induced dose-dependant inhibition of testosterone production in Leydig cells.

High concentrations of H<sub>2</sub>O<sub>2</sub> are likely to affect the survival of cells. Despite the fact that comparatively low concentrations of H<sub>2</sub>O<sub>2</sub> were used, Leydig cells apoptosis was enhanced. Following exposure of the cells to H<sub>2</sub>O<sub>2</sub> at a concentration of 200 μM, an increase in cell apoptosis (>2-fold) was observed. The present study hypothesized that apoptotic

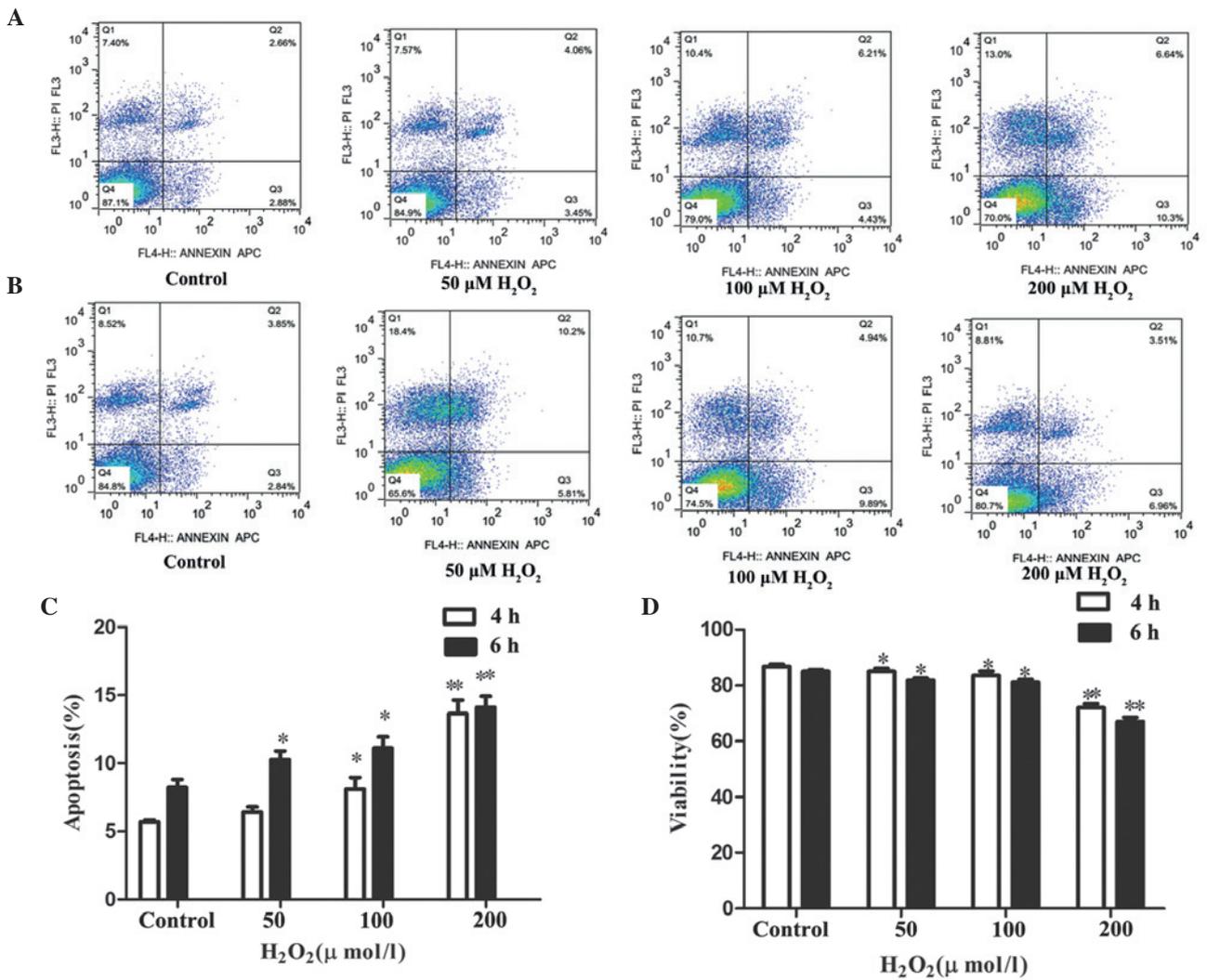


Figure 2. Analyses of Leydig cell viability and apoptosis using flow cytometry using with PI and Annexin V staining. (A) Percentages of cell viability and apoptosis following treatment with H<sub>2</sub>O<sub>2</sub> for 4 h. (B) Percentages of cell viability and apoptosis following treatment for 6 h with different concentrations of H<sub>2</sub>O<sub>2</sub>. Graphs showing the percentages of (C) apoptosis and (D) cell viability. The data are representative of three independent experiments and expressed as the mean ± standard error of the mean. \*P<0.05 and \*\*P<0.01, compared with the untreated control. PI, propidium iodide; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide.

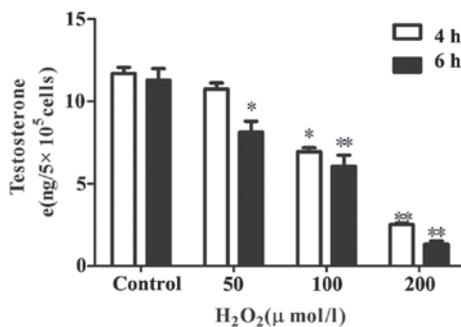


Figure 3. Effect of H<sub>2</sub>O<sub>2</sub> on hCG-stimulated testosterone production in Leydig cells. The graph shows the levels of testosterone produced by Leydig cells following hCG stimulation and treatment with different concentrations of H<sub>2</sub>O<sub>2</sub> for two durations. The data are representative of three independent experiments and expressed as the mean ± standard error of the mean. \*P<0.05 and \*\*P<0.01, compared with the untreated control. H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide.

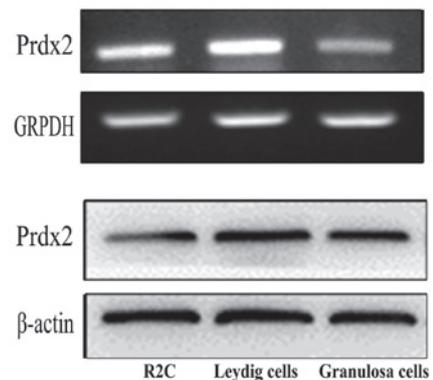


Figure 4. Analysis of the expression of Prdx2 in Leydig cells. The expression of Prdx2 was analyzed using (A) reverse transcription-polymerase chain reaction and (B) western blot analyses in R2C cells (Leydig tumor cell line), primary Leydig cells and Granulosa cells (positive control). Prdx2, peroxiredoxin 2.

induction may also have a negative effect on steroidogenic capacity. These observations suggested that H<sub>2</sub>O<sub>2</sub> at low levels modulates Leydig cell apoptosis and testosterone production.

These results are consistent with the previous findings of Gautam *et al* (25).

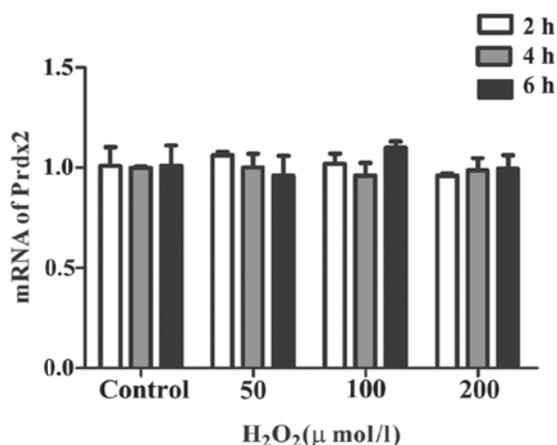


Figure 5. mRNA levels of Prdx2 following H<sub>2</sub>O<sub>2</sub> treatment. The mRNA levels of Prdx2 were measured using reverse transcription-quantitative polymerase chain reaction analysis in Leydig cells treated with control or different concentration of H<sub>2</sub>O<sub>2</sub> for 2, 4 and 6 h. Data are presented as bar graphs showing no significant difference. Prdx2, peroxiredoxin 2; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide.

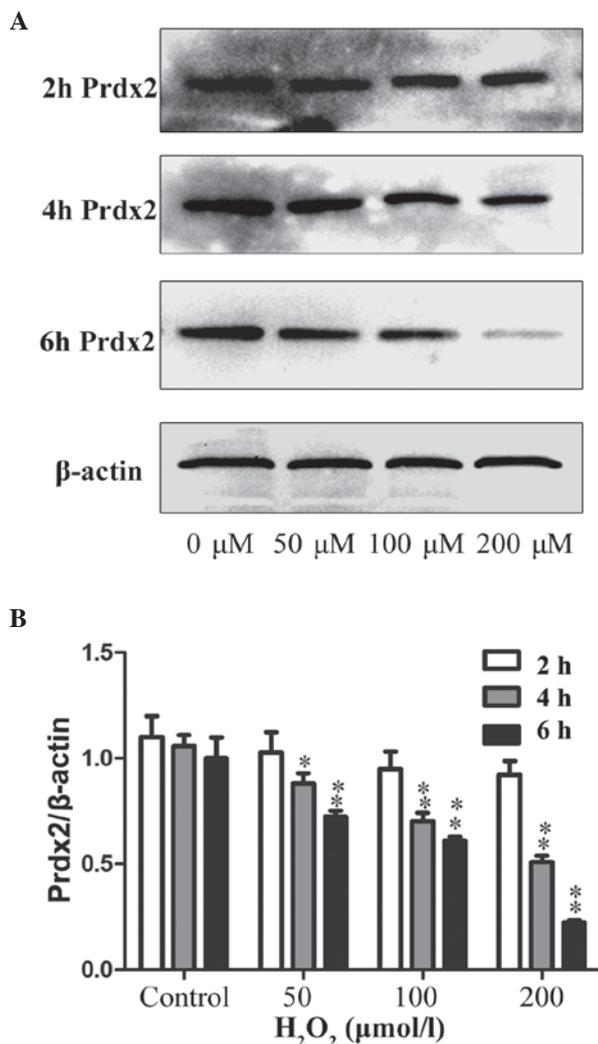


Figure 6. Protein expression levels of Prdx2 following H<sub>2</sub>O<sub>2</sub> treatment. (A) Protein levels of Prdx2 in primary Leydig cells treated with various concentrations of H<sub>2</sub>O<sub>2</sub> for 2, 4 and 6 h, analyzed using Western blot analysis. (B) Protein expression data. Data are representative of three independent experiments and expressed as the mean ± standard error of the mean. \*P<0.05 and \*\*P<0.01, compared with the untreated control. Prdx2, peroxiredoxin 2; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide.

Prdx2, a member of the Prdx family, has a crucial function in eliminating the H<sub>2</sub>O<sub>2</sub> produced during cell metabolism. The protein eliminates H<sub>2</sub>O<sub>2</sub> with reducing equivalents provided by the thioredoxin system. Considering the fact that Prdx2 protects cells from attack by ROS, and the fact that ROS induced apoptosis and reduced testosterone levels in the present study, it was suggested that Prdx2 may be significant in modulating Leydig cell function.

The present study examined the mRNA and protein levels of Prdx2 in Leydig cells. No significant changes were observed in the mRNA levels of Prdx2 following H<sub>2</sub>O<sub>2</sub> treatment. However, the protein expression of Prdx2 decreased as the concentration of H<sub>2</sub>O<sub>2</sub> increased, and this effect may have been due to the induction of molecular structural transformation. The thiol (Cys-SH) group of Prdx2 is oxidized to disulfide in the presence of H<sub>2</sub>O<sub>2</sub>. As the level of H<sub>2</sub>O<sub>2</sub> increases, Prdx2 undergoes further oxidation to the sulfinic (Cys-SO<sub>2</sub>H) or sulfonic (Cys-SO<sub>3</sub>H) acid forms (26). This hyperoxidation results in the transition from monomeric Prdx2 to its dimeric form (27). In addition, when Prdx2 is oxidized to sulfinic or sulfonic acid, it cannot eliminate H<sub>2</sub>O<sub>2</sub>, and this may explain why apoptosis was evident following treatment with 200 μM H<sub>2</sub>O<sub>2</sub>.

The results of the present study are consistent with those of Zhao *et al* (27), which showed that H<sub>2</sub>O<sub>2</sub> stimulation resulted in a significant decrease in the expression of Prdx2 in cardiomyocytes, along with reduced cell viability. Furthermore, the overexpression of Prdx2 protected cardiomyocytes from oxidative stress-induced cell death and apoptosis, whereas its ablation impaired these protective effects. Thus, the present study hypothesized that Prdx2 may have a protective effect against H<sub>2</sub>O<sub>2</sub> oxidative damage in Leydig cells.

In conclusion, the results of the present study demonstrated that low concentrations of H<sub>2</sub>O<sub>2</sub> induced oxidative stress, and modulated cell apoptosis and the production of testosterone in Leydig cells. In addition, stimulation with H<sub>2</sub>O<sub>2</sub> resulted in dose- and time-dependent decreases in the expression levels of Prdx2, which may have been caused by the induction of molecular structure transformation due to the elimination of H<sub>2</sub>O<sub>2</sub>. Therefore, it was hypothesized that Prdx2 may be pivotal in protecting Leydig cells from ROS damage and preventing the reduction of testosterone. Further investigations are required to confirm this hypothesis, and further examine the protective mechanism of Prdx2, which may provide novel insights to assist in the diagnosis and treatment of LOH.

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