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·*Complementary Medicine*·

Cytoprotective effects of *Morinda officinalis* against hydrogen peroxide-induced oxidative stress in Leydig TM3 cells

Mun-Seog Chang, Won-Nam Kim, Woong-Mo Yang, Hyu-Young Kim, Ji-Hoon Oh, Seong-Kyu Park

Department of Prescriptionology, College of Oriental Medicine, Kyung Hee University, Seoul 130-701, Korea

Abstract

Aim: To investigate the antioxidant effects of *Morinda officinalis* (*Morindae radix*, MR) on H_2O_2-induced oxidative stress in cultured mouse TM3 Leydig cells. **Methods:** We carried out 2,2-diphenyl-1-picrylhydrazyl free radical scavenging, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, lipid peroxidation, testosterone enzyme immunoassay, superoxide dismutase (SOD), and catalase (CAT) assays in Leydig TM3 cells. **Results:** MR showed a 47.8% 2,2-diphenyl-1-picrylhydrazyl radical scavenging effect in TM3 cells with no significant cytotoxicity. Oxidative stress was induced in TM3 cells with 100 μmol H_2O_2, and treatment of the cells with 250 μg/mL MR showed the most significant protective effect (64%, $P < 0.001$) in the cell viability assay with a decreased lipid peroxidation level (1.75 nmol/mg protein, $P < 0.05$), increased testosterone production (43.5 pg/mL), and improvements in SOD activity (7.49 units of SOD/mg protein, $P < 0.001$) and CAT activity (74.6 units of CAT/mg protein, $P < 0.001$). **Conclusion:** These findings indicate that MR, as an antioxidant, protects functions of cultured mouse TM3 Leydig cells from H_2O_2-induced oxidative stress. *(Asian J Androl 2008 Jul; 10: 667–674)*

Keywords: *Morindae radix*; Leydig cell; testosterone; hydrogen peroxide; antioxidant

1 Introduction

Oxidative damage is a consequence of excessive oxidative stress, insufficient antioxidant potential, or a combination of both. Oxidative damage induced by reactive oxygen species (ROS) is implicated as an important contributing factor in the pathogenesis of more than 100 conditions [1]. ROS include oxygen radicals, such as superoxide anions and hydroxyl radicals, reactive oxygen, and species that are not radicals in nature but are capable of radical formation in cellular environments including hydrogen peroxide (H_2O_2), nitric oxide, and peroxynitrite anion. ROS cause tissue damage by a variety of mechanisms including DNA damage, lipid peroxidation, protein oxidation, depletion of cellular thiols, and activation of pro-inflammatory cytokine release. Several cellular antioxidant systems help protect against free radical damage. These antioxidant systems include antioxidant molecules such as α-tocopherol, ascorbic acid, and glutathione, and antioxidant enzymes such as glutathione peroxidase and superoxide dismutase (SOD) [2]. In steroidogenic cells, ROS production is expected to be particularly high because, in addition to

Correspondence to: Seong-Kyu Park, M.D., Ph.D., Department of Prescriptionology, College of Oriental Medicine, Kyung Hee University, Seoul 130-701, Korea.
Tel: +82-2-961-0330 Fax: +82-2-961-0536
E-mail: comskp@khu.ac.kr
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the mitochondrial electron transport chain, ROS are also produced as by-products of steroid hydroxylation by cytochrome P450 enzymes [3, 4].

Previous studies have indicated that ROS also inhibit steroidogenesis in mouse MA-10 tumor Leydig cells at the level of cholesterol transfer [5]. ROS might also be important to the senescence of Leydig cell function during aging [6–8]. Culturing Leydig cells with vitamin E or giving vitamin E to rats shows protective effects on steroidogenic function [9].

The roots of *Morinda officinalis* (*Morindae radix*, MR), have been used in traditional medicine in northeast Asia to treat impotence, menstrual disorders, and inflammatory diseases such as rheumatoid arthritis and dermatitis [10]. An investigation of the hypoglycemic and antioxidant activities of the dried MR roots in streptozotocin-induced diabetic rats showed hypoglycemic, hyperglycemic, and antioxidant properties [11]. However, there is no report on the antioxidant activity of MR on testicular Leydig cells or a Leydig cell line. We previously reported that natural compounds such as *Panax* ginseng can be cytoprotective against gallic acid-induced cytotoxicity and induce spermatogenesis through cAMP-responsive element modulator in rat testes [12]. Here we investigated the effects of MR on H_2O_2-induced oxidative stress in the mouse Leydig TM3 cell line and subsequently evaluated its antioxidant effects *in vitro*.

2 Materials and methods

2.1 Cell culture

TM3 cells (mouse Leydig cells) were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, penicillin G sodium (100 units/mL), streptomycin sulfate (100 mg/mL), and amphotericin B (250 ng/mL). Cells were maintained with 5% CO_2 in a humidified chamber (Sanyo, Tokyo, Japan) at 37ºC.

2.2 Preparation of MR extract

MR was purchased from the Anguo Herbal Medicine Market (Anguo, Beijing, China). A sample of 150 g dried MR was boiled in 1 L water for 2 h. The suspension was then filtered and concentrated under reduced pressure. The filtrate was lyophilized and yielded 61.4 g of powder.

2.3 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The radical scavenging activity of MR extract against stable DPPH was determined as described elsewhere [13]. Test samples were added to DPPH of equal volumes in 96-well microplates and incubated for 30 min. The absorption of DPPH was then measured at 517 nm on a microplate spectrophotometer at room temperature. The radical scavenging activity was calculated by the following formula: DPPH radical scavenging activity (%) = $[(A_C - A_T) / A_C] \times 100$, where A_C is the absorption of the control reaction and A_T is the absorption of the tested sample.

2.4 Effect of MR extract on the growth of Leydig TM3 cells

Cell viability was assessed by a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as described elsewhere [14, 15]. Equal volumes of predetermined concentrations of MR extract (5, 10, 50, 100, and 250 μg/mL in phosphate-buffered saline [PBS]) and medium were added to the cells and incubated for 24 h at 37ºC in a 5% CO_2 incubator. Four hours before the end of the incubation, 20 μL MTT (5 mg/mL in PBS) was added. After 4 h, 100 μL dimethylsulfoxide was added to each well. After incubating the plate for 2 h at 37ºC, the intensity of the developed color was measured at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 37ºC. Cell viability was determined using the formula: cell viability (%) = $100 \times A_T/A_C$, where A_C is the absorption of the control and A_T is the absorption of the tested extract solution.

2.5 Effect of MR extract against H_2O_2-induced cytotoxicity

Equal volumes of predetermined concentrations of MR extract (10, 50, 100, and 250 μg/mL in PBS) and 100 μmol H_2O_2 in fetal bovine serum (FBS)-free DMEM was added to each well, and the plate was incubated for 24 h. Four hours before the end of the incubation, 20 μL MTT (5 mg/mL in PBS) was added. After 4 h, 100 μL dimethylsulfoxide was added to each well. After incubating the plate for 2 h at 37ºC, the intensity of the developed color was measured at 570 nm using a microplate reader at 37ºC.

2.6 Measurement of lipid peroxidation

Equal volumes of predetermined concentrations of MR extract (10, 50, 100 and 250 μg/mL in PBS) and

100 μmol H_2O_2 in FBS-free DMEM were added to each well, and the cell plate was incubated for 24 h. Lipid peroxidation products were measured by the thiobarbituric acid (TBA) assay as described elsewhere [16] with minor modifications. Briefly, cells were lysed using a freezing-thawing method. After lysis, 0.2 mL cell suspension was added to the TBA reagent (1.5 mL of 20% acetic acid, 1.5 mL of 8.1% sodium dodecylsulfate, and 1.5 mL of 0.8% TBA). This mixture was incubated at 90ºC for 1 h then cooled. Four milliliters of a mixture of n-butanol and pyridine (15 : 1, v/v) was added, and the whole mixture was centrifuged (15 min at 1 500 × *g*). The absorbance of the upper phase was measured at 532 nm. The concentration of TBA reactive substances was calculated from a standard calibration curve generated with known amounts of malondialdehyde (MDA). MDA values were expressed as nmol per mg protein.

2.7 Determination of testosterone level

Equal volumes of predetermined concentrations of MR extract (10, 50, 100, and 250 μg/mL in PBS) and 100 μmol H_2O_2 in FBS-free DMEM were added to each well, and the plate was incubated for 24 h. The collected medium was assayed for testosterone by an enzyme immunoassay kit (Assay Designs, Ann Arbor, MI, USA).

2.8 Antioxidant enzyme assay

The SOD activity was assayed according to the method described by Crapo *et al*. [17] with modifications. Equal volumes of predetermined concentrations of MR extract (10, 50, 100 and 250 μg/mL in PBS) and 100 μmol H_2O_2 in FBS-free DMEM were added to each well, and the plate was incubated for 24 h. Briefly, 20 μL of samples with the same amount of protein were mixed with 870 μL of solution A (50 mmol phosphate buffer (pH 7.8) with 0.1 mol ethylenediaminetetraacetic acid, 0.001 N NaOH with 5 μmol xanthine, and 2 μmol cytochrome C) and 20 μL of solution B (50 mmol phosphate buffer [pH 7.8] and 0.2 μmol xanthine oxidase). Enzyme activity in the sample was calculated from a standard curve with the range from 0.05 to 12.5 units/mg protein using SOD enzyme (Sigma, Saint Louis, MO, USA). One unit of SOD activity is defined as the enzyme concentration required to inhibit chromogen production by 50% in 5 min under the assay conditions.

CAT activity was assayed by the method of Aebi [18] with modifications. Briefly, 50 μL of samples with the same amount of protein were mixed with 1 mL of 0.01 mol phosphate buffer (pH 7.0) and 15 mmol H_2O_2. The mixture was immediately read at 240 nm for 1 min on a spectrophotometer. Enzyme activity was expressed as units of CAT/mg protein.

2. 9 RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Fenozol was added to TM3 cells. The samples were then homogenized and incubated for 5 min at 50ºC. Chloroform was added and the samples were centrifuged at 12 000 × *g* for 10 min at room temperature. The aqueous phase was transferred to fresh tubes and isopropanol was added. The supernatant was incubated for 10 min at room temperature and centrifuged at 12 000 × *g* for 15 min at 4ºC. Then the RNA pellets were washed with 70% ethanol, air dried, and resuspended in diethylpyrocarbonate-treated water. Total RNA was analyzed using gel electrophoresis and the amount of RNA was estimated by determining the optical density at 260 nm. Subsequently, cDNA was synthesized from 2 μg total RNA with reverse transcription (Promega, Madison, WI, USA) carried out at 42ºC for 1 h following incubation at 95ºC for 5 min. cDNA amplification was carried out according to the temperature profile: 95ºC for 1 min; 55ºC (Cu-Zn SOD and CAT) and 56ºC (β-actin) for 1 min; and 72ºC for 1 min. At the end of 30 cycles, the reaction was prolonged for 10 min at 72ºC as PCR amplification was carried out. The sequences of the Cu-Zn SOD primers were 5'-AAGGCCGTGTGCGTGCTGAA-3' (forward) and 5'-CAGGTCTCCAACATGCCTCT-3' (reverse). The CAT primers were 5'-GCAGATACCTGTGAACTGTC-3' (forward) and 5'-GTAGAATGTCCGCACCTGAG-3' (reverse), and those for β-actin were 5'-ACC GTG AAA AGA TGA CCC AG-3' (forward) and 5'-TAC GGA TGT CAA CGT CAC AC-3' (reverse). The PCR products were separated on 1.5% agarose gels, visualized by ethidium bromide staining using the i-MAX gel image analysis system (CoreBioSystem, Seoul, Korea), and analyzed using Alpha Ease FC software (Alpha Innotech, San Leandro, CA, USA).

2.10 Western blot analysis

Proteins from cells were separated using lysis buffer (Cell Signaling Technology, Billerica, NY, USA) according to the manufacturer's protocol with minor modifications. The protein concentrations were determined by the Bradford method [19]. Equivalent amounts

(50 μg) of protein extracts were loaded onto 10% Tris-glycine sodium dodecyl sulfate–polyacrylamide gels and separated, then electrophoretically transferred to nitrocellulose membranes using 25 mmol Tris and 250 mmol glycine containing 20% methanol (pH 8.3). Transfer was carried out at a constant voltage of 120 mA for 1 h. After transfer, the membranes were blocked in PBS containing 0.05% Tween-20 with 5% skim milk for 1 h at room temperature and incubated with the primary antibody (1 : 1 000; rabbit anti-Cu/Zn SOD polyclonal antibody and rabbit polyclonal to CAT–peroxisome marker (Assay Designs, Ann Arbor, MI, USA)). After incubation, the membranes were rinsed three times with 1 × PBS and incubated with secondary antibody (antirabbit peroxidase-conjugated immunoglobulin G) at a dilution of 1 : 1 000 for 2 h at room temperature. The membranes were then rinsed three times with 1 × PBS. Chemiluminescence was developed using a SuperSignal West Pico kit from Pierce (Benebiosis, Seoul, Korea) and medical blue X-ray film (Agfa, Mortsel, Belgium).

2.11 Statistical analysis

Values are presented as means ± SD. The significance of the differences between groups was determined by ANOVA with the aid of SPSS 11.0 for Windows (SPSS, Chicago, IL, USA).

3 Results

3.1 DPPH radical scavenging activity of MR extract

DPPH radicals react with suitable reducing agents causing color loss, and the number of electrons consumed is measurable by a spectrophotometer at 517 nm. The DPPH radical scavenging activity of MR extract is shown in Figure 1. MR extracts showed effective free radical scavenging activities of 41.2%, 47.8% and 46.8% at concentrations of 100, 500 and 1 000 μg/mL, respectively.

3.2 Effect of MR extract on viability of TM3 cells

The cytotoxicity of MR extract in TM3 cells was evaluated by the MTT test. The results in Figure 2A show that TM3 cells were grown with no significant cytotoxicity at concentrations ranging from 5 μg/mL to 250 μg/mL.

3.3 Protective effect of MR against H_2O_2-induced cytotoxicity

The cytoprotective effect of MR extract (10, 50, 100 and 250 μg/mL) in TM3 cells was examined (Figure 2B). Viabilities of cells exposed to 100 μmol H_2O_2 decreased below 50% and increased to a statistically significant extent up to 64.0% in the MR-treated group at 250 μg/mL.

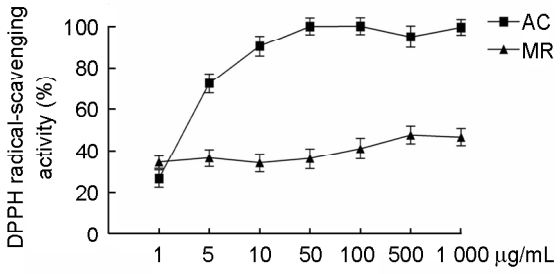

Figure 1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity of ascorbic acid (AC) and aqueous extract from *Morindae radix* (MR). Results are presented as the mean ± SD.

3.4 Effect of MR on lipid peroxidation

An inhibitory effect of MR on H_2O_2-induced lipid peroxidation was observed through the formation of MDA (Figure 2C). MR extract reduced lipid peroxidation production in a dose-dependent manner. As shown in Figure 2, the levels of MDA concentration in H_2O_2-induced cells were significantly increased compared to control cells (0.68 nmol/mg protein *vs.* 4.31 nmol/mg protein, $P < 0.001$). The treatment of cells with 10, 50, 100 and 250 μg/mL MR extract significantly reduced the MDA production to 2.60, 2.20, 1.87 and 1.75 nmol/mg protein, respectively ($P < 0.05$).

3.5 Effect of MR on testosterone production

The protective effect of aqueous extract of MR on H_2O_2-induced testosterone production was examined (Figure 3A). MR extract increased testosterone production in a dose-dependent manner. As shown in Figure 3A, the levels of testosterone production in H_2O_2-induced cells were significantly reduced compared to control cells (33.0 pg/mL to 26.4 pg/mL, $P < 0.001$). The treatment of cells with 10, 50, 100 and 250 μg/mL MR extract significantly increased testosterone production to 35.2, 38.4, 43.1 and 43.5 pg/mL, respectively ($P < 0.001$).

3.6 Effect of MR on antioxidant enzymes

The protective effect of aqueous extract of MR on H_2O_2-induced SOD and CAT activity was examined (Figure 3B, C). MR extract alone or as co-treatment

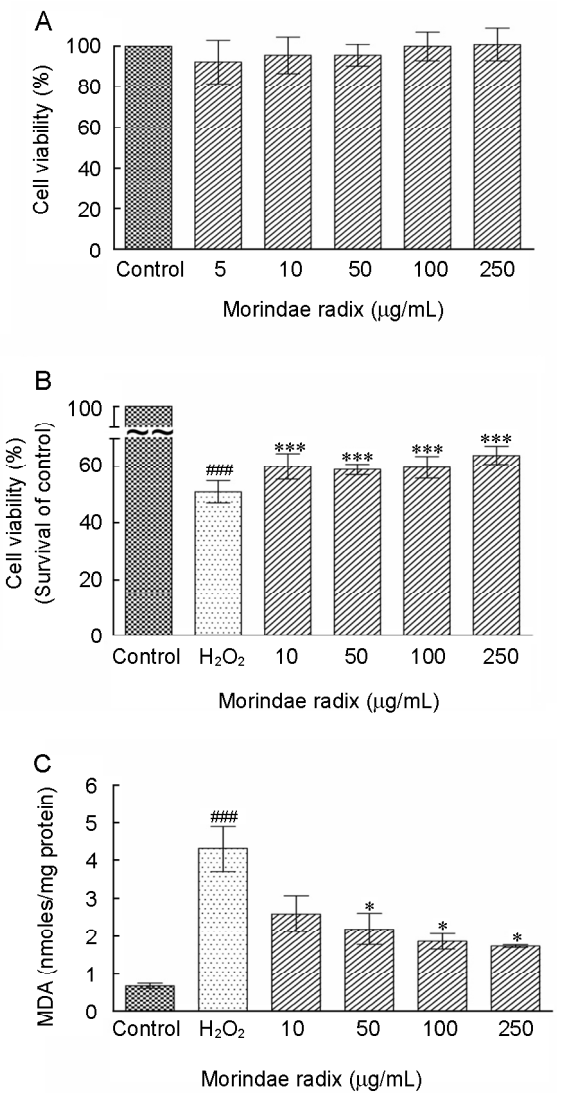

Figure 2. (A): Effect of aqueous extract from *Morindae radix* (MR) on mouse TM3 Leydig cells. TM3 cells were treated with MR at 37ºC for 24 h. (B): Protective effect of aqueous extract from MR on H_2O_2-induced cytotoxicity. TM3 cells treated with MR were incubated in the presence or absence of 100 μmol H_2O_2 at 37ºC for 24 h. (C): Protective effect of aqueous extract of MR on H_2O_2-induced lipid peroxidation. TM3 cells treated with MR were incubated in the presence or absence of 100 μmol H_2O_2 at 37ºC for 24 h. Total cell lysate from cultured cells was analyzed for malondialdehyde (MDA) formation. Results are presented as the mean ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$, compaired to cells exposed to H_2O_2 alone; $^{###}P < 0.001$, compared to control.

with H_2O_2 induced SOD and CAT activities. Western blot and reverse transcription–polymerase chain reaction analyses showed that both protein and mRNA levels of SOD and CAT were induced on MR treatment in a dose-dependent manner. As shown in Figure 3B, the levels of SOD activity in H_2O_2-induced cells were significantly reduced compared to the control (4.49 to 1.31 units of SOD/mg protein, $P < 0.001$). The treatment of cells with 10, 50, 100 and 250 μg/mL MR extract significantly increased SOD activity to 2.37, 2.55, 5.90, and 7.49 units of SOD/mg protein, respectively ($P < 0.001$). As shown in Figure 3C, the levels of CAT activity in H_2O_2-induced cells were significantly reduced compared to the control (76.2 to 54.0 units of CAT/mg protein, $P < 0.001$). The treatment of cells with 10, 50, 100, and 250 μg/mL MR extract significantly increased the CAT activity to 66.7, 68.3, 73.0 and 74.6 units of CAT/mg protein, respectively ($P < 0.001$).

4 Discussion

Oxidative stress has not been routinely investigated, even though it plays an important role in male infertility [20–22]. However, the source of the generation of this stress varies, as observed in men with clinical confirmation of varicocele. Those with a significantly higher number ($> 1 \times 10^6$/mL) of contaminated leukocytes, or those with a higher percentage of morphologically abnormal spermatozoa manifest increased ROS levels in semen [23]. An imbalance between ROS production and its disposal through naturally occurring antioxidants might also lead to a rise in ROS levels in semen [24]. A higher level of ROS would not only be detrimental to the unique ability of male germ cells to move forward, but would probably also affect their ability to fertilize the oocyte. The fertilizing ability of human spermatozoa is inversely proportional to the sperm ROS production [25].

The antioxidant activity of MR was determined using DPPH as a free radical resource [26]. This assay provided information on the reactivity with a stable free radical (DPPH), by a strong hydroxyl radical ($OH^{\bullet}$) scavenging effect, and was independent of any enzymatic activity. Aqueous extract of MR showed a 47.8% DPPH radical scavenging effect.

H_2O_2 is one of the major ROS associated with oxidative stress. It readily penetrates into cells and reacts with intracellular metal ions, such as iron or copper, to generate highly reactive hydroxyl radicals that successively attack cellular components including lipids, proteins, and DNA to cause a wide variety of oxidative insults. We examined the protective effect of MR against H_2O_2-in-

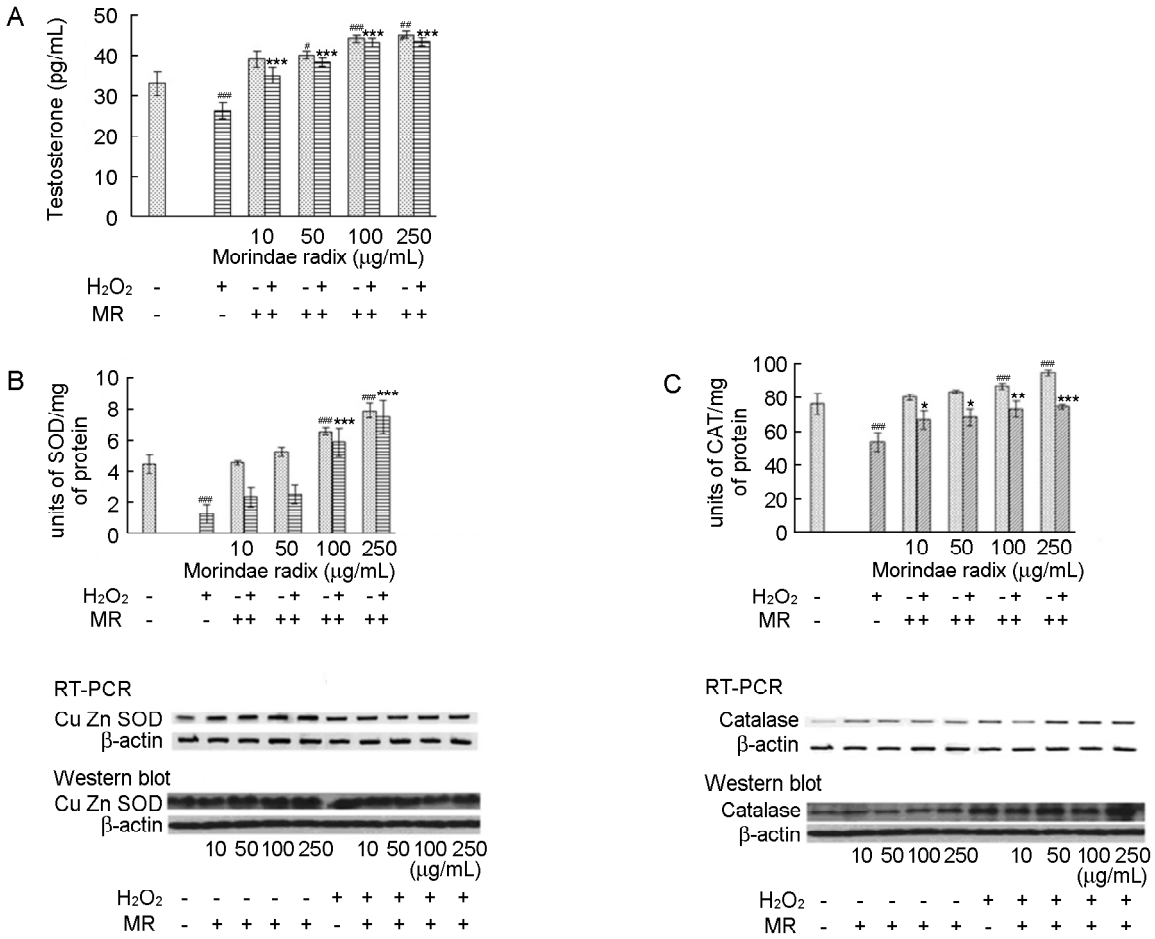

Figure 3. Mouse TM3 Leydig cells treated with Morindae radix (MR) alone or in co-treatment with H_2O_2 were incubated in the presence or absence of 100 μmol H_2O_2 at 37°C for 24 h. (A): The protective effect of aqueous extract of MR on H_2O_2-induced testosterone production. (B): The protective effect of aqueous extract of MR on H_2O_2-induced superoxide dismutase (SOD) activity. Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analyses were carried out. (C): The protective effect of aqueous extract of MR on H_2O_2-induced catalase (CAT) activity. RT-PCR and Western blot analyses were carried out. Results are presented as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to cells exposed to H_2O_2 alone; $^{\#\#\#}P < 0.001$, compared to control.

duced oxidative insults in Leydig TM3 cells. Because H_2O_2 induces lipid peroxidation, MDA formation in response to H_2O_2 was measured as a reflection of the peroxidation of membrane lipids in Leydig cells. Increased MDA accumulation has been noted in response to H_2O_2 [27], and the cytotoxic effects of H_2O_2 on Leydig TM3 cells were shown by its strong inhibition of cell growth (Figure 2B) and MDA formation (Figure 2C). These results indicate that MR is capable of reducing H_2O_2-induced cytotoxicity and lipid peroxidation.

Leydig cells in the interstitium, located between the seminiferous tubules of the testis, are the major source of androgenic steroids [7, 28]. Testosterone is synthesized by Leydig cells in the interstitial compartment of the testis and is mainly bound to androgen binding protein, produced by Sertoli cells. Androgen binding protein might be required to maintain high levels of testosterone inside the tubular compartment because of the lack of storage capacity inside the seminiferous tubules [29]. Testosterone is needed for maintenance of the spermatogenic process and for inhibition of germ cell apoptosis [30]. Complete inhibition of intratesticular testosterone, however, results in complete failure of spermatogenesis [31–33]. The assessment of testosterone production in our study using an enzyme immunoassay confirmed that H_2O_2 inhibits testosterone production by Leydig TM3 cells

and that MR has a protective effect on testosterone production *in vitro*.

SOD catalyzes the dismutation of the superoxide anion ($O2^-$) to produce H_2O_2. Although it recycles the superoxide anion free radical, SOD can be considered more as a pro-oxidant because it converts a rather short-lived and confined molecule ($O2^-$) into a quite stable and invasive molecule, H_2O_2. CAT transforms H_2O_2 into a harmless product, H_2O. CAT only uses H_2O_2 as a substrate and functions when its concentration is largely above physiological levels, as can happen in oxidative bursts characteristic of stress responses [33, 34]. The results showing that MR significantly increased SOD and CAT activity revealed how MR is capable of protecting Leydig TM3 cells from H_2O_2-induced cytotoxicity and lipid peroxidation.

The present observations indicate that testosterone production in Leydig TM3 cells is reduced by H_2O_2-induced cytotoxicity and lipid peroxidation. Aqueous extract of MR increased the testosterone production in Leydig TM3 cells and protected the cells from H_2O_2-induced cytotoxicity and lipid peroxidation through the activation of antioxidant enzymes such as SOD and CAT. The expression levels of SOD and CAT, both mRNA and protein, were increased on treatment with MR alone or co-treatment with H_2O_2. Therefore, as a natural compound, MR shows antioxidant activities and increases testosterone production in oxidative stress conditions, for cultured mouse TM3 Leydig cells. From this, it can be suggested that MR and other natural compounds with similar effects might be candidate agents for use in male infertility treatments. However, it remains to be tested if MR can exert an effect on Leydig cells *in vivo*.

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