Resveratrol Reduces Oxidative Damage in TM3 Mouse Leydig Cells via the Sirtuin 1-Uncoupling Protein 2 Signaling Pathway [Post-Print]

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Abstract

This study aimed to investigate the protective effects of resveratrol (RES) on oxidatively damaged mouse testicular Leydig cells TM3 and explore its potential mechanisms of action. First, mouse testicular Leydig cells TM3 were treated with different concentrations (0, 150, 200, 250, 300 mol/L) of hydrogen peroxide (H2O2) for 8 h to determine the appropriate H2O2 concentration for establishing an oxidative damage cell model. An oxidative damage cell model was established using the appropriate concentration of H2O2; then, normal cells and oxidatively damaged cells were treated with different concentrations (0, 2.5, 5.0, and 10.0 mol/L) of RES for 24 h to determine the safe concentration of RES; finally, oxidatively damaged cells were treated with the safe concentration of RES for 24 h. Throughout the culture process, an iCELLigence real-time label-free cell function analyzer was used to monitor cell proliferation; after treatment of oxidatively damaged cells with the safe concentration of RES was completed, the content of reactive oxygen species (ROS) in cells was detected using the 2' ,7' -dichlorofluorescein diacetate (DCFH-DA) probe method, and the relative expression levels of SIRT1 and UCP2 mRNA and protein, key factors in the sirtuin 1 (SIRT1)/UCP2 signaling pathway, were detected by real-time fluorescence quantitative PCR (RT-qPCR) and Western blotting, respectively. The results showed that: 1) Treatment of cells with H2O2 at concentrations of 150 mol/L and above for 8 h extremely significantly reduced cell viability ($P<0.01$); therefore, 150 mol/L was determined as the appropriate H2O2 concentration for establishing the oxidative damage cell model. 2) After normal cells were treated with RES at concentrations of 10.0 mol/L and below for 24 h, cell viability showed no significant changes ($P>0.05$); after oxidatively damaged cells were treated with 2.5, 5.0, and 10.0 mol/L RES for 24 h, cell viability was significantly increased ($P<0.05$), with no significant differences among the

RES groups at various concentrations (P>0.05). Therefore, 5.0 mol/L was selected as the safe concentration of RES. 3) After oxidatively damaged cells were treated with 5.0 mol/L RES for 24 h, the ROS content in cells was extremely significantly reduced (P<0.01); the relative expression levels of SIRT1 mRNA and protein in cells were extremely significantly increased (P<0.01), whereas the relative expression levels of UCP2 mRNA and protein were significantly or extremely significantly decreased (P<0.01 or P<0.05). Thus, appropriate concentrations of RES can activate SIRT1 while inhibiting UCP2 expression, and UCP2 reduces intracellular ROS generation through negative feedback regulation, thereby inhibiting oxidative damage to TM3 cells to a certain extent.

Full Text

Resveratrol Attenuates Oxidative Injury in Mouse Leydig Cell TM3 via Silent Information Regulator 1/Uncoupling Protein 2 Signaling Pathway

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Abstract: This study investigated the protective effect of resveratrol (RES) on hydrogen peroxide (H O)-induced oxidative damage in mouse Leydig cell TM3 and explored its underlying mechanisms. Initially, TM3 cells were treated with various concentrations of H O (0, 150, 200, 250, and 300 mol/L) for 8 h to determine the optimal concentration for establishing an oxidative damage model. Subsequently, both normal and oxidative-damaged cells were treated with different concentrations of RES (0, 2.5, 5.0, and 10.0 mol/L) for 24 h to identify a safe concentration. Finally, oxidative-damaged cells were treated with the selected safe concentration of RES for 24 h. Cell proliferation was monitored in real-time using an iCELLigence label-free cell function analyzer throughout the culture period. After RES treatment, intracellular reactive oxygen species (ROS) levels were measured using the 2' ,7' -dichlorodihydrofluorescein diacetate (DCFH-DA) probe method, while mRNA and protein expression levels of silent information regulator 1 (SIRT1) and uncoupling protein 2 (UCP2)—key factors in the SIRT1/UCP2 signaling pathway—were determined by real-time quantitative PCR (RT-qPCR) and Western blotting, respectively.

The results showed that: 1) Treatment with H O at concentrations of 150 mol/L or higher for 8 h extremely significantly reduced cell viability (P<0.01). Therefore, 150 mol/L was selected as the optimal concentration for establishing the oxidative damage model. 2) Treatment of normal cells with RES at concentrations up to 10.0 mol/L for 24 h did not significantly alter cell viability (P>0.05). In contrast, treatment of oxidative-damaged cells with RES at 2.5, 5.0, and 10.0 mol/L significantly increased cell viability (P<0.05), with no significant differences among these RES concentration groups (P>0.05). Thus, 5.0

mol/L was selected as the safe concentration of RES. 3) Treatment of oxidative-damaged cells with 5.0 mol/L RES for 24 h extremely significantly reduced intracellular ROS content ($P<0.01$), extremely significantly increased SIRT1 mRNA and protein expression ($P<0.01$), and significantly or extremely significantly decreased UCP2 mRNA and protein expression ($P<0.05$ or $P<0.01$). These findings demonstrate that appropriate concentrations of RES can activate SIRT1 while inhibiting UCP2 expression, and that UCP2 reduces ROS generation through negative feedback regulation, thereby attenuating oxidative damage in TM3 cells.

Key words: resveratrol; oxidative injury; SIRT1; UCP2; mouse Leydig cell TM3

Introduction

When organisms are exposed to various harmful stimuli, the balance between oxidative and antioxidant systems is disrupted, leading to excessive accumulation of reactive oxygen species (ROS) and subsequent oxidative damage. Studies have shown that oxidative damage can cause severe spermatogenic dysfunction in animals, resulting in significant pathological changes in testicular tissue, decreased testicular index and sperm count, increased sperm abnormality rate, and ultimately male infertility [1-2].

Resveratrol (RES), a natural polyphenolic compound widely found in grapes, peanuts, *Polygonum cuspidatum*, *Cassia tora*, and other plant-based foods, exhibits extensive biological activities and has been applied in treating oxidative damage, inflammation, allergies, tumors, and cardiovascular diseases [3-4]. In livestock production, RES is widely used to improve production performance, carcass and meat quality, and to enhance immunity and antioxidant capacity [5]. Previous studies have demonstrated that high-glucose and high-cholesterol diets impair testosterone synthesis in mouse Leydig cells, and RES supplementation can exert protective effects by alleviating oxidative stress and activating silent information regulator 1 (SIRT1) while influencing hypothalamic-pituitary-gonadal axis regulation [6]. Cheng et al. [7] found that RES promotes apoptosis in bovine subcutaneous adipocytes by activating the SIRT1/AMPK signaling pathway. Zhou et al. [8] reported that RES may inhibit oxidative stress injury in vascular endothelial cells by regulating the SIRT1/UCP2 signaling pathway. SIRT1, a deacetylase, regulates transcription of genes and proteins associated with oxidative stress, apoptosis, and inflammatory responses through post-translational modification (deacetylation) of histones, transcription factors, and transcriptional co-regulators [9]. UCP2, a mitochondrial uncoupling protein, affects mitochondrial membrane potential by reducing hydrogen ion (H) concentration across the inner membrane when membrane potential increases, thereby promoting oxygen consumption and inhibiting ROS production [10]. While the antioxidant effects of RES have been established, its specific mechanisms remain incompletely understood. Therefore, this study investigated the therapeutic effects of RES on H O -induced oxidative stress in mouse Leydig cell TM3 and

elucidated its potential mechanisms, providing a theoretical basis for preventing and treating male reproductive system diseases caused by oxidative damage.

Materials and Methods

1.1 Experimental Materials

The mouse Leydig cell TM3 cell line was obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences. Resveratrol, hydrogen peroxide, goat anti-UCP2 polyclonal antibody, and horseradish peroxidase (HRP)-conjugated rabbit anti-goat immunoglobulin G (IgG) were purchased from Sigma-Aldrich (USA). Rabbit anti-SIRT1 polyclonal antibody and rabbit anti- -actin polyclonal antibody were purchased from Abcam (UK). HRP-conjugated donkey anti-rabbit IgG was purchased from Santa Cruz Biotechnology (USA). Trizol reagent was purchased from Life Technologies (USA). HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) and ChamQTM SYBR® qPCR Master Mix kits were purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). Bicinchoninic acid (BCA) protein assay kit was purchased from Beyotime Biotechnology Institute. ROS detection kit was purchased from Nanjing Jiancheng Bioengineering Institute. Radioimmunoprecipitation assay (RIPA) lysis buffer and phenylmethylsulfonyl fluoride (PMSF) were purchased from Beijing Solarbio Science & Technology Co., Ltd. Fetal bovine serum was purchased from Gibco (USA). DMEM/F12 medium was purchased from Hyclone (USA).

1.2.1 Cell Culture

TM3 cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-amphotericin B at 37 °C in a 5% CO incubator. The medium was changed daily. When cells reached logarithmic growth phase (80–90% confluence), they were digested with trypsin to prepare cell suspensions.

iCELLigence Real-Time Cell Proliferation Monitoring For real-time monitoring of cell proliferation, cells were digested, centrifuged, and resuspended in DMEM/F12 medium containing 1% fetal bovine serum. Cell density was adjusted to 2.5×10 cells/mL. E-Plate L8 plates were pre-loaded with 150 L/well of DMEM/F12 medium containing 1% fetal bovine serum and placed in the iCELLigence real-time cell function analyzer (Roche, Germany). After automatic baseline measurement, 300 L of cell suspension was added to each well with three replicate wells per group. Following 30 min of static incubation, the plate was returned to the analyzer for continuous monitoring. The system recorded data every 15 min and generated proliferation curves expressed as proliferation index (PI) to reflect real-time cell proliferation under different treatment conditions.

Three experimental protocols were employed: 1) For determining the optimal H O concentration, after cells entered logarithmic growth phase (24 h post-

seeding), 100 L of medium was gently removed and replaced with 100 L of medium containing H O at final concentrations of 0 (control), 150, 200, 250, and 300 mol/L. The plate was returned to the analyzer for an additional 8 h monitoring (total 32 h). 2) For determining RES safety concentration, after 24 h of culture, cells were treated with RES at 0 (control), 2.5, 5.0, and 10.0 mol/L for 24 h (total 48 h monitoring). 3) For RES intervention in oxidative-damaged cells, after 24 h of culture, cells were first treated with 150 mol/L H O for 8 h, then treated with RES at 0 (H O group), 2.5, 5.0, and 10.0 mol/L for 24 h, with an untreated normal control group included (total 56 h monitoring). Cell viability was determined in each plate at the end of monitoring.

1.2.3 Detection of Intracellular ROS Content by DCFH-DA Probe

After RES treatment (5.0 mol/L) of oxidative-damaged cells, intracellular ROS content was measured using the ROS detection kit according to the manufacturer' s instructions. Briefly, the culture supernatant was removed and fresh medium containing 10 mol/L DCFH-DA probe was added. After 40 min incubation at 37 °C, cells were digested with trypsin and centrifuged at 1,000 r/min for 10 min. The supernatant was discarded, and cells were washed once with phosphate-buffered saline (PBS), centrifuged, and resuspended in PBS at a density of 6×10 cells/mL. Fluorescence intensity was measured using a fluorescence microplate reader at excitation wavelength of 485 nm and emission wavelength of 525 nm. DCFH-DA is non-fluorescent; upon entering cells, it is hydrolyzed by esterases to DCFH, which is then oxidized by intracellular ROS to produce the highly fluorescent DCF. Therefore, fluorescence intensity is proportional to ROS content.

1.2.4 Real-Time Quantitative PCR (RT-qPCR) for SIRT1 and UCP2 mRNA Expression

After RES treatment (5.0 mol/L) of oxidative-damaged cells, total RNA was extracted using Trizol reagent, and RNA concentration and purity were determined using NanoDrop 2000. Total RNA was reverse-transcribed using HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) to obtain cDNA for qPCR amplification. RT-qPCR was performed on an ABI StepOne system using ChamQTM SYBR® qPCR Master Mix. The 20 L reaction mixture contained 10 L of 2×ChamQTM SYBR® qPCR Master Mix, 0.4 L each of forward and reverse primers (10 mol/L), 0.4 L of 50×ROX, 2.0 L of cDNA, and 6.8 L of double-distilled water (ddH O). Thermal cycling conditions were: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Melting curve analysis was performed at 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. -actin served as the internal reference, and relative mRNA expression was calculated using the 2^(-ΔΔCt) method, with the control group normalized to 1. Primers were designed and synthesized by BGI Genomics Co., Ltd. (Table 1).

Table 1. Primer sequences for RT-qPCR

Gene	Accession number	Primer sequence (5´-3´)
-actin	NM_007393.5	F: CATCCGTAAAGACCTCTATGC-CAACR: ATGGAGCCACCGATCCACA
SIRT1	NM_001159589.2	F: GCTTCATGATGGCAAGTGGR: TCGTGGAGACATTTTTAATCAGG
UCP2	NM_011671.5	F: TCGGACACAGCCTTCTR: CTGGGAGACGAAACACTTA

F and R represent forward and reverse primers, respectively.

1.2.5 Western Blotting for SIRT1 and UCP2 Protein Expression

After RES treatment of oxidative-damaged cells, the medium was removed and cells were washed twice with ice-cold PBS. Cells were lysed in 200 L RIPA buffer containing 1% PMSF on ice for 30 min with vigorous vortexing three times (30 s each). The lysate was centrifuged at 12,000 r/min for 10 min at 4 °C, and the supernatant was collected. Protein concentration was determined using the BCA protein assay kit. Protein samples were mixed with 5×Loading Buffer and denatured at 100 °C for 5 min. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 30 g protein loaded per well, then transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 0.2% gelatin for 1 h at room temperature, incubated overnight at 4 °C with primary antibodies, washed three times with Tris-buffered saline containing Tween-20 (TBST) for 10 min each, incubated with HRP-conjugated secondary antibodies for 1 h at room temperature, and washed again three times with TBST for 10 min each. Protein bands were visualized using ECL chemiluminescence reagent and detected using a Chemi-DocTM XRS+ imaging system. Band densities were analyzed using Quantity One software, with -actin as the internal reference.

1.3 Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA in SPSS 17.0 software, followed by LSD post-hoc test for pairwise comparisons. $P<0.05$ was considered statistically significant, and $P<0.01$ was considered extremely significant.

Results

2.1 Effect of H O on TM3 Cell Proliferation

Real-time monitoring by the iCELLigence cell function analyzer showed that treatment with various concentrations of H O for 8 h extremely significantly reduced cell viability compared with the normal control group ($P<0.01$) in

a concentration-dependent manner (Figure 1 [Figure 1: see original paper]). Treatment with 150 mol/L H O decreased cell viability to approximately 75%, achieving the desired level of damage. Therefore, 150 mol/L H O was selected to establish the oxidative damage model for subsequent experiments.

Figure 1. Effects of different concentrations of H O on TM3 cell proliferation

A: Real-time monitoring of H O effects on TM3 cell proliferation by iCELLigence cell function analyzer. B: Effects of different H O concentrations on TM3 cell proliferation after 8 h treatment.

Value columns marked with "**" indicate extremely significant difference compared with the normal control group ($P<0.01$). The same applies to subsequent figures.

2.2 Effect of RES on TM3 Cell Proliferation

To investigate the protective effect of RES against H O -induced oxidative damage, we first examined the effect of RES on TM3 cell proliferation. As shown in Figure 2 [Figure 2: see original paper], treatment of normal cells with RES at 2.5, 5.0, and 10.0 mol/L for 24 h did not significantly affect cell viability compared with the normal control group ($P>0.05$). Figure 3 [Figure 3: see original paper] demonstrates that treatment of oxidative-damaged cells with RES significantly increased cell viability compared with the H O group ($P<0.05$), although levels did not return to those of the normal control group, with no significant differences among the different RES concentration groups ($P>0.05$). Therefore, 5.0 mol/L RES was selected for subsequent experiments.

Figure 2. Effects of different concentrations of RES on TM3 cell proliferation

A: Real-time monitoring of RES effects on TM3 cell proliferation by iCELLigence cell function analyzer. B: Effects of different RES concentrations on TM3 cell proliferation after 24 h treatment.

Figure 3. Effects of RES on proliferation of H O -induced oxidative-damaged TM3 cells

A: Real-time monitoring of RES effects on proliferation of H O -induced oxidative-damaged TM3 cells by iCELLigence cell function analyzer. B: Effects of RES on proliferation of H O -induced oxidative-damaged TM3 cells after 24 h treatment.

Value columns marked with "#" indicate significant difference compared with the H O group ($P<0.05$).

2.3 Effect of RES on ROS Content in Oxidative-Damaged TM3 Cells

As shown in Figure 4 [Figure 4: see original paper], H O treatment extremely significantly increased relative fluorescence intensity in normal cells (P<0.01), indicating elevated ROS content. In contrast, RES treatment extremely significantly decreased relative fluorescence intensity in oxidative-damaged cells (P<0.01), although levels remained higher than in the normal control group. These results demonstrate that RES can inhibit ROS production in oxidative-damaged TM3 cells.

Figure 4. Effects of RES on ROS content in H O -induced oxidative-damaged TM3 cells

Value columns marked with "##" indicate extremely significant difference compared with the H O group (P<0.01). The same applies to subsequent figures.

2.4 Effect of RES on SIRT1 and UCP2 mRNA and Protein Expression in Oxidative-Damaged TM3 Cells

Figure 5 [Figure 5: see original paper]-A shows that H O treatment extremely significantly decreased SIRT1 mRNA expression and extremely significantly increased UCP2 mRNA expression in normal cells (P<0.01). RES treatment of oxidative-damaged cells extremely significantly increased SIRT1 mRNA expression and extremely significantly decreased UCP2 mRNA expression (P<0.01). As shown in Figure 5-B, the protein expression patterns of SIRT1 and UCP2 mirrored their mRNA expression trends. H O treatment extremely significantly reduced SIRT1 protein expression and extremely significantly increased UCP2 protein expression (P<0.01), while RES treatment extremely significantly increased SIRT1 protein expression and significantly decreased UCP2 protein expression (P<0.01 and P<0.05, respectively).

Figure 5. Effects of RES on mRNA and protein expression levels of SIRT1 and UCP2 in H O -induced oxidative-damaged TM3 cells

A: Detection of SIRT1 and UCP2 mRNA relative expression levels by qPCR. B: Detection of SIRT1 and UCP2 protein relative expression levels by Western blotting.

Values in the same row with "**" superscript indicate extremely significant difference compared with the normal control group (P<0.01); with "##" superscript indicate extremely significant difference compared with the H O group (P<0.01); with "#" superscript indicate significant difference compared with the H O group (P<0.05).

Discussion

3.1 Establishment of H O -Induced Oxidative Damage Model in TM3 Cells

H O is commonly used as an inducer in cell oxidative damage models. However, different cell types exhibit varying sensitivity to the inducer, making concentration and treatment duration critical parameters [11-12]. Excessive concentration or prolonged treatment causes severe cell damage that is unsuitable for studying antioxidant mechanisms, whereas insufficient damage may allow cellular self-repair, confounding results. While the MTT assay is commonly used to assess cell proliferation and determine damage extent based on absorbance values, it only provides single time-point data. In contrast, the iCELLigence cell function analyzer enables real-time monitoring, providing more intuitive assessment of cell proliferation without requiring special reagents, thereby reducing errors from multiple manipulations. In this study, real-time monitoring revealed that treatment with 150 mol/L H O for 8 h reduced cell viability to approximately 75%, meeting the requirements for subsequent antioxidant experiments. Therefore, 150 mol/L H O was selected to establish the oxidative damage model.

3.2 Protective Effect of RES on Oxidative-Damaged Cells

Approximately 90% of ROS in animals originates from the mitochondrial electron transport chain, with ROS production closely linked to mitochondrial energy metabolism. Increased ROS content damages mitochondrial structure and function, which in turn promotes ROS generation, creating a vicious cycle that exacerbates oxidative damage [13-14]. Uncoupling proteins (UCPs) are transporters located in the mitochondrial inner membrane that mediate "proton leak," dissipating energy as heat [15]. UCP2, a member of the UCP family, is the primary form expressed in the reproductive system [16-17].

Numerous studies have demonstrated that UCP2 regulates ROS generation and participates in antioxidant defense in various tissues. Brand [18] proposed a negative feedback model for UCP2 regulation of ROS: when ROS content increases, certain substances produced during chemical conversion of peroxides activate UCP2 proton leak activity, causing mitochondrial membrane potential decline and limiting ROS generation through negative feedback. This suggests that during oxidative stress, UCP2 protects against oxidative damage by reducing ROS production. Similar conclusions were reported by Zhang et al. [19] and Wang et al. [20]. Zhang et al. [19] showed that heat-induced apoptosis increased ROS content and UCP2 protein expression in mouse testis. Wang et al. [20] found that UCP2 protein expression in sperm positively correlated with H O concentration, with UCP2 expression increasing to counteract elevated ROS. SIRT1, a nuclear protein, can transcriptionally inhibit UCP2 activity by binding to the UCP2 promoter, suppressing UCP2 gene expression, reducing mitochondrial inner membrane protein production, and inhibiting mitochondrial uncoupling [21].

Studies in drug-induced oxidative stress models in rats and mice have shown low SIRT1 expression, with SIRT1 activation leading to decreased UCP2 expression [8,22-23]. Based on these findings, we hypothesized that RES exerts protective effects against oxidative damage in mouse Leydig cell TM3 by modulating the SIRT1/UCP2 signaling pathway.

Our results showed that compared with the normal control group, H O treatment extremely significantly increased ROS content, extremely significantly decreased SIRT1 mRNA and protein expression, and extremely significantly increased UCP2 mRNA and protein expression. This suggests that H O -induced damage decreased SIRT1 content, which may have induced increased UCP2 expression to counteract elevated ROS. Although increased UCP2 expression should uncouple oxidative phosphorylation, reduce ATP production, and decrease ROS generation, oxidative stress persisted despite enhanced UCP2 expression, possibly because the increased UCP2 was insufficient to counteract excessive ROS production. Compared with oxidative-damaged cells, RES treatment inhibited H O -induced downregulation of SIRT1 and upregulation of UCP2, while extremely significantly reducing ROS content. This suggests that RES, as a SIRT1 activator, promotes SIRT1 expression. Activated SIRT1 inhibits UCP2 expression, which theoretically should increase ROS; however, RES treatment actually decreased ROS in oxidative-damaged cells. We speculate this may be due to negative feedback regulation of ROS by UCP2—when UCP2 expression is inhibited, ROS content initially increases, triggering UCP2-mediated negative feedback to limit ROS production.

Additionally, we observed abundant UCP2 mRNA expression in normal TM3 cells, while UCP2 protein was difficult to detect. However, UCP2 protein expression became extremely significant after H O damage. This discrepancy may be related to antibody specificity or may indicate that under physiological conditions, UCP2 mRNA in certain organs is not translated into protein, whereas pathological conditions can initiate UCP2 mRNA translation. Similar findings were reported by Fisler et al. [24], who detected abundant UCP2 mRNA in pancreas, spleen, heart, hypothalamus, lung, testis, and macrophages, but detected UCP2 protein by Western blotting only in hypothalamus, macrophages, white adipose tissue, spleen, and pancreatic islet cells.

Conclusion

The SIRT1/UCP2 signaling pathway mediates H O -induced oxidative damage in TM3 cells. RES attenuates this damage by activating SIRT1 and inhibiting UCP2 expression, with UCP2 reducing ROS generation through negative feedback regulation, thereby protecting TM3 cells from oxidative injury.

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Note: Figure translations are in progress. See original paper for figures.