



Resveratrol protects against high glucose-induced steroidogenesis and apoptosis in murine granulosa cells

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ABSTRACT

Resveratrol (Res) is a polyphenolic compound that exhibits a diverse array of biological effects. Herein, we detected the ability of Res on murine granulosa cells (GCs) against impaired steroidogenesis and apoptotic death in response to high glucose levels. Ovarian GCs were harvested from C57BL/6 mice and cultured in steroidogenic media supplemented with follicle-stimulating hormone (FSH, 30 ng/mL), Res (50 μ mol/L), and low or high glucose concentrations (5 mM or 30 mM). After culture for 24 h, cell supernatants were harvested and the levels of progesterone and estradiol therein were measured. Also, caspase-3 activity and the expression of genes associated with apoptosis and steroidogenesis were assessed. High-glucose treatment suppressed steroidogenesis in this assay system, resulting in the impaired expression of steroidogenesis-related genes including Cyp11a1, Cyp19a1, 3 β HSD, and StAR and a concomitant decrease in progesterone and estradiol production. Cells exposed to high glucose also exhibited apoptotic phenotypes characterized by Bax upregulation, Bcl-2 downregulation, and increased caspase-3 activity levels. However, Res treatment was sufficient to reverse this high glucose level-induced apoptotic and steroidogenic phenotypes with improving progesterone and estradiol production, and these maybe related the effects of Res on Cyp11a1, Cyp19a1, 3 β HSD, and StAR expressions. These data suggested that Res is well suited to overcoming the negative effects of hyperglycemia of GC functionality.

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Introduction

Diabetes mellitus (DM) is a metabolic disease that can result in adverse fertility-related outcomes in women such as amenorrhea, oligomenorrhea, abnormal folliculogenesis, ovulation failure, and infertility (1,2). Persistently elevated glucose levels can adversely impact ovarian structural characteristics and function, with particularly deleterious effects on the granulosa cells (GCs) that surround oocytes. GCs are essential producers of steroids, growth factors, and cytokines that regulate the development of oocytes. GC apoptosis influences ovarian cell growth and follicular atresia. When dysregulated, however, GC apoptosis can adversely affect ovarian follicular development. A growing body of evidence suggests that ovaries from individuals with diabetes exhibit oocytes expressing higher levels of cell cycle inhibitors and a concomitant increase in GC apoptosis and cell cycle arrest (3), consistent with the ability of hyperglycemia to induce apoptotic death in GCs (4). A recent report also has shown that higher glucose levels decrease glucose transporters level, leading to disruption of GCs glucose uptake (5). Moreover, cell cycle arrest of GCs and granulosa cell apoptosis were found in oocytes from diabetic ovaries (6).

Natural bioactive compounds have emerged as effective

treatments for reproductive complications owing to their ready availability, low levels of side effects, and efficacy (7). Prior work suggests that several bioactive compounds can benefit diabetes patients owing to their antiapoptotic, anti-inflammatory, and antioxidant properties. However, the specific mechanisms whereby these compounds function remain to be fully clarified in the context of ovarian health.

Resveratrol (3,5,49-trihydroxystilbene, Res) is a polyphenolic compound present in a range of plants and plant-derived products such as red wine that exhibits robust antiapoptotic and antioxidant properties (8,9). Prior reports have shown Res to have a beneficial effect on the proliferation, viability, and apoptosis of ovarian cells (10). Moreover, Res can impact the secretory activity of ovarian cells, decreasing plasma levels of insulin-like growth factor 1, anti-mullerian hormone (11), and insulin (12). Here, we sought to examine the impact of Res treatment on steroidogenic and apoptotic activity in murine under high-glucose conditions.

Materials and Methods

Experimental animals

Female mice (21 days old) were from the Jinan Pengyue

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experimental animal breeding Co., Ltd (Jinan, China) and were housed with free access to water and food in a facility with a 12 h light/dark schedule. The research ethics committee of Yantai Yuhuangding Hospital approved all studies, which were consistent with institutional guidelines for the care and use of laboratory animals.

Murine ovarian GC isolation and culture

Follicular growth was induced by injecting mice with 20 IU of pregnant mare serum gonadotropin (PMSG). At 48 h post-injection, mice were euthanized and ovaries were harvested, separated from fat, oviducts, and the bursa ovary. Ovaries were then transferred to DMEM/F-12 (pH 7.4) on ice supplemented with 10 mM HEPES, 1% penicillin/streptomycin, and 10% FBS. A 26-G needle was then used to harvest GCs from the antral follicle, after which the resultant cell suspension was dissociated through repeated influx and efflux using a glass pipette (13). Cells were washed two times with culture media and counted with Trypan blue to assess viability.

Experimental procedures

GCs were added to 6-well plates (10^6 cells/mL) for 24 h, after which media was exchanged for fresh growth media supplemented with appropriate reagents. To assess the effects of Res on GC steroidogenesis, cells were cultured in the presence of Res (50 μ mol/L) for 12 h in either basal media (5 mM glucose) or high-glucose media (30 mM glucose). Media was then harvested and stored at -20°C for subsequent analysis, while GCs were stored at -80°C prior to subsequent qPCR and caspase-3 activity assays (14). Experiments were conducted in triplicate.

MTT assay

Cells were added to 96-well plates containing appropriate stimuli for 24 h, after which 10 μ L of 5 mg/ml MTT reagent was added per well. Following an additional 3 h incubation at 37°C , media was exchanged for 100 μ L DMSO, after which absorbance at 570 nm with a microplate reader. All samples were analyzed in triplicate.

qPCR

Relative gene expression was assessed via qPCR, with GAPDH serving as a normalization control. Briefly, samples were analyzed in triplicate, with cDNA-free samples being assessed as controls. The $2^{-\Delta\Delta C_t}$ method was used to evaluate gene expression. A five-fold dilution series prepared with control template cDNA (1, 1:2, 1:4, 1:8, 1:16) was used to validate target and reference gene amplification efficiency. Standard curves were constructed by graphing the log-transformed input cDNA dilution factor against the mean CT value, with the slope of the resultant line then being determined.

ELISAs

Commercial ELISA kits (Beyotime Institute of Biotechnology, Shanghai, China) were used to measure levels of estradiol and progesterone in cell culture media at the end of appropriate experiments based on provided directions. All samples were run in a single assay to minimize variation, with intra-assay variation coefficients for both hormones being $< 5\%$. Samples were analyzed in duplicate.

Caspase-3 activity assay

A caspase-3 colorimetric assay kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to assess caspase-3 activity based on provided directions in order to examine the effects of Res on this cell death-related pathway.

Western blotting

Cells were rinsed with chilled phosphate buffered saline (PBS) and homogenized using lysis buffer supplemented with proteinase inhibitors. Protein quantification was performed with a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA), and equal amounts of protein (50 μ g/sample) were separated via 6-12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes that were blocked using 5% bovine serum albumin (BSA) for 2 h at 37°C . Samples were then probed overnight with polyclonal antibodies specific for Bcl-2, Bax, and β -actin at 4°C , followed by incubation for 1 h at room temperature with appropriate secondary antibodies.

Statistical analysis

Data are means \pm SD and were compared via Student's t-tests and one-way ANOVAs as appropriate, with Newman's test being used for further comparisons when ANOVAs exhibited significant results. $P < 0.05$ was the significance threshold.

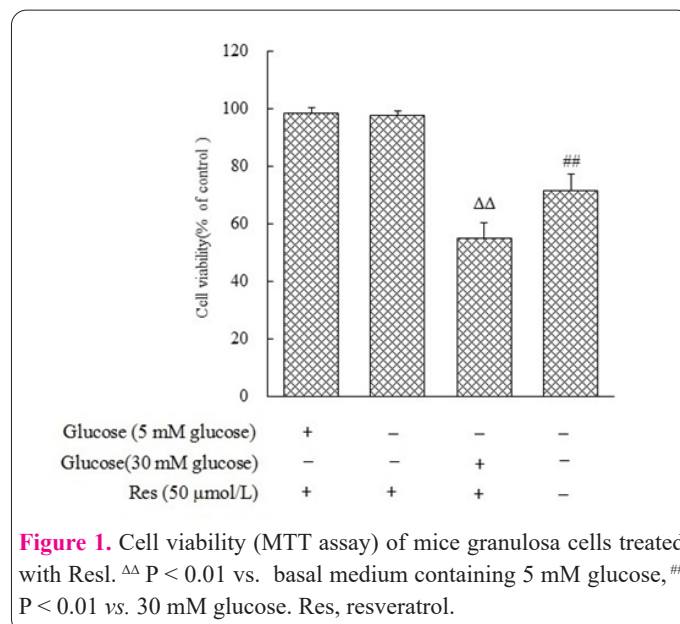
Results

The impact of Res on GC viability

When GCS were cultured in media containing high glucose concentrations (30 mM), their viability was reduced as compared to that of cells grown in basal media (5 mM glucose) in an MTT assay ($P < 0.01$; Figure 1). Res treatment (50 μ mol/L) enhanced the survival of these GCs under high-glucose conditions ($P < 0.01$; Figure 1), but had no apparent impact on the survival of cells cultured in basal media.

Res treatment protects against hyperglycemia-induced changes in hormone secretion

FSH was sufficient to induce GCs to secrete progester-



one and estradiol (Figure 2), while high-glucose conditions (30 mM) suppressed the secretion of these hormones relative to that for cells cultured in basal media ($P < 0.01$; Figure 2). Res treatment (50 $\mu\text{mol/L}$), however, improved the ability of GCs to secrete estradiol and progesterone in response to FSH when cultured under high-glucose conditions (Figure 2).

Res alters steroidogenesis-related gene expression

Consistent with the above results, high-glucose culture conditions (30 mM) resulted in the impaired expression of steroidogenesis-related genes including 3 β HSD, Cyp19a1, Cyp11a1, and StAR in the presence of FSH as compared to levels observed for cells culture in basal media (5 mM) with FSH (Figure 3). Res treatment (50 $\mu\text{mol/L}$) reversed the observed changes in the glucose-mediated downregulation of these steroidogenesis-related genes relative to levels for untreated cells ($P < 0.01$; Figure 3). Res also increased the expression of steroidogenesis-related gene expression in GCs cultured in the basal media (5 mM glucose) ($P < 0.01$; Figure 3).

Res alters GC apoptosis-related gene expression

To assess the ability of Res to protect GCs against hyperglycemia-induced cell death, we next assessed apoptosis-related gene expression and caspase-3 activity levels in these cells in the presence of high glucose levels (30 mM) without any FSH. Marked Bcl-2 downregulation was observed following high-glucose treatment for 24 h, with a concomitant increase in pro-apoptotic Bax expression. Consistently, the Bcl-2/Bax ratio declined under these conditions (Figure 4, $P < 0.01$ vs. control), while Res treatment (50 $\mu\text{mol/L}$) was sufficient to largely reverse these glucose-induced changes ($P < 0.01$ vs. glucose).

Caspase-3 activity levels were also elevated when GCs were exposed to high levels of glucose, consistent with the ability of hyperglycemia to induce apoptotic cell death. However, Res treatment (50 $\mu\text{mol/L}$) ($P < 0.05$). In contrast, Res had no effect on the expression of apoptosis-as-

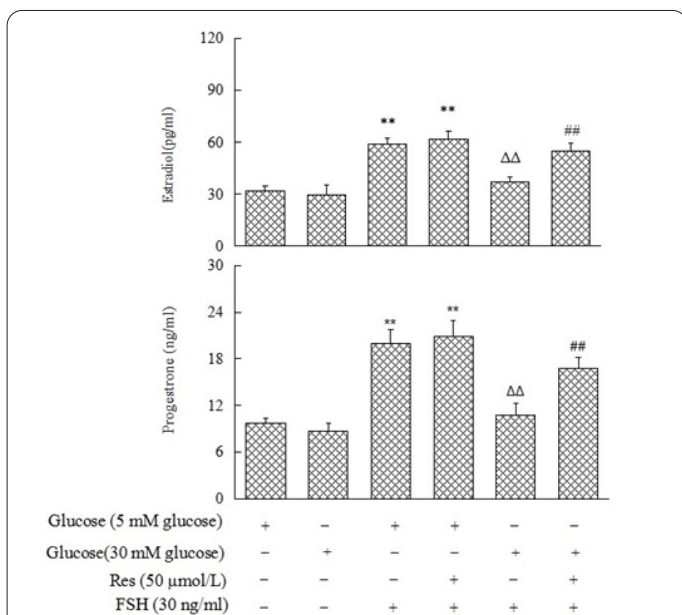


Figure 2. Effect of Res treatment on basal and FSH stimulated progesterone. ****** $P < 0.01$ vs. basal medium containing 5 mM glucose; **$\Delta\Delta$** $P < 0.01$ vs. basal medium containing 5 mM glucose +FSH (30 ng/ml), **##** $P < 0.01$ vs. 30 mM glucose+FSH (30 ng/ml). Res, resveratrol.

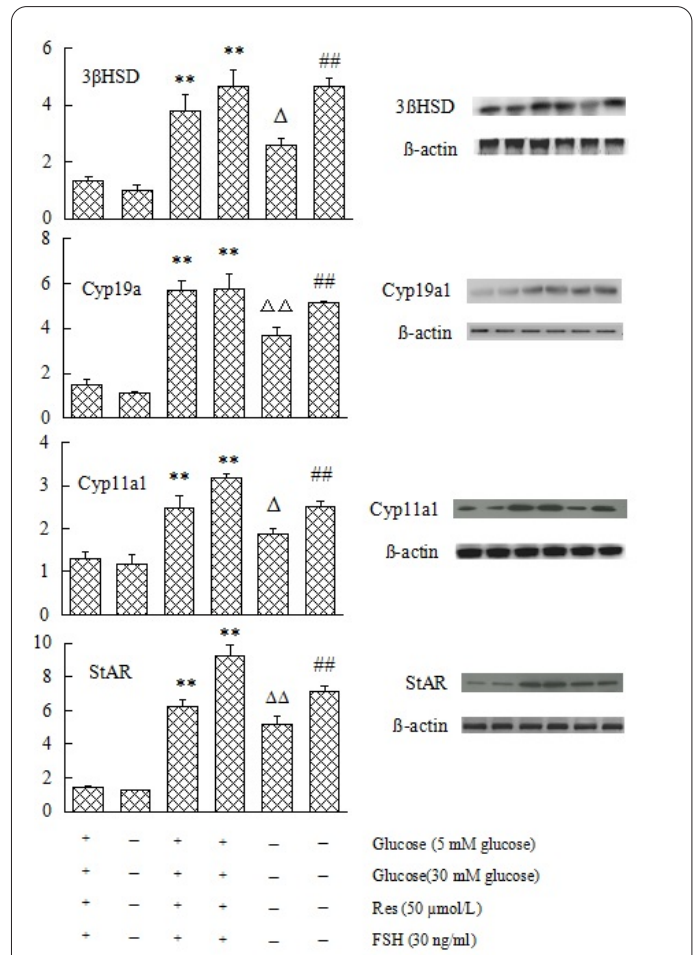


Figure 3. Effect of Res on the expression of key genes in steroidogenesis. ****** $P < 0.01$ vs. basal medium containing 5 mM glucose; **$\Delta\Delta$** $P < 0.01$, **Δ** $P < 0.05$ vs. basal medium containing 5 mM glucose +FSH (30 ng/ml), **##** $P < 0.01$, vs. 30 mM glucose+FSH (30 ng/ml). Res, resveratrol.

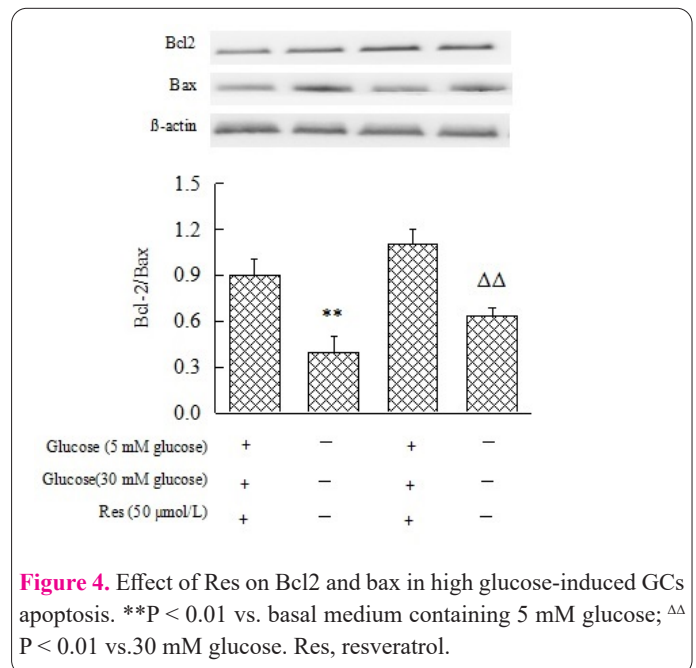
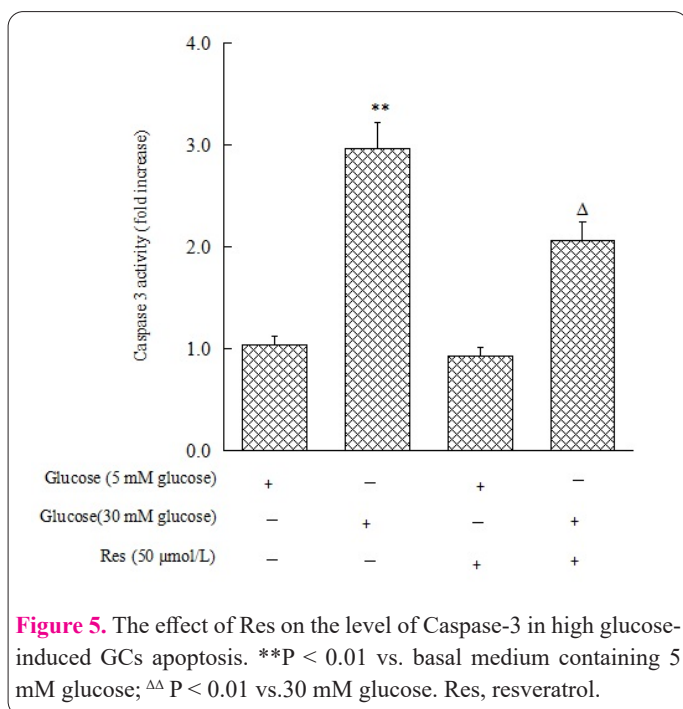


Figure 4. Effect of Res on Bcl2 and bax in high glucose-induced GCs apoptosis. ****** $P < 0.01$ vs. basal medium containing 5 mM glucose; **$\Delta\Delta$** $P < 0.01$ vs.30 mM glucose. Res, resveratrol.

sociated genes for cells cultured in basal medium (Figure 5).

Discussion

Hyperglycemia has been repeatedly demonstrated to



adversely impact female fertility (15). Res is a polyphenolic compound found in a range of plants that exerts beneficial effects on human health. Herein, we explored the ability of Res to affect steroidogenesis and apoptotic cell death-related activity in murine GCs in response to hyperglycemic conditions.

Herein, we found that hyperglycemic conditions induced apoptotic cell death in GCs characterized by Bcl-2 downregulation, Bax upregulation, and increased caspase-3 activity. When GCs were cultured in the presence of high levels of glucose for 24 h, this was sufficient to decrease the expression of steroidogenesis-related genes (Cyp11a1, StAR, 3βHSD, and Cyp19a1) and associated production of progesterone and estradiol production.

Çolakoğlu et al found that exposing primary rat GCs to elevated glucose levels was sufficient to reduce progesterone and estradiol production while downregulating 3βHSD, Cyp11a1, StAR, and Cyp19a1 at the protein level (16). Consistently, a recent study exploring the impact of type 2 diabetes *in vivo* in mice revealed a time-dependent reduction in ovarian steroidogenic enzyme expression (17). Both our results and these prior studies indicate that high glucose levels can induce GC apoptosis and reduce steroidogenic enzyme expression, thereby causing ovarian dysfunction and negative reproductive outcomes such as amenorrhea, oligomenorrhea, PCOS, and/or infertility.

Overall, our results suggest that the treatment of GCs exposed to hyperglycemic conditions with Res was sufficient to largely obviate the negative impact of high glucose levels on steroidogenesis and apoptotic cell death. Res-treated cell viability was elevated as compared to that of untreated cells in the presence of 30 mM glucose, with a concomitant drop in caspase-3 activity and Bax expression together with increases in Bcl-2 expression and a drop in the Bax/Bcl-2 ratio. These results thus strongly indicate that Res can protect FCs from hyperglycemia-mediated cytotoxic cell death.

Multiple factors may contribute to the protective benefits of Res treatment in the context of hyperglycemia. When present at high levels, glucose can act as a serum osmolyte that impairs absorption within the extracellular

space (18). Diabetes and associated hyperglycemia can increase cellular susceptibility to hypertonic stress and associated shrinkage (19). Shrinkage, in turn, can function as a form of mechanical stressor that induces endoplasmic reticulum (ER) stress, thereby promoting cytochrome release, caspase-3/12 activation, and apoptotic cell death (20).

By influencing unfolded protein response (UPR)-related gene expression, Res can modulate ER stress, which has been identified as a promising target for therapeutic intervention in individuals with PCOS (21). Res can also protect against hyperglycemia-induced renal tubular cell apoptosis in the context of DN by inhibiting ER stress (22).

Overall, these results suggest that Res treatment may be able to improve GC steroidogenic activity in hyperglycemic contexts. Specifically, Res enhanced the GC-mediated production of progesterone and estradiol while simultaneously increasing the expression of Cyp11a1, StAR, 3βHSD, and Cyp19a1. In contrast, the addition of Res to basal GC culture media supplemented with GSH and low levels of glucose (5 mM) had no impact on estradiol or progesterone production relative to GCs culture in FSH-free Res-containing media, with similar changes being observed for the expression of the 3β-HSD and Cyp19a1 genes. These results suggest that Res has no impact on GC steroidogenic pathway activity at baseline, whereas it can enhance FSH-driven steroid production in the context of hyperglycemia.

Given the role of Res as an inhibitor of oxidative stress, inflammation, and hyperhomocysteinemia (23-30), it may also serve to counteract the negative effects of hyperglycemia as observed in the present study, thereby reversing the impaired GC steroidogenesis observed following high-glucose treatment. However, further research will be essential to clarify the molecular mechanisms governing these Res-mediated improvements in GC function in order to better explore the potential clinical utility of this natural compound.

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Conflicts of interest

The authors declare no conflicts of interest.

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