Mechanisms of Yangjing capsule in Leydig cell apoptosis and testosterone synthesis via promoting StAR expression

Dalin Sun, Weihang Dong, Baofang Jin, Guanghui Chen, Bin Cai, Weimin Deng, Yugui Cui, Yihan Jin

1 Andrology Department of Integrative Medicine, Zhongda Hospital, School of Medicine, Southeast University, Nanjing 210009, China
2 Medical College of Qinghai University, Xining 810001, China
3 Hebei Provincial Hospital of Traditional Chinese Medicine, Shijiazhuang 050011, China
4 State Key Laboratory of Reproductive Medicine, Clinical Center of Reproductive Medicine, First Affiliated Hospital, Nanjing Medical University, Nanjing 210029, China

*Correspondence to: Baofang Jin, Andrology Department of Integrative Medicine, Zhongda Hospital, School of Medicine, Southeast University, 87# Dingjia Bridge, Nanjing 210009, China, Email: hexiking@126.com.
Abstract
The present study aims to investigate the roles of StAR in Yangjing Capsule (YC) induced anti-apoptotic effects on Leydig cells and the related mechanism. Leydig tumor cells (MLTC-1) were cultured and treated with YC, and immunofluorescence assay was performed to examine the expression of StAR; furthermore, luciferase reporter assay was conducted to evaluate the impact of YC on StAR promoter; next, MLTC-1 cells were treated with StAR siRNA, and flow cytometry was carried out to examine the effect of StAR siRNA on the apoptosis of the cells; furthermore, qRT-PCR and western blot methods was used to determine the expression of StAR and apoptosis related molecules Bcl-2, Bax and Caspase-3 on both mRNA and protein levels in different groups; finally the secretion of testosterone in different groups was examined by radioimmunoassay. We observed that the YC can increase the expression of StAR in a dose-dependent manner, and YC can activate the promoter of StAR; moreover, transfection of StAR siRNA can block YC induced anti-apoptotic effects and increased production of testosterone. In conclusion, our results suggested that YC might suppress the apoptosis of MLTC-1 cells and enhance the production of testosterone through regulating the expression of StAR.

Key words: YC, Leydig cell, apoptosis, testosterone, StAR
Introduction

It is well known that adult Leydig cells (ALCs) distribute in the interstitial compartment of the testis, and Leydig cells are neighbors of the testicular seminiferous tubules (1, 2). In adult males, Leydig cells are of great importance, because the synthesis of androgen and testosterone most occur in Leydig cells (3), and the levels of androgen were correlated with the male phenotype, spermatogenesis development and maintenance (4). Thus, the proliferation and differentiation of Leydig cells are responsible for testosterone production (4). It has been reported that the intratesticular hormones, including desert hedgehog and platelet-derived growth factor can induce the proliferation and differentiation of Leydig cells (5). Previous study demonstrated that calretinin protects Leydig cells by promoting Leydig cell viability and proliferation (6). Several other factors, including Smad4, CDKN1A, ALK3 and MEF2 may also play important roles in Leydig cell proliferation and differentiation (7-9).

The steroidogenic acute regulatory protein (StAR) is a nuclear encoded mitochondrial protein. Results of previous studies indicated that StAR may serve as a key regulator for the synthesis of steroid hormone and development of steroidogenic cells (10). Additionally, StAR may also facilitate the process of cholesterol translocation from mitochondrial outer membrane to inner membrane (11). It has been reported that the steroidogenic machinery such as luteinizing hormone receptor (LHCGR), the cholesterol transporting unit—high-density lipoprotein receptor (SCARB1), CYP11A1, 3β-HSD1, CYP17A1 and also StAR are involved in the progress of Leydig cell differentiation and proliferation (12).

Yangjing capsule is primarily composed of Herba Epimedii Brevicornus, Radix Rehmanniae Preparata, Rhizoma Polygonati Sibirici, Placenta Hominis, and Angelica sinensis (13). In our previous studies, we have reported that Yangjing Capsule (YC) has the benefits for the reproductive system by increasing the production of androgen, maintaining the balance of the hormones, and reducing the apoptosis of the Leydig cell (14). However, the underlying mechanism of the protective roles of YC in Leydig cell development remain unclear. In the present study, we aimed to explore the roles of StAR in YC induced anti-apoptotic effects on Leydig cell apoptosis. Our results may provide theoretical basis for the clinical application of YC.
Materials and methods

Cell line and chemicals. The mouse Leydig tumor cells (MLT C-1) were purchased from the Cell Institute of Shanghai (Shanghai, China) and incubated with RPMI-1640 (Thermo Fisher Scientific) containing 10% fetal bovine serum humidified with 5% CO₂ at 37 °C. The YC was prepared according to the method explained in our previous publications (15, 16). Furthermore, Cells were treated with low (0.01 mg/ml), medium (0.1 mg/ml) and high concentrations (1 mg/ml) of YC. 0.1 U/mL hCG was used as the positive control.

Confocal fluorescence microscopy for examining the expression of StAR expression. MLT-C-1 cells with cultured until it reach the 70% confluence. Then, the YC were added to MLT-C-1 cells and incubated for 24 h. The cells were then collected and fastened with 4% PFA for 15 min. In addition, we incubated the cells with primary antibodies (Abcam, MA, USA) and then with FITC-conjugated secondary antibodies (Abcam, MA, USA) for 1 h at room temperature. Subsequently, the stained cells were detected by confocal microscopy (Thorlabs) according to the manufacturer’s protocols (17).

Luciferase reporter assay. MLT-C-1 cells (5×10³) were seeded onto the 96-well plates and incubated in RPMI-1640 medium with 5% CO₂ at 37 °C to reach 70% confluence. Then, pGL3-StAR-Luc plasmids (containing sequence of StAR promoter) and phRL-TK internal plasmids were transfected into MLT-C-1 cells with Lipo3000 (Thermo Fisher, MA, USA) and incubated for 24 h. Subsequently, the YC (1 mg/ml) and hCG (0.1 U/ml) were added to the transfected cells and incubated for 48 h in 5% CO₂ at 37 °C. Additionally, the dual-luciferase reporter assay kit (Promega) was applied for determination of the luciferase activity, and the activity of the luciferase was normalized to the activity of the Renilla.

Cell transfection. MLT-C-1 cells with 60% confluence were transfected with si-StAR using Lipo3000 and incubated for 24 h in RPMI-1640 medium at 37 °C with 5% CO₂. Then, the medium was changed to serum free medium. At the same time, the YC (1 mg/ml) was added to si-StAR groups and incubated for 24 h in RPMI-1640 medium at 37 °C with 5% CO₂.

Flow cytometry analysis for cell apoptosis. MLT-C-1 cells were transfected with si-StAR vector and treated with YC for 24 h at 37 °C in 5% CO₂. Then, cells (5×10⁶) were stained with propidium iodide (10 μg/mL; Sigma, USA) and Annexin V-FITC (50 μg/mL, BD, USA)
for 15 min at room temperature in dark. Subsequently, the apoptosis of the cells were examined and analysis by a flow cytometer (FACScan; BD Biosciences).

qRT-PCR. qRT-PCR was performed to evaluate the expression of StAR. After 24 h of incubation, the total RNAs were extract using Trizol reagent (Thermo Fisher). After that, the concentration of RNA was determined by Nanodrop2000 (Thermo Fisher). The GoTaq® DNA Polymerase (Thermo Fisher) and M-MLV RT (Thermo Fisher) were used for cDNA synthesis and PCR reaction. cDNA was synthesized at 95 °C for 12 min. The PCR reaction conditions were as followed: 96 °C for 5 min, 95 °C for 30 sec, 63 °C for 30 sec and 75 °C for 1 min for 36 cycles. Furthermore, the primers of StAR (Forward/Reverse: 5’CGAGGAGACAGAAATGGGTCA3’/5’TCCACTGCTTTGCTGTGAAT3’) were employed for PCR reactions. The expressions of mRNAs were normalized to β-actin (Forward/Reverse: 5’AAGGAGCCCCACGAGAAAAAT3’/5’ACCGAACTTGCATTGATTCAG3’). The 2−ΔΔCq method was used for quantification (18).

Western blot. Western blot assay was carried out to examine the protein expression of StAR, Bax, Bcl-2 and Caspase-3. The total proteins were isolated from transfected MLTC-1 cells with RIPA lysis buffer (Thermo Scientific™) and BCA Kit (Beyotime Biotechnology, China) was applied to determine the concentration of the proteins in each sample. Subsequently, the protein samples (2 μl/per lane) were separated via 15% SDS-PAGE. Then, the proteins were transfered onto PVDF membranes (Millipore, Billerica, MA, USA) and blocked with 5% non-fat milk for 2 h, and incubated with the primary antibodies (anti-StAR, -Bax, -Bcl-2, -caspase and -β-actin, all purchased from Abcam, Cambridge, MA, USA) overnight. On day 2, the membranes were washed and incubated with the secondary antibodies for 45 min at room temperature. Next, ECL kit (#32209, Suzhou Biotsith Bioscience Co., Ltd, Suzhou, China) was applied for visualization and the image was captured with ChemiDoc™XRS+ imaging system (Bio-Rad, Hercules, CA, US) and analyzed using ImageJ.

Testosterone production. The concentration of testosterone was examined using Iodine [125I] and the Testosterone Radioimmunoassay Kit (Cisbio) according to the manufacturer’s
Statistical methods. Each experiment was performed in triplicate and all quantitative data were displayed as mean ± standard deviation (SD). The statistical analysis was conducted with GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA, USA). Student-T test was performed for two groups comparisons and one-way ANOVA analysis was applied for multiple groups comparisons. p<0.05 was regarded as the statistical significance.

Results

YC promoted StAR expression. First, MLTC-1 were treated with low (0.01 mg/ml), medium (0.1 mg/ml) and high concentrations (1 mg/ml) of YC and incubated for 24 h, and the expression of StAR was determined by immunofluorescence methods. As shown in Figure 1 (200 ×), YC treatment induced increased expression of StAR in a dose-dependent manner.

YC activate the promoter region of StAR. Next, The plasmids containing StAR promoter sequence were constructed and luciferase reporter assay was conducted to determine whether YC can activate the promoter of region of StAR. It was observed that the activity of the luciferase in the STAR group was significantly higher than the control group (Figure 2, p<0.01), suggesting that YC may have the potential impacts on StAR expression by activating the promoter region of StAR.

YC may inhibit the apoptosis and the expression apoptosis-related protein through regulating the expression of StAR in MLTC-1 cells. Moreover, Si-StAR was applied to study the roles of StAR in cells apoptosis of MLTC-1. In addition, YC (1 mg/ml) was added to MLTC-1 cells. The non-treated groups were considered as the blank control (E) groups and the negative control (NC) was transfected with empty plasmids. As shown in Figure 3A, the expression of StAR was dramatically decreased in si-StAR treated groups compared as the E groups as well as NC groups. Moreover, in Figure 3B, the cell apoptosis rates were measured by flow cytometry. We observed that Si-StAR induced increase in the apoptosis of MLTC-1 cells compared with E and NC groups. Additionally, YC treatment can inhibit the apoptosis of MLTC-1 cells, while transfection of Si-StAR can partially block YC induced anti-apoptotic effects(p<0.001).
Furthermore, in Figure 4, the results indicated that the expression of Bax and caspase-3 were significantly increased and Bcl-2 was dramatically decreased in si-StAR groups compared to E and NC groups. On the other hand, YC treatment decreased the expression of Bax, caspase-3 and increased the expression of Bcl-2. Moreover, transfection of StAR siRNA partially affected YC induced alternation in the expression of Bcl-2, Bax and Caspase-3.  

YC may increase the production of testosterone through regulating the expression of StAR. Finally, the production of testosterone in each group was examined. The results indicated that the production of testosterone was lower in si-StAR group than E and NC groups (Figure 5, p<0.01). On the other hand, YC treatment can increase the production of testosterone in MLTC-1 cells, while transfection of Si-StAR can decrease the production of testosterone in YC treated MLTC-1 cells (p<0.01). (Figure 5).

Discussion

Leydig cells are able to secrete testosterone which plays essential roles in spermatogenesis (4). Previous studies indicated that human chorionic gonadotropin (hCG) excreted by the placenta can promote the transmission of cholesterol to the inner mitochondrial membrane through StAR dependent signaling pathway (19). Therefore, StAR is important in lipid metabolism in Leydig cells (19). We previously reported that YC can affect the steroidogenesis and apoptosis of Leydig cells (13,14), however, the underlying mechanism has not yet been discussed. In the present study, we observed that YC can increase the expression of StAR in a dose-dependent manner, and transfection of Si-StAR can partially block YC induced anti-apoptotic effects. Taken together, our results confirmed the previous conclusion that YC can induce anti-apoptotic effects on Leydig cells, and more importantly, we first reported that the YC induced anti-apoptotic effects was StAR-dependent.

The activation of StAR can be affect by various factors, including increased production of reactive oxygen species(ROS) induced by cAMP analogs, and phosphorylation of ERK1/2 in the Leydig cells (20). Furthermore, results of previous studies showed that stress was associated with the mitochondrial function of Leydig cells referring the basal oxygen.
consumption, cytochrome c, transition pores formation and caspase-3 pathway activation (21), which can lead to the increased apoptosis of Leydig cells (21). Caspase-3 is also the necessary for the process of Leydig cell apoptosis induced by ethane dimethanesulfonate (22). In our present study, we found that YC can decrease the expression of Bax, caspase-3 and increase the expression of Bcl-2. while, transfection of StAR siRNA can partially affect YC induced alternation in the expression of Bcl-2, Bax and Caspase-3. Our results suggested that YC can alleviate the apoptosis of Leydig cells through StAR/Caspase-3 signaling pathway.

In conclusion, we first reported that YC might induce production of testosterone and inhibit cell apoptosis of MLTC-1 via increasing the expression of StAR. Therefore, the identification of StAR as a target of YC might provide potential and promising ways in the treatment of male reproductive diseases.

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Conflict of Interest
The authors declare no conflict of interest.

Supplementary Materials
The online version of this article contains supplementary materials.
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**Figure legends**

**Figure 1 YC promoted StAR expression**
After treatment with YC for 24 h, the expression of StAR was determined by immunofluorescence. Upper panels: merged image with StAR stained in green and DAPI stained in blue; Lower panels: StAR stained in green. Control group: non-treated group; 0.01 mg/ml group: cells treated with 0.01 mg/ml YC; 0.1 mg/ml group: cells treated with 0.1 mg/ml YC; 1 mg/ml group: cells treated with 1 mg/ml YC. (Color figure can be accessed in the online version.)

**Figure 2 YC promoted the activate the promoter of StAR**
The luciferase reporter assay was conducted to investigate the impact of YC on StAR expression. Cells were treated with YC and HCG respectively. Compared with Control group, the luciferase activity of StAR in YC group and HCG group was significantly increased. **p<0.01

**Figure 3 StAR inhibited the apoptosis of MLTC-1 cells in vitro**
(A) Expression of StAR by qRT-PCR and western blot after si-StAR. (B) Cell apoptosis rate by flow cytometry after si-StAR and YC treatment. E group: control group; NC group: cells treated with control plasmids; SI group: cells treatment with si-StAR; E+YC group: cells treated with 1 mg/ml YC; SI+YC group: cells treated with both si-StAR and 1 mg/ml YC. *p<0.05 vs. SI group; **p<0.001 vs. SI; #p<0.05 vs. E+YC group, ###p<0.001 vs. E+YC groups. &p<0.05 vs. SI+YC group; &&p<0.01 vs. SI+YC groups; &&&p<0.001 vs. SI+YC group. (Color figure can be accessed in the online version.)

**Figure 4 StAR inhibited the expression of apoptosis-related proteins in MLTC-1 cells in vitro**
Expression of StAR, Bax, Caspase-3 and Bcl-2 after si-StAR and YC treatment by western blot. E group: control group; NC group: cells treated with control plasmids; NC+YC group:
cells treated with 1 mg/ml YC + control plasmids; SI group: cells treatment with si-StAR; SI+YC group: cells treated with both si-StAR and 1 mg/ml YC. *p<0.05 vs. E group; **p<0.01 vs. E group; ***p<0.001 vs. E group.

**Figure 5 Facilitated testosterone production by YC**

After si-StAR and YC treatment, the testosterone concentration was measured by radioimmunoassay. E group: control group; NC group: cells treated with control plasmids; SI group: cells treatment with si-StAR; E+YC group: cells treated with 1 mg/ml YC; SI+YC group: cells treated with both si-StAR and 1 mg/ml YC. *p<0.05 vs. SI group; ***p<0.001 vs. SI; #p<0.05 vs. E+YC group, ###p<0.001 vs. E+YC groups. &p<0.05 vs. SI+YC group; &&p<0.01 vs. SI+YC groups; &&&p<0.001 vs. SI+YC group.
Fig. 1.
Fig. 2.

![Graph showing the relative luciferase activity for StAR promoter-Luc with treatments: Control, YC (1mg/ml), and HCG (0.1U/ml).](image)

The relative luciferase activity

- Control
- YC (1mg/ml)
- HCG (0.1U/ml)
Fig. 3.
Fig. 4.

30KD StAR
21KD Bax
32KD caspase-3
26KD Bcl-2
42KD β-actin

YC (1 mg/ml)  -  -  +  -  +
SI          -  -  -  +  +
NC          -  +  +  -  -

Relative protein expression

StAR  BAX  Caspase-3  Bcl-2

E  NC  NC+YC  SI  SI+YC
Fig. 5.