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Original Research Article

Yangjing capsule attenuates cyclophosphamide-induced deficiency of testicular microcirculation in mice

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Abstract

Purpose: *To explore the protective effects of Yangjing capsule (YC) on testicular microcirculation in a mouse model of deficiency of testicular microcirculation.*
Methods: *Immunohistochemistry was applied to determine the effects of YC on microvascular density of mice. The protein level of CD34 and vascular endothelial growth factor A (VEGF A) was measured by western blot. The viability of Testicular cell line (TM4 cells) was examined by CCK-8 assay.*
Results: *Histopathological changes demonstrated that CP-induced decrease of microvascular density of the mice was rescued by YC dose-dependently ($p < 0.5$). Western blot data showed that the protein levels of CD34 and VEGF A in CP group were significantly decreased, but dose-dependently increased by YC, respectively, following co-administration of CP + YC, compared with those in CP group ($p < 0.5$). The results from CCK-8 assay showed that the cell viability of TM4 cells increased with the amount of YC administered, and that high concentrations of YC (0.1 and 1 mg/mL) showed significant effects ($p < 0.5$). Moreover, YC showed little effect on VEGF A mRNA and protein expression in TM4 cells.*
Conclusion: *YC may be considered an alternative therapeutic agent for the management of testicular microcirculation disease. However, further studies are required to ascertain this.*

Keywords: *Yangjing Capsule, Testicular microcirculation, Cyclophosphamide, Vascular endothelial growth factor A*

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INTRODUCTION

Testicular microcirculation is featured with a high-amplitude vasomotion and a low capillary pressure [1]. It possesses a series of temporal variation for complicated physiologic or pathophysiologic disturbances [1-3]. Testicular microcirculation play key roles in transporting nutritive compounds and removal of cellular waste products in the testis [4-5]. Testicular tissue microcirculation had a close relationship with sperm production and sperm function [6-8]. Abnormal testicular microcirculation might cause male infertility, especially in those with weak sperm [9].

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Vascular endothelial growth factor (VEGF) is crucial regulator of phenotypic behaviors of vascular endothelial cells [10]. VEGF participates in angiogenesis, and could stimulate growth and development of vascular endothelial cells [11]. VEGFA, as the most efficient angiogenic factor in the VEGF family, has a crucial effect on embryonic lethality [12]. The downregulation of VEGFA in vascular endothelial cells induced postnatal mortality for vascular degeneration, which suggested an important role of VEGFA in vascular homeostasis [13]. Moreover, depletion of VEGFA isoforms mediates the expression of apoptotic testicular genes in mice, induces subfertility, decreased the number of sperms [14]. Meanwhile, it is known that VEGFA is involved in the chemotherapy of breast cancer cells [15]. However, whether VEGFA participates in the effects of YC on CP induced deficiency of testicular microcirculation is still lacking.

Recently, traditional Chinese medicine (TCM) was shown to have a huge advantage in the treatment of male infertility [16]. Yangjing capsule (YC) is a type of TCM [17]. It plays key roles in stimulating kidney activity [18]. YC could increase the number of sperm and sperm vitality for patients with male infertility [19]. YC promotes testosterone progression via steroidogenic enzymes and mediates male infertility and sexual dysfunction [20]. In previous research, it was indicated that YC could promote sperm concentration and motility in infertile males [17]. In addition, the YC might enhance androgen synthesis and hormonal balance [18]. However, whether YC affect the expression of VEGFA protein and testicular microcirculation remains unclear.

In the present study, the roles of YC in CP-induced deficiency of testicular microcirculation and expression of VEGFA was investigated. These findings may provide a novel strategy for CP-mediated deficiency of testicular microcirculation.

EXPERIMENTAL

Animals and treatments

Adult male (8 - 10 weeks old) Balb/c mice (weighing 20 ± 2 g) were acquired from Model Animal Research Center of Nanjing University (Nanjing, China). This study was approved by the Research Ethics Committee of Nanjing Medical University, China (20160623). This study strictly observed the principles of the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1985). The mice were then randomly classified into: control, cyclophosphamide (CP, Pude Mecicine, Nanjing, China), CP + YC with 3 different doses (630, 1260, and 2520 mg/kg), with 12 mice in each group. For the first 7 days, the mice in groups treated with CP were injected intraperitoneally with CP (50 mg/kg/day). In addition, mice in the YC treatment groups were intragastrically administered with YC (630, 1260, and 2520 mg/kg/day) for consecutive 30 days. The animals' general health was observed daily. Finally, the testes tissues were rapidly excised and weighed followed by storing in liquid nitrogen or applying for the isolation of RNA and protein.

YC extraction and cell culture

YC extract was obtained as described previously [21,22]. YC was extracted from Yinyanghuo, Muli, Wangbuliuxing ect., and diluted with 333 mL of distilled water. Then the compounds were ultrasonicly extracted for 45 min. The supernatants were collected at 13,000 g at 4 °C for 30min to 100 mg/mL of the crude herb.

TM4 cell lines were provided by ATCC (Rockville, MD, USA). Cells were incubated with DMEM, supplemented with 10 % FBS and 1% penicillin/streptomycin in 5% CO_2 at 37°C for 24 h. Cells were subjected to 0, 0.01, 0.1, and1mg/mL YC in 5% CO_2 at 37°C for 24 h. After the above different treatments, TM4 cells were collected for the use of the following experiment.

Immunohistochemistry

Testis were embedded in paraffin. The sections were cultured a citrate buffer. The sections were subjected to primary antibodies against CD34 (Dako, Glostrup, Denmark) and then to the secondary antibody. Thereafter, the slices were incubated with DAB. Immunostaining was confirmed with a light microscope (K5007, Dako).

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNAs were collected from tissues and reversely transcribed into cDNA with PrimeScript RT Master Mix. qRT-PCR was conducted as previously described [18]. The relative expression was determined with $2^{-\Delta\Delta Ct}$ method. GAPDH acted as an internal control. The the sequences of the primers used were as followed: VEGFA, forward: 5'-ATCCAATCGAGACCC TGGTG-3' and reverse: 5'-ATCTCTCCTATGTG CTGGCC-3'; GAPDH, forward: 5'-TGGGCTAC CGTGTTCCATC-3' and reverse: 5'-AGGTTGGT GAAGAAGTCGCA-3'.

Western blot

The testes tissues or cells were lyzed with RIPA lysis buffer. Protein was isolated by 12% SDS-PAGE and moved onto PVDF membranes (Millipore) and incubated with specific antibodies for GAPDH (ab181602, 1: 10000, Abcam, USA), CD34 (ab81289, 1: 10000, Abcam, USA) and VEGFA (ab52917, 1: 10000, Abcam, USA) at 4°C overnight and then with secondary antibody (ab6721, 1: 5000, Abcam, USA). GAPDH served as loading control.

Cell viability

CCK-8 was conducted to determine the cell viability after treatment with YC (0.01, 0.1 and 1 mg/mL). Cells ($2x10^3$ cells/well) were plated into 96-well plates. After that, CCK-8 kit was supplemented with 10 μL of CCK8 solution. The absorbance rate at the wavelength of 450 nm was determined with a microplate reader (Bio-Rad Laboratories, Inc.).

Statistical analysis

Data are presented as mean ± standard deviation (SD, n = 3). Difference between various groups were determined with one-way ANOVA. $P < 0.05$ was deemed statistically significant.

RESULTS

Effect of YC on microvascular density in mice

Testicular micro vessels were closely distributed in microtubules. As shown in Figure 1, compared with control group (Figure 1 A), the intensity of CD34 staining in CP group was decreased (Figure 1 B); compared with CP group, YC dose-dependently (630, 1260 and 2520 mg/kg) increased the intensity of CD34 staining in CP + YC groups (Figure 1C-E). In addition, the intensity of CD34 staining in CP + YC group (2520 mg/kg) was similar with that of control group. A previous study suggested that immunoexpression of CD34 was used to analyze testicular microvascular density [23]. To further clarify the effects of YC on the microvascular density of the mice, we examined the expression of CD34 protein levels in each group of testicular tissues as well. The western blot result showed that CD34 protein level in control group was significantly lower; compared with CP group, YC dose-dependently (630, 1260 and 2520 mg/kg) increased the CD34 protein level in CP + YC groups; in addition, the CD34 protein level in CP + YC group (2520 mg/kg) was similar with that of control group (Figure 2 A), which showed a protective role of YC in CP induced decrease of microvascular density of mice.

Effect of YC on expression of VEGFA in mice testis

The qRT-PCR results indicated that the expression of VEGFA in the CP group and control group was of no significant difference, which was paralelled with YC (630, 1260, and 2520 mg/kg) treatment group (Figures 3 A). Interestingly, western-blot results suggested that compared with control group, CP significantly decreased VEGFA protein levels, which were dose-dependently reversed by YC treatment, and the protein level of VEGFA in CP + YC group (2520 mg/kg) was similar with that of control group (Figure 3 B and C).

YC increased TM4 cell viability

CCK-8 assay was performed to examine TM4 cell viability. At 48-hour post treatment of YC extract (0.01, 0.1 and 1 mg/mL), the cell viability of TM4 cells was increased in a dose-dependent manner, and high doses (0.1 and 1 mg/mL) of YC significantly increased the cell viability (Figure 4).

Effect of YC on expressions of VEGFA in TM4 cells

When TM4 cells were treated with YC at different concentrations (0.01, 0.1 and 1 mg/mL), we examine the expression of VEGFA in TM4 cells. The mRNA expression level of VEGFA was not changed even with gradually increased concentration of YC (Figure 5 A). Similarly, western blotting results indicated that VEGFA protein expressions were also not changed with the increase of the YC concentration (Figures 5 B and C).

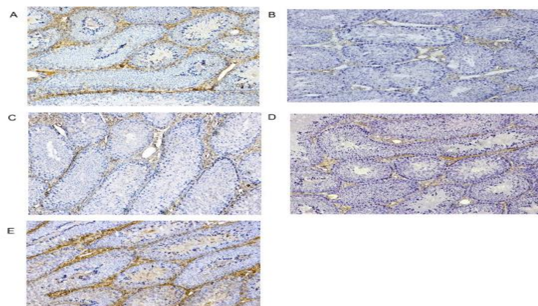

Figure 1: YC rescued CP induced decrease of CD34 staining in mice testis. The intensity of CD34 staining was lower in CP group (B) than in control group (A), which was increased dose-dependently by YC (630, 1260 and 2520 mg/kg) (C - E)

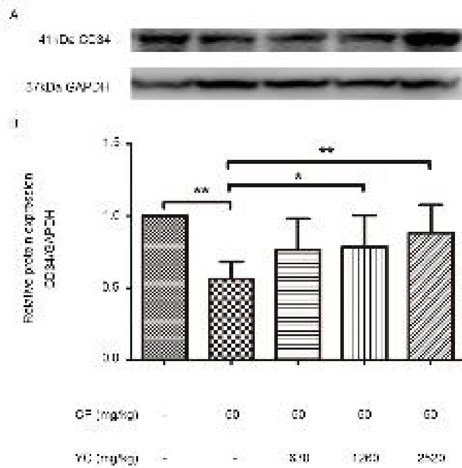

Figure 2: YC rescued CP induced decrease of CD34 protein level in mice testis. The protein level of CD34 was lower in CP group than in control group, which was increased dose-dependently by YC (630, 1260 and 2520 mg/kg) (A). The statistical data are also presented (B); *$p < 0.05$, **$p < 0.01$

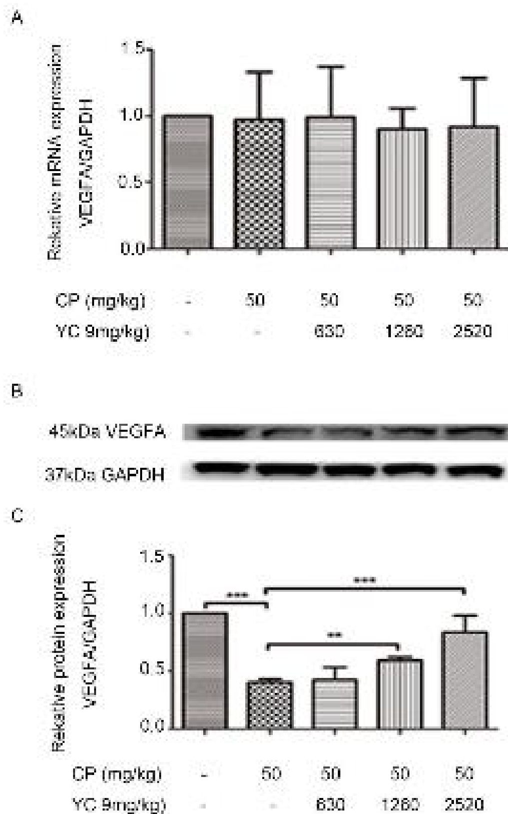

Figure 3: Effects of YC on the mRNA and protein level of VEGFA in mice testis. Neither CP nor YC affected the mRNA level of VEGFA (A). The protein level of VEGFA was lower in CP group than in control group, which was increased by YC (630, 1260 and 2520 mg/kg) in a dose-dependent manner (B and C); **$p < 0.01$

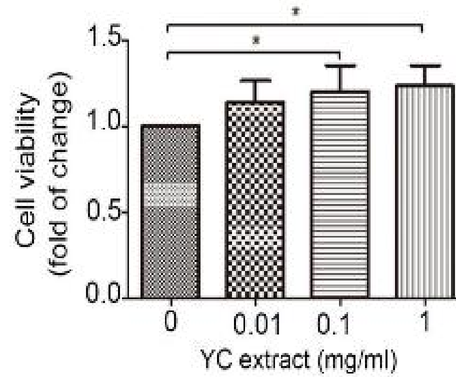

Figure 4: Effects of YC on TM4 cell viability. YC (0.01, 0.1 and 1 mg/mL) improved the cell viability of TM4 in a concentration-dependent manner compared to control group.

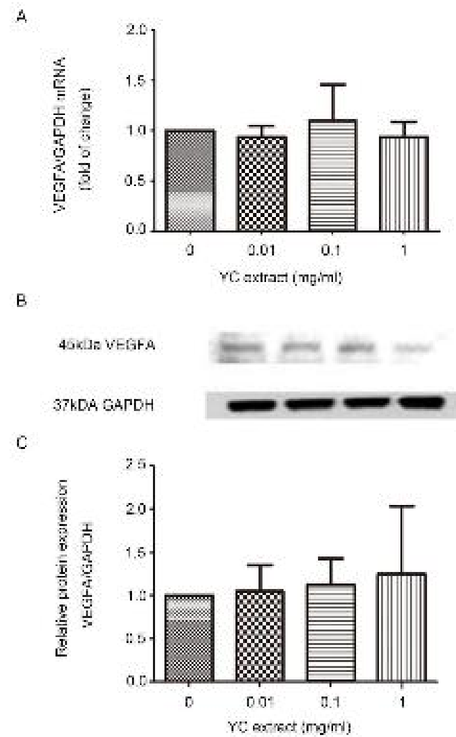

Figure 5: Effects of YC on VEGFA expression in TM4 cells. qRT-PCR suggested that the mRNA expression level of VEGFA was not changed with the treatment of YC (A). Similarly, western blotting results indicated that VEGFA protein expression changes were similar with that of mRNA (B and C)

DISCUSSION

So far, low sperm count or poor motility and quality of sperm induced male infertility still affected male (24). Testicular microcirculation induced change of spermatogenesis is a common cause for male infertility [25]. Usually, infertile males are treated with hormones or

empiric medical treatment, but the corresponding therapeutic effect is often not satisfactory [8]. Traditional Chinese formulations have achieved great advantages in the treatment of male infertility [17]. However, their molecular mechanisms of action on testicular microcirculation in infertile males remain unclear. In this study, we studied the roles of YC in testicular microcirculation in a mouse model whose dysregulation of testicular microcirculation was induced by CP.

Vascularization is an important part of microenvironment for the testis [4]. Increased microvessel density is a crucial factor in many physiologic and pathologic conditions [4]. In this study we found that administration of CP significantly decreased micro vessel density compared with the control group which was evidenced by decreased IHC staining for CD34 as well as decreased CD34 mRNA and protein levels. Interestingly, compared with CP group, YC significantly dose-dependently increased CD34 staining as well as in decreased CD34 mRNA and protein levels in testis sections of mice, indicating the protective effect of YC on micro vessel density and the development of blood vessels to improve testicular microcirculation in mice exposed to CP. The results are paralleled with Arena et al study [23].

In the current study, results indicated that VEGFA protein but not mRNA level was significantly decreased by CP, which was significantly upregulated by YC in mice testis, suggesting the involvement of VEGFA in testicular microcirculation, which is consistent with Zhao et al study [20].

In addition, in this study, YC concentration-dependently increased the TM4 cell viability, suggesting that YC could promote the viability of TM4 cells in a concentration-dependent manner. Moreover, we found that VEGFA expression did not significantly changed the mRNA or protein level after treatment with different concentrations of YC in TM4 cells, indicating that YC alone had little effect on VEGFA expression in TM4 cells. Therefore, the YC exerted a beneficial effect on TM4 cells. The findings of YC in cells are consistent with Gu et al study, which revealed that YC stimulates testosterone synthesis [17].

Traditional Chinese medicine is drug mixture plays key role in in the treatment of male infertility via suppressing the progression of teratospermia and improving the sperm acrosin activity [16]. YC consisted of 11 traditional Chinese drugs [17]. Therefore, additional studies will be required to understand the pharmacological mechanisms of action of YC on the testes microenvironment in our future work.

In conclusion, the present study suggested that YC could rescue CP-induced decrease in the density of testicular microcirculation in male mice, which is likely due to the enhancement of TM4 cell viability. In addition, high expression of CD34 and VEGFA might be involved in the process of micro circulation in testis tissues, but YC had little effect on VEGFA expression in TM4 cells. Therefore, YC might be regarded as an alternative therapeutic treatment for testicular microcirculation in the future.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Baofang Jin provided the idea and prepared the manuscript; Weihang Dong, Dalin Sun, and Bin Cai collected materials; Weimin Deng, Yugui Cui, Yihan Jin, and Li Tong conducted the experiment and analyzed the data; Ping Wu corrected the manuscript.

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