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Effects of Liver-Regulating Herb Compounds on Varicocele-Associated Testicular Dysfunction Through Restoring Hormones and Spermatocytes Apoptosis

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ABSTRACT

Purpose: Varicocele is considered as one of the causes for male infertility. Though varicocelectomy is suppose to improve semen parameters in adult infertile men, some patients with varicocele were still infertile after varicocelectomy.. Previous studies showed Traditional Chinese Medicine, Liver-regulating herb compounds (LRHC) could improve the semen quality and increase fertility rates of infertile patients with varicocele. This study aimed to throw light on the mechanism of LRHC on varicocele-associated infertility.

Materials and methods: Rats with varicocele-induced were treated with LRHC at dosage of 1mL/100g by intragastric administration for 90 days. The effects of LRHC on hormones and spermatocytes apoptosis were examined using ELISA assay, Western blotting, and flow cytometry.

Results: Rats induced with varicocele showed higher level of follicle stimulating hormone (FSH) in serum, which was brought back to normal level by LRHC. After treating with LRHC, both testicular tissue *in vivo* and Sertoli cell TM4 cells *in vitro* showed elevated expressions of FSHR. Cell viabilities of TM4 cells and spermatocyte GC-2 cells were improved by LRHC treatment under normoxia and hypoxia condition. Moreover, LRHC protected GC-2 cells from apoptosis induced by hypoxia. The expression of Bax reduced, while that of Bcl-2 increased after treating with LRHC.

Conclusion: This study revealed that LRHC had protective effects on spermatogenic disturbance caused by varicocele through regulating hormones and reducing spermatogenic cell apoptosis under hpoxia condition.

INTRODUCTION

Varicocele is an abnormal dilation, elongation and tortuosity of the tendril venous plexus within the spermatic cord; it is prevalent worldwide with 10%-15% overall incidence. Varicoceles are found in 19%-41% of men with primary infertility and 45%-81% of those with secondary infertility.[1] Among infertile patients with poor semen quality, the incidence of varicocele is 25%, while that in normal people is 12%.[2] Varicocele has been considered as one of the causes of male infertility.

The current treatment for varicocele-associated infertility is varicocelectomy, which may improve pregnancy rates and sperm concentration in adult infertile men.[3] However, during our clinical practice, we found that some patients are still infertile after surgery. Therefore, it is of great practical significance to find alternative treatment for varicocele-associated infertility. Liver-regulating herb compounds (LRHC) is a Chinese herb mixture including *Cyperus rotundus*，seeds of *Litchi chinensis Sonn*，*Angelica sinensis*，*Cynanchum otophyllum*，*Pericarpium citri reticulatae*，and *Citrus aurantium*. Our previous clinical practice suggested that LRHC achieved a good therapeutic effect for varicocele-associated infertility.[4] The average sperm motility and sperm count of infertile patients with varicocele significantly improved after taking LRHC for 2 to 4 courses. In rats with varicocele, LRHC could ameliorate histological appearance and ultrastructure of seminiferous tubules damaged by varicocele.[5] However, the effects of LRHC on hormones and spermatocytes apoptosis are still not clear.

Spermatogenesis failure caused by varicocele is considered as the cause for male infertility.[6] Spermatogenesis is regulated by neuroendocrine, especially the hypothalamic-pituitary-gonadal axis. The hypothalamus secretes gonadotropin-releasing hormone (GnRH), which stimulates the pituitary gland to secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Then LH promotes the secretion of testosterone (T) by Leydig cells. FSH binds with FSH receptor (FSHR) on the surface of Sertoli cells, affecting the maturation, proliferation and function of Sertoli cells independently or in synergy with T.[7] Except for affecting hormones, testicular hypoxia induced by varicocele could promote testicular cell apoptosis. Spermatogenic cells, Sertoli cells and Leydig cells are sensitive to hypoxia; cell apoptosis induced by hypoxia have a negative effect on spermatogenesis.[8]

In this study, we tried to verify whether LRHC could regulate the hormone levels in rats with varicocele which may be helpful to understand the molecular mechanisms of treating varicocele-associated infertility by LRHC.

Materials and Methods

Animals

A total of twenty-three eight-week-old male Sprague-Dawley rats weighing about 250g were included in this study. All of rats were maintained under controlled conditions with a 12/12 h light-dark cycle at 24℃ and were given free access to water and food. Twenty-three rats were randomly divided into three groups: control group (rats undertaken sham operation, n = 8),

varicocele group (rats with varicocele-induced, n = 8), and LRHC group (rats with varicocele-induced treated by LRHC, n = 7). Rats were sacrificed at the end of the study by intraperitoneal injection of pentobarbital sodium. All rat experiments were performed in compliance with the Guide for Care and Use of Laboratory Animals and were approved by Independent Ethics Committee of Shanxi Provincial People's Hospital (ethics committee approval number: Provincial Medical Opinions 2021 No.8).

Animal experiment design

For varicocele group, experimental rat model of varicocele was made as previously described.[4] Briefly, the rats were anesthetized by intraperitoneal injection of 40 mg/kg pentobarbital sodium. After making an abdominal midline incision and exposing the left kidney, left renal vein, adrenal vein, and testicular vein, we loosely tied the left renal vein using a 3-0 silk suture in a diameter of 0.64 mm. Partial ligation of the left renal vein decreased its diameter to approximately half. The abdominal midline incision was sewed up. Then the rats were fed for 48 days to develop varicocele.

For control group, rats underwent a sham operation which is similar to the above procedure without the left renal vein ligation.

For LRHC group, rats were induced varicocele as above described. After 48 days of varicocele induction, rats were treated with LRHC (1mL/100g) by intragastric administration for 90 days. Physiological saline (1mL/100g) was used in the control group and varicocele

group instead of LRHC.

Collection of serum and testes

After 90 days of treatment, all of rats were sacrificed and blood was collected from heart and then centrifuged at 4000 rpm for 10 min to separate the serum. The serum was used to examine the concentration of FSH, LH, T, and also used for cell treatment. The serum was stored at -80℃ before performing ELISA. The left testes were collected by orchiectomy. The testes were homogenized for protein extraction.

Enzyme Linked Immunosorbent Assay (ELISA)

Testicular protein was extracted by homogenizing testicular tissue in physiological saline. The content of FSH, LH, T in serum and testicular tissues were examined using commercially available ELISA kits (Elabscience Biotechnology Co.,Ltd, Wuhan, China), according to the manufacturer's instructions. The absorbance at 450nm was read using a SynergyTM 4 Multi–Mode Reader (BioTek, Winooski, USA).

Western blotting

The procedure of Western blotting was as previously described.[4] The antibodies used were as follows: anti-FHSR antibody (abcam, Cambridge, USA), anti-β-Actin antibody (Boster Biotechnology Co., Wuhan, China), anti-Bax antibody (Boster Biotechnology Co., Wuhan, China), anti-Bcl-2 antibody (Boster Biotechnology Co., Wuhan, China), anti-GAPDH (Boster

Biotechnology Co., Wuhan, China). The relative intensity of protein bands was quantified using Image J software (National Institute of Health, MD, USA).

Cell culture and CCK-8 assay

The Sertoli cell line TM4 and spermatocyte cell line GC-2 were purchased from American Type Culture Collection (ATCC). TM4 cells were cultured in DMEM/F12 medium, supplemented with 10% fetal bovine serum (FBS). GC-2 cells were cultured in DMEM medium supplemented with 10% FBS. All cells were kept at 37℃ with 5% CO_2.

For CCK-8 assay, TM4 or GC-2 cells were plated at 50-70% confluence and were treated with 10 % rat serum from experimental rats. LRHC-containing serum was used at the concentration of 0%, 5% and 10%. Control rat serum was used to supplement with LRHC-containing serum to reach the final concentration of 10%. Cells treating with rat serum were cultured under normoxia or hypoxia (1% oxygen concentration) condition for 24h. Then Cell viabilities were tested using a commercial CCK-8 kit (Dojindo, Shanghai, China), following the manufacturer's instructions.

Real-time PCR

Total RNA were extracted from TM4 cells that treated by rat LRHC-containing serum. Then cDNA was synthesized and real-time PCR was performed. The primers used were as followings: FSHR forward primer: 5' GGCGGCAAACCTCTGAACTT 3'; reverse primer: 5'

TCAGATCCTTTTCCATAACTGGGT 3'. β-actin forward primer: 5' CTAGGCACCAGGGTGTGATG 3'; reverse primer: 5' TCTCCATGTCGTCCCAGTTG 3'.

Cell apoptosis analyses

Cell apoptosis of GC-2 cells treating with different concentrations of LRHC-containing serum was analyzed by flow cytometry using an Annexin V/PI apoptosis detection kit (KeyGEN BioTECH, Jiangsu, China). The proportion of cells in different apoptosis status was determined using the FC500 flow cytometer. The excitation and emission wavelength of flowcytometry for Annexin and PI were as follows, Annexin V-FITC (Ex=488 nm, Em=530 nm); PI (Ex=488nm,Em=630nm).

Statistical analyses

Each experiment was replicated three times. The statistical analyses were performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The results were shown as the mean with 95% confidence intervals (CIs). One-way ANOVA were used for statistical analysis. Shapiro-Wilk test and Levene test were used to test the normality and homogeneity of variance, respectively. LSD test was used for multiple comparisons between groups if they met homogeneity of variance; otherwise Dunnett's T3 test was used. *P* values of $< .05$ were considered statistically significant.

RESTULTS

The effect of LRHC on the hormone secretion of rats with varicocele

FSH, LH and T are important hormones involved in spermatogenesis. We examined the concentrations of FSH, LH and T in testicular tissues and serum from rats using ELISA (**Figure 1**). We found that the content of FSH in testicular tissue was not affected by varicocele inducing and LRHC treatment. However, in serum, higher level of FSH was observed in rats with varicocele as compared with control rats (6.59 [95% CI: 4.26-8.91] VS 2.31 [95% CI: 1.85-2.77]); and the increased level was attenuated by LRHC application. The content of LH was higher in testicular tissues from rats with varicocele than that in control rats (0.24 [95% CI: 0.19-0.29] VS 0.14 [95% CI: 0.11-0.19]), which was not influenced by LRHC. In serum, there is no difference in the content of LH among groups. The concentration of T was higher in serum from rats with varicocele than that in control rats (7.78 [95% CI: 7.02-8.55] VS 2.80 [95% CI: 2.07-3.53]). Rats with varicocele treated with LRHC showed the highest level of T both in testicular tissue and in serum.

The effect of LRHC on the expression of FSHR in vivo and in vitro

FSHR is the molecular marker of Sertoli cell, and it affects the function of Sertoli cells. After treating varicocele-induced rats with LRHC, we analyzed the effect of LRHC on expression of FSHR. In testicular tissues, Western blotting results showed that the expression of FSHR in varicocele-induced rats did not alter. However, after taking LRHC, the varicocele-induced rats

showed elevated expression level of FSHR than control rats (**Figure 2A-B**).

In mouse Sertoli TM4 cells, the FSHR expression was detected by Real-time PCR after treating with 0% (control), 5% and 10% LRHC-containing serum. The results showed significantly up-regulated expression of FSHR in TM4 cells treating with 10% LRHC-containing serum, when compared with control cells (**Figure 2C**).

The effect of LRHC on cell viability and apoptosis

It has been found that varicocele induced hypoxia and apoptosis of testicular tissue which contributed to the pathophysiology of varicocele. We consequently tested the effects of LRHC on testicular cell viability under normoxia and hypoxia condition. In GC-2 cells, LRHC treatment improved the cell viability significantly under both normoxia and hypoxia condition, especially at the 10% concentration (**Figure 3A**). In TM4 cells maintained at normoxia, LRHC treatment improved the cell viability slightly, while the alteration did not reach to statistically significant. Under the hypoxia condition, LRHC treatment at the 10% concentration dramatically increased the TM4 cell viability (**Figure 3B**).

Since GC-2 cell viability was more sensitive for LRHC treatment, the cell apoptosis of GC-2 cells affected by LRHC was further analyzed. The results of flow cytometry showed that the amount of apoptotic cells increased significantly after hypoxia (1% oxygen) treatment for 24h. The apoptosis rate increased from (11.45 ± 0.50) % to (15.51 ± 0.81) %. After treating with LRHC at the 5% and 10% concentration, the cell apoptosis rate reduced to (13.34 ±

0.74) % and (11.85 ± 0.74) %, respectively (**Figure3C-D**).

The expressions of apoptosis related proteins were detected by Western blotting. Under the hypoxia condition, the expression of pro-apoptotic protein Bax increased slightly, while that of anti-apoptotic protein Bcl-2 increased significantly. After treating with LRHC, the Bax expression was significantly reduced, while the expression Bcl-2 was dramatically up-regulated (**Figure 3E-F**).

DISCUSSION

In varicocele, hormonal perturbation is induced and thus spermatogenesis impaired, which is considered as the pathogenesis of varicocele-associated infertility. High FSH level has a relationship with worse sperm quality[9], making FSH an indicator for the diagnosis of impaired spermatogenesis, with a sensitivity of 88.9% and a specificity of 94.1%.[10] It has been reported that varicocele could increase the FSH and LH levels, while decrease T level in the serum, which may contribute to poor sperm quality.[11,12] Traditional Chinese Medicines turned to regulate the hormones to improve infertility rate. In this study, we found an up-regulated level of FSH in varicocele-induced rats. The damage of spermatogenesis in varicocele results in the compensatory high level of FSH. LRHC treating brought FSH level back to a normal level, implying LRHC could improve spermatogenesis and the decrease in FSH level might be a result of negative feedback loop activation. Additionally, LRHC could elevate T level dramatically. Varicocele is associated with hypogonadism; and low level of T

could affect the process of spermatogenesis. Dramatically high level of T elevated by LRHC meant this medicine could improve the testicular function. This result should further be verified in clinical patients. Taken together, LRHC may play positive effects on varicocele-associated infertility in a direct testis-oriented action.

In testicular tissue, smoothly spermatogenesis relies on the cooperation of several functional cell types, including spermatogenic cells, Sertoli cells, and Leydig cells. Sertoli cells form a niche for germ cell maturation, and they construct the blood-testis barrier through tight junction formation.[13] Moreover, Sertoli cells function as "nurse cells" and provide nutritional support for developing germ cells, which is pivotal during spermatogenesis.[14] The proliferation, maturation, and function of Sertoli cells are regulated by FSH. By binding with FSHR, FSH promotes Sertoli cell proliferation and maturation, increases Sertoli cell number, and determines testicular size.[15] Absence of FSH or FSHR considerably decreases the Sertoli cell number by 30–45%, compared with normal testicular development.[7] In this study, we found that LRHC could improve the FSHR level in varicocele testes as well as in TM4 cells. Providing more FSHR for FSH to bind with, the serum level of FSH may reduce dramatically. Thus, LRHC may exert active roles on spermatogenesis through supporting and protecting the functions of Sertoli cells.

Amounts of studies indicated that varicocele could induce tissue hypoxia and apoptosis which contributed to the pathophysiology of varicocele-associated infertility.[16] Hypoxia could result in severe chronic oxidative and nitrosative stress in testicular tissues of patients with

varicocele. Consequently, this chronic stress leads to impaired spermatogenesis and produces poor sperms.[17] In vitro, hypoxia induced the spermatocyte cell apoptosis in a time-dependent manner.[18] In this study, we cultured GC-2 cells under the hypoxia condition to mimic the testicular physiological environment of varicocele. We found that LRHC could improve the cell viabilities and protect GC-2 cells from apoptosis induced by hypoxia. Oxidative stress (OS) has been considered as a major contributory factor to varicocele-associated infertility.[19] Excessive OS causes alteration in testicular microenvironment and sperm DNA fragmentation, which further induces germ cell damages. It is possible that LRHC could decrease oxidative stress and thus protect sperms cells from apoptosis.The exact mechanism that LRHC improves cell viability and protects cells from apoptosis induced by hypoxia needs further studies.

CONCLUSION

In conclusion, LRHC can restore the damaged testicular function. It also exerts a function on FSH/FSHR system, improves cell viability and protects cell from apoptosis. This study may provide some insight into the molecular mechanism of LRHC treating the varicocele-associated infertility.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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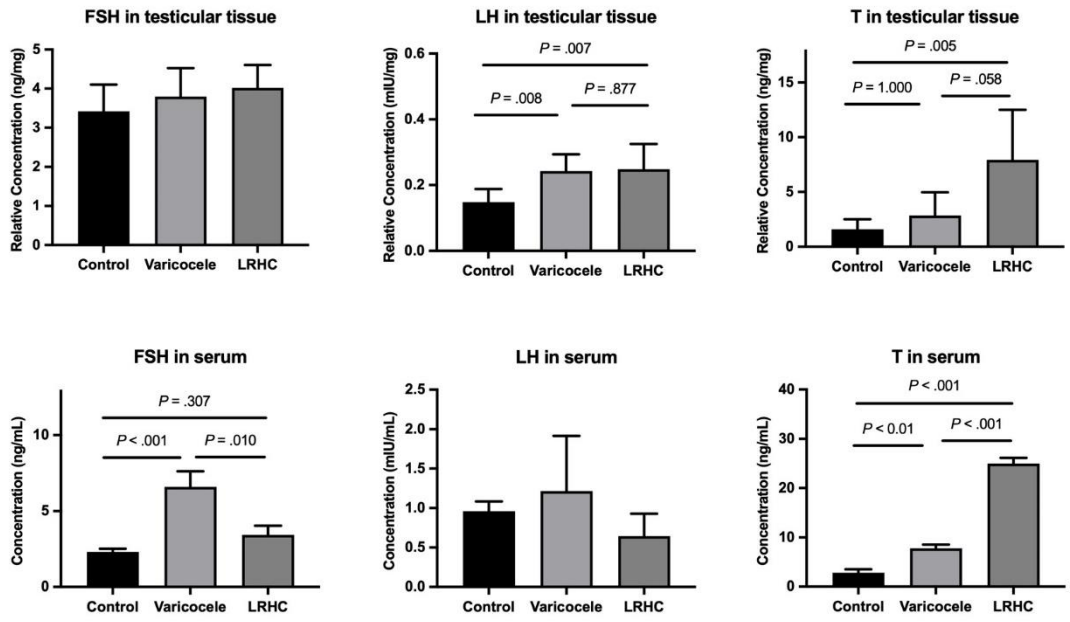

Figure1: The effect of LRHC on the hormone secretion of rats with varicocele. The levels of FSH, LH and T in serum as well as testicular tissues were examined by ELISA. LRHC abrogated the up-regulated level of FSH in serum caused by varicocele. LRHC increased the level of T in testicular tissue as well as in serum from rats with varicocele.

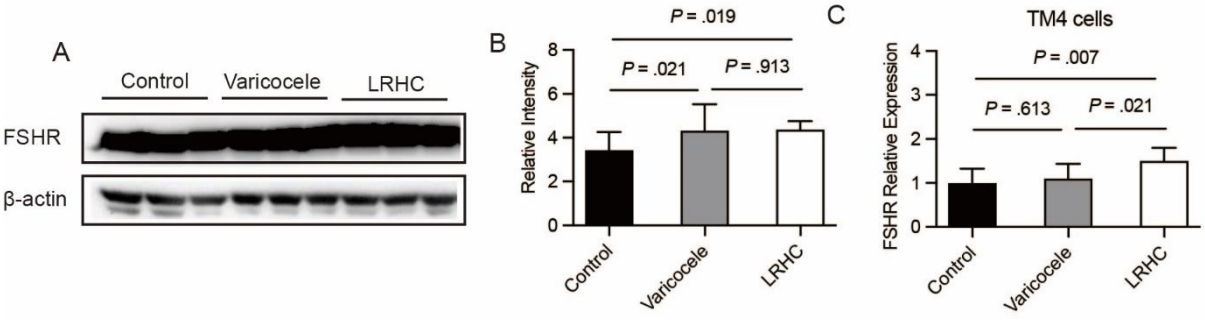

Figure 2: The effect of LRHC on the expression of FSHR. (A) The expression of FSHR in testicular tissues from control rats, rats with varicocele, and rats with varicocele treated with LRHC were detected by Western blotting. (B) The result of Western blotting was quantified by Image J software. Higher expression of FSHR was detected in LRHC treating group than that in control group. (C) Mouse Sertoli cells TM4 were treated with LRHC-containing rat serum at concentrations of 0% (control), 5% and 10%. The expression of FSHR was detected by Real-time PCR, showing higher expression in cells treated with 10% LRHC-containing serum, than that in control cells.

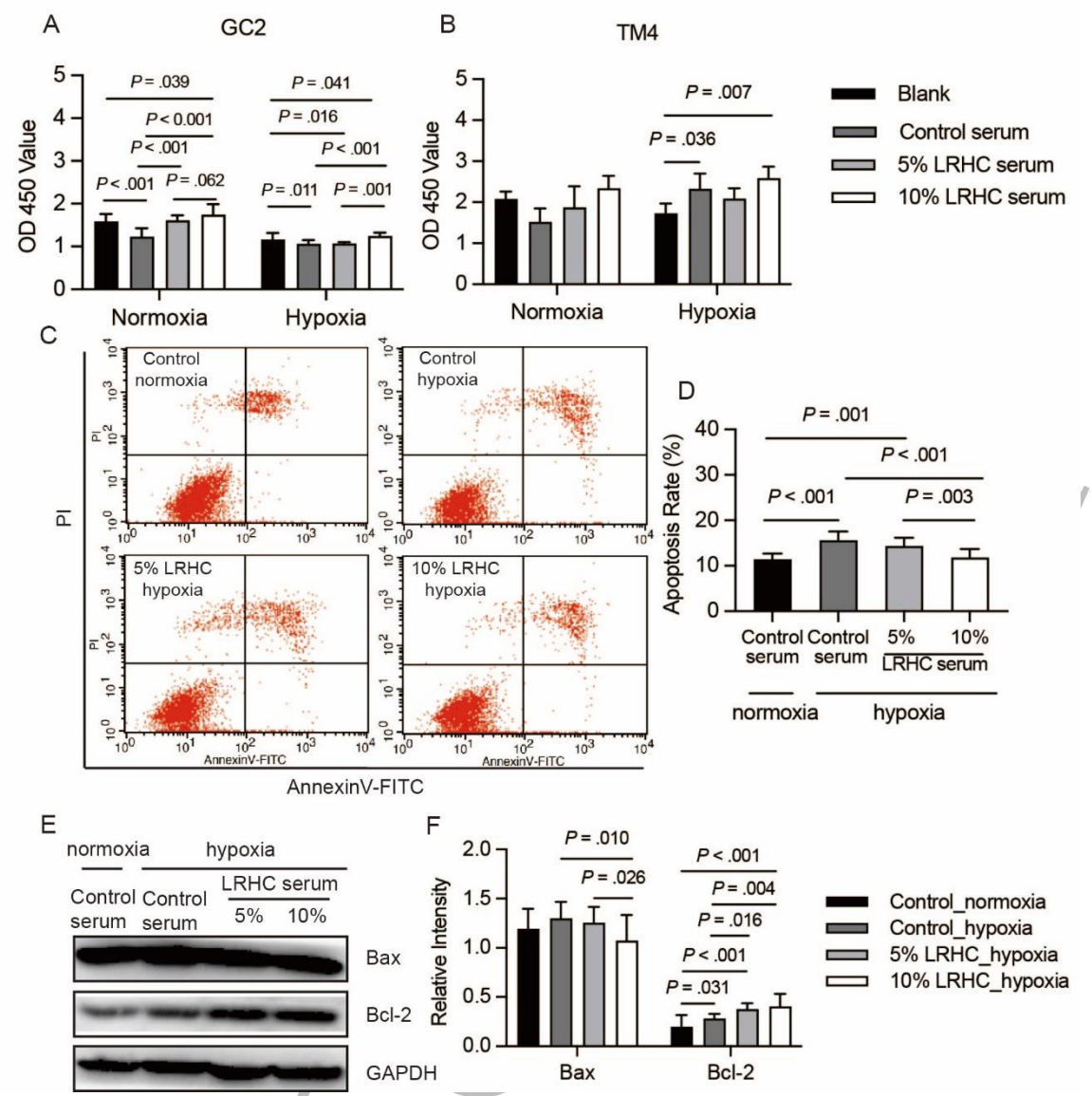

Figure 3: The effect of LRHC on the cell viability and cell apoptosis. (A) Mouse spermatocyte GC-2 cell viability was tested using CCK-8 assay. Blank group indicated GC-2 cells cultured in the regular medium (containing 10% FBS). Control serum group indicated cells treated with 10% control rat serum. 5% LRHC serum group indicated cells treated with 5% LRHC-containing serum, supplement with 5% control rat serum. 10% LRHC serum group indicated cells treated with 10% LRHC-containing rat serum. (B) Cell viability of mouse Sertoli cell TM4 cells was tested. (C) The cell apoptosis of GC-2 cells was examined

by Annexin V/PI staining followed by flow cytometry. (D) Statistical analysis of the flow cytometry results. Cell apoptosis was induced by hypoxia, while LRHC-treating significantly reduced the apoptosis rate in a concentration-related pattern. (E) The protein expressions of Bax and Bcl-2 in GC-2 cells treated with control serum or LRHC-containing serum. (F) The results of Western blotting were quantified. The expression of Bax was decreased by LRHC treating, while that of Bcl-2 was increased.