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EXPERIMENTAL STUDY

Effect of Roucongrong (*Herba Cistanches Deserticolae*) on reproductive toxicity in mice induced by glycoside of Leigongteng *(Radix et Rhizoma Tripterygii)*

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Abstract

OBJECTIVE: The purpose of this research is to study the effect of Roucongrong (*Herba Cistanches Deserticolae*) on reproductive toxicity in mice induced by a glycoside extracted from Leigongteng (*Radix et Rhizoma Tripterygii*) (GRT).

METHODS: Forty-eight BALB/c mice were randomly divided into two groups in the ratio of 1 : 3, 12 in one group and 36 in the other. The 12-mouse group was the control group that was intragastrically administered physiological saline for 3 weeks. The 36 mice in the other group were given 30 mg · kg $^{-1}$ · d $^{-1}$ GRT for 3 weeks, then randomly divided into 3 subgroups: the model group, GRT group and Roucongrong (*Herba Cistanches Deserticolae*) group, with 12 mice in each group. In the model group, 0.25 mL physiological saline was intragastrically administered; in the GRT group, GRT, 0.25 mL at 30 mg · kg $^{-1}$ · d $^{-1}$ was intragastrically administered once a day; in the Roucongrong (*Herba Cistanches Deserticolae*) group, mice were administered Roucongrong (*Herba Cistanches Deserticolae*) decoction equivalent to 0.25 mL at a final dose of 10 g · kg $^{-1}$ · d $^{-1}$ crude drug (calculated as per 20 times of 0.5 g · kg $^{-1}$ · d $^{-1}$ for adults), and GRT 0.25 mL at 30 mg · kg $^{-1}$ · d $^{-1}$ daily. After another 3 weeks of exposure, expression levels of the reproduction-related genes DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked, B-cell CLL/lymphoma 6 and Signal transducer and activator of transcription 3 were evaluated.

RESULTS: After 6 weeks of GRT treatment, the spermatogenic cell population in the convoluted tubule of testis was in disorder and the tubule cavity expanded. Sertoli cell and Leydig cells exhibited atrophy or disappeared. The number of sperm decreased. The spermatogenic cell level of testis for male mice was ranked in order and sperm was produced in the cavity of the spermatogenic cell. The expression levels of DDX3Y, BCL6 and STAT3 were up-regulated.

CONCLUSION: GRT affected reproduction-related genes. Roucongrong (*Herba Cistanches Deserticolae*) reversed reproductive toxicity in mice induced by GRT.

Key word: Cistanche deserticola; Tripterygium wilfordii; Toxicity; Reproduction-related genes; Hypogonadal model

INTRODUCTION

Glycoside of Leigongteng (*Radix et Rhizoma Tripterygii*) (GRT) is extracted from the root of the euonymus Leigongteng (*Radix et Rhizoma Tripterygii*). Currently, it is one of medications that is used to treat some kidney diseases. However, its reproductive toxicity limits its clinical application.[1] It was found that after intragastric administration of GRT for 3 weeks, hair of male mice became, matted and disheveled, and their fertility had declined. The pregnancy rate for female mice mating with these male mice fell in a dose-dependent manner of GRT dose.[2] In another study, it was found that GRT caused hypogonadism in male mice by regulating reproduction-related genes affecting the secretion of sex hormones. Roucongrong (*Herba Cistanches Deserticolae*), in terms of the theory of Traditional Chinese Medicine (TCM), can moderately invigorate the kidney *Yang* as well as enrich and nourish essence and blood, which to some extent can reduce the reproductive toxicity of GRT.[2-3] This study was conducted to investigate the mechanisms underlying these effects.

MATERIALS AND METHODS

Animals

Forty-eight adult BALB/c male mice were provided by Sino-British Sippr/BK Lab Animal Ltd., Shanghai, China, with lab animal license No. SYXK (HU) 2004-2005 (Certificate of inspection No. for the lab animal: 0058668). They were kept in separate cages with a 12 h light/dark cycle and relative humidity of 45% and ad libitum access to food and water. This study was approved by the IRB of shuguang hospital affiliated with shanghai university of TCM.

Materials

SuperScript Ⅲ reverse transcriptase was purchased from Invitrogen Corporation (Invitrogen Corporation, Carlsbad, CA, USA). Quantitative PCR (polymerase chain reaction) reagents (SYBR Green) were purchased from Shanghai GenePharma Co., Ltd., Shanghai, China. Bouin fluid was prepared by mixing a saturated solution of picric acid (Sinopharm Chemical Reagent Co., Ltd., SCRC, Shanghai, China) 40% methanol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), and glacial acetic acid (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) pro rata. Bouin fluid was used for fixing specimens that were flushed with 70% ethanol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Specimens were then stored in 70% ethanol 12 to 24 h post-fixing.

Experimental procedure

Modeling and intervention: BALB/c mice were acclimated for 1 week and then randomly divided into two groups in the ratio of 1∶3. 12 in one group and 36 in the other. The 12-mouse control group was intragastrically administered physiological saline. The 36 mice in the other group were utilized as the toxicity groups by intragastric administration of 30 $mg \cdot kg^{-1} \cdot d^{-1}$ GRT calculated as 20 times of 1.5 $mg \cdot kg^{-1} \cdot d^{-1}$ for adults for 3 weeks. Then they were randomly divided into 3 groups: the model group, GRT group and Roucongrong (*Herba Cistanches Deserticolae*) group, with 12 mice per group. For the model group, 0.25 mL physiological saline was intragastrically administered. In the GRT group, GRT, 0.25 mL at 30 $mg \cdot kg^{-1} \cdot d^{-1}$was intragastrically administered once a day. In the Roucongrong (*Herba Cistanches Deserticolae*) group, mice were administered Roucongrong (*Herba Cistanches Deserticolae*) decoction equivalent of 0.25 mL at a final dose of 10 $g \cdot kg^{-1} \cdot d^{-1}$ of crude drug (calculated as per 20 times of 0.5 $g \cdot kg^{-1} \cdot d^{-1}$ for adults), and GRT 0.25 mL at 30 $mg \cdot kg^{-1} \cdot d^{-1}$ daily for anther 3 weeks.

Measurement and methods

After 6 weeks, mice were euthanized, and the testes of the male mice were harvested instantly. The testes without envelopes were stored in liquid nitrogen for further examination (within 10 min). Some of the testes were fixed in 70% ethanol after 24 h of Bouin fluid treatment. They were then prepared for examination by morphological observation.

Real-time quantitative PCR

After the frozen testes were ground and homogenated, they were extracted by the standard chloroform method, and total RNA was stored at - 80℃. RNA (5 μg) was placed into sterilized Eppendorf tubes (Eppendorf, Hamburg, German); then 1 μL Oligo $(dT)_{12-18}$ (500 μg/mL), 1 μL random primer (500 μg/mL), 1 μL 10 mmol/L dNTPs (solarbio, Beijing solarbio science & technology Co., Ltd., Beijing, China)and 12 μL double distilled water were added to the each tube. Samples were vibrated, and tubes were placed at 65℃ in a water bath for 5 min. The tubes were then put on ice immediately. After a short centrifugation, 5 proportions of 4 μL first strand buffer solution, 2 μL 0.1 mol/L DTT (SCRC, Shanghai, China) and 1 μL RNase inhibitor were added with moderate mixing. Tubes were placed in a water bath at 42℃ for 2 min, and then 1 μL reverse transcriptase(solarbio, Beijing solarbio science & technology Co., Ltd., Beijing, China) was added with

gentle mixing. The tubes were placed in a water bath of 52℃ for 50 min followed by 70℃ for 15 min to terminate the reactions. Real-time PCR MasterMix (solarbio, Beijing solarbio science & technology Co., Ltd., Beijing, China) (SYBR Green) was used on an ABI real-time PCR System StepOnePlus to amplify the expressed genes. β-actin was used as the housekeeping gene. Optimized reaction conditions were adopted to perform the real-time quantitative PCR reaction. The 5-μL reaction was then prepared and included 2 proportions of 2.5 μL Mix, 0.5 μL cDNA template and 0.5 μL of each differential [DDX3Y, BCL6 and signal transducer and activator of transcription 3 (STAT3)]. The reaction conditions were as follows: denaturation at 95℃ for 10 s, followed by 40 cycles of denaturation at 95℃ for 15 s, annealing at 60℃ for 15 s, and extension at 72℃ for 15 s. The reactions for each specimen were performed in triplicate. Melting curve analysis was performed following the amplification to verify that there was no non-differential amplification. Upon the completion of the reactions, the system software automatically provided Ct values of amplification for each specimen. Based on the Ct values of the target genes i.e., DDX3Y, BCL6 and STAT3 as well as β-actin, the relative expression levels of the target genes were calculated using the 2 (-Delta Delta C (T) method.

Statistical analysis

SPSS 16.0 (SPSS Inc. Chicago, IL, USA) was used to analyze data. The measured quantity was indicated by $\bar{x} \pm SD$. Student's *t*-test was conducted to compare the means for two groups, and χ^2 test was performed for the sperm counts. $P<0.05$ was the significant level.

RESULTS

After 6 weeks, male mice testes were stained as shown in Figure 1.

For mice in the control group, the testes were full and smooth. Different levels of spermatogenic cells were observed inside the convoluted tubule (spermatogonium, primary spermatocyte, secondary spermatocyte, spermatoblast and sperm in turn from epithelium to lumen), which were arranged in an orderly fashion with 6-8 distinct levels. Primary spermatocytes were active for early stage of seminiferous epithelium, and spermatoblasts were rich in the later period spermatogenic cells, which were distributed in groups. Sertoli cells were set in between spermatogenic cells of all levels and sperms, with tight connections between them. Blood vessels were rich in the interstitium. Leydig cells were normal in form and arranged in order (Figure 1A).

For mice in the model group, atrophy was observed in the convoluted tubule of the testes. The number of the spermatogenic cells was increased. Sertoli cells were connected between different levels of spermatogenic cells, and there were small numbers of sperm in the lumen. However, the Leydig cells were arranged in an orderly fashion (Figure 1B). One mouse died in the 4th week of the intragastric administration and another one at the time of urine collection.

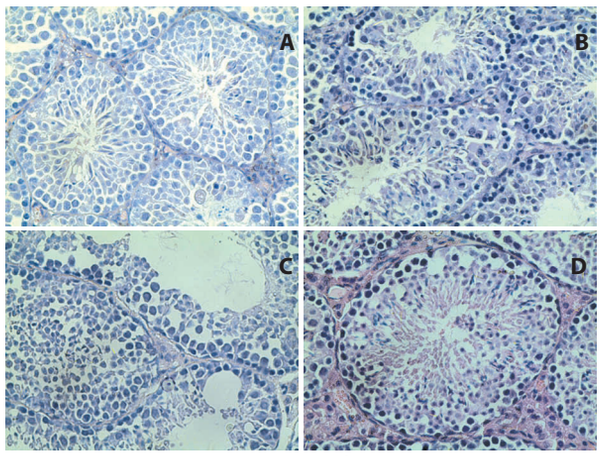

Figure 1 the testes' expression of hematoxylin-eosin staining in groups (×40)

A: control group; B: model group; C: GRT group; D: Roucongrong (*Herba Cistanches Deserticolae*) group. Control group was intragastrically administered 0.25 mL physiological saline. Model group was intragastrically administered GRT 0.25 mL (30 mg·kg^{-1}·d^{-1}) for 3 weeks, then was given 0.25 mL physiological saline for another 3 weeks. GRT group was intragastrically administered GRT 0.25 mL (30 mg·kg^{-1}·d^{-1}), once a day for 6 weeks. Roucongrong (*Herba Cistanches Deserticolae*) group was intragastrically administered GRT 0.25 mL (30 mg·kg^{-1}·d^{-1}) for 3 weeks, then were administered Roucongrong (*Herba Cistanches Deserticolae*) decoction equivalent of 0.25 mL at a final dose of 10 g·kg^{-1}·d^{-1} of crude drug and GRT 0.25 mL at 30 mg·kg^{-1}·d^{-1} daily for anther 3 weeks. GRT: Leigongteng (*Radix et Rhizoma Tripterygii*).

For mice in the GRT group, atrophy of testes and degradation of most convoluted tubules were observed. For the spermatogenic cells inside the lumen, there was abnormal proliferation. The cells were not arranged in an orderly fashion. The cellular levels of the cells decreased, and the sperm disappeared. The degraded and deciduous spermatogenic cells fell off and dispersed around the lumen. Sertoli cells were arranged in an extremely sparse manner. Some of them even disappeared, and vacuoles were observed. The basilar membrane around the seminiferous tubule thickened, and the interstitial cells were not arranged in an orderly fashion (Figure 1C).

For mice in the Roucongrong (*Herba Cistanches Deserticolae*) group, the lumen forms were inconsistent. The existing spermatogenic cells were not arranged in an orderly fashion. Sperm was observed in the lumen. The sign in the GRT group was not observed. The interstitial cells proliferated (Figure 1D). One mouse died at the time of urine collection.

Changes of reproduction-related genes

Real-time quantitative PCR results (Figure 1) demonstrated that compared with the control group, the reproduction-related gene DDX3Y and the proto-oncogenes BCL6 and STAT3 for mice in the model group and GRT group were significantly up-regulated. Com-

pared with the control group, genes in mice in the model group were up-regulated by more than 1.5 fold. Expression levels of DDX3Y, BCL6 and STAT3 were further up-regulated in mice in the GRT group.

Real-time quantitative PCR results demonstrated that compared with the control group, the reproduction-related gene DDX3Y, BCL6 and STAT3 for mice in the model group and GRT group were significantly up-regulated. The expression level changes ratio between control group and normal group in DDX3Y, BCL6 and STAT3 is 1.99, 1.65 and 1.86 individually. The expression level changes ratio between GRT group and normal group in DDX3Y, BCL6 and STAT3 is 4.62, 3.43 and 2.78 individually.

After exposure to Roucongrong (*Herba Cistanches Deserticolae*), the expression levels of DDX3Y, BCL6 and STAT3 were significantly down-regulated. The expression levels of those genes were similar to those in the model group.

The expression levels of DDX3Y, BCL6 and STAT3 changes ratio between the GRT group and model group of DDX3Y, BCL6 and STAT3 is 2.32, 2.80, 1.50. The expression level changes ratio between Roucongrong (*Herba Cistanches Deserticolae*) group and model group in DDX3Y, BCL6 and STAT3 is 0.73, 0.75 and 0.83 individually.

DISCUSSION

GRT has been widely used to treat renal diseases or autoimmune diseases due to its significant immune suppression effect. Nevertheless, to a certain extent, GRT-induced damage to the reproductive system limits its clinical application.[4] We discovered from a previous prophase study[2] that GRT-induced symptoms were similar to the symptoms of Renal-*Yang* asthenia in terms of TCM. Meanwhile, other studies have shown that the use of this medicine for dissolving stasis alleviated the degree of injury to the male testis and sperm caused by Leigongteng (*Radix et Rhizoma Tripterygii*).[5,6]

Based on our research, it was shown that GRT-induced reproductive injury was dose dependent. In the Roucongrong (*Herba Cistanches Deserticolae*) group, the level of the spermatogenic cells of testes were in clear order, and sperm were generated in the lumen of the convoluted tubule. These data suggested that Roucongrong (*Herba Cistanches Deserticolae*) could reverse the reproductive toxicity induced by GRT.

By real-time quantitative PCR, our results demonstrated that genes related to testes reproduction, including the proto-oncogene BCL6 and signal transducer and activator of transcription 3 STAT3, were up-regulated by GRT exposure. Our findings suggested that GRT might cause the testes to be distorted and hindered the generation of normal sperm by affecting meiosis of the spermatocyte, which gave rise to reproductive toxicity.

Located at chromosome translocation banding 3q27, the BCL6 gene encodes and transcribes this inhibitor, which is a proto-oncogene.[11] It was found that BCL6 prevents apoptosis of spermatocytes.[12] STAT are critical transcriptional regulatory factors in the cell. Similar to other STAT proteins, STAT3 is activated after being phosphorylated. It is the transcription activator of BCL6.[13] Our results suggested that GRT exposure induced testes tissue damage and distortion, which affected normal fertility. Moreover, STAT3 functions together with other oncogenes, such as Piwil2 gene and Bcl-XL. Meanwhile, it is highly expressed in spermocytoma and produces a possible carcinogenic effect.[14] Even if apoptosis is not inhibited, STAT3 and BCL6 may also be highly expressed and result in tissue damage and chemotaxis of inflammatory cells.[15,16] Our study results suggested that GRT might damage testes tissue, cause distortion, and inhibit normal differentiation and apoptosis, thus affecting normal fertility.

In our study, results showed that Roucongrong (*Herba Cistanches Deserticolae*) not only reversed the reproductive toxicity of GRT, but also down-regulated gene expression levels of DDX3Y, BCL6 and STAT3 similar to the levels in the model group. These results suggest that Roucongrong (*Herba Cistanches Deserticolae*) had an antagonistic effect on the reproductive toxicity caused by GRT.

In conclusion, GRT caused dysgenesis in male mice and Roucongrong (*Herba Cistanches Deserticolae*) had an antagonistic effect on the reproductive toxicity caused by GRT. Future experiments are needed to elucidate the mechanism(s) underlying the abnormal gene expression caused by GRT.

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