Journal Pre-proof

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PII: S0378-8741(19)32323-2

DOI: https://doi.org/10.1016/j.jep.2019.112500

Reference: JEP 112500

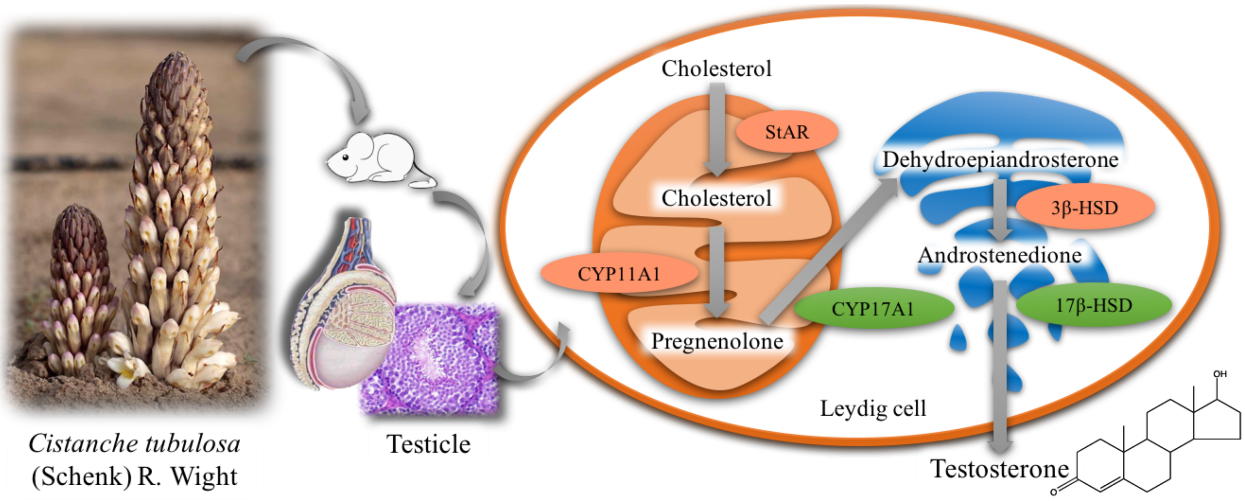
To appear in: *Journal of Ethnopharmacology*

Received Date: 12 June 2019

Revised Date: 10 December 2019

Accepted Date: 21 December 2019

Please cite this article as: Wang, Q., Dong, J., Lu, W., He, H., Sun, X., Zhang, K., Song, Q., Jiang, Y., Wang, Y., Li, C., Tu, P., Phenylethanol glycosides from *Cistanche tubulosa* improved reproductive dysfunction by regulating testicular steroids through CYP450-3β-HSD pathway, *Journal of Ethnopharmacology* (2020), doi: https://doi.org/10.1016/j.jep.2019.112500.



Cistanche tubulosa
(Schenk) R. Wight

Phenylethanol glycosides from *Cistanche tubulosa* improved reproductive dysfunction by regulating testicular steroids through CYP450-3β-HSD pathway

Qixin WANG[1a], Jianteng DONG[2a], Wenji LU[2], Hao HE[3], Xiaoqian SUN[2], Ke ZHANG[1], Qingqing SONG[1], Yong JIANG[4], Yong WANG[3*], Chun LI[1*], Pengfei TU[1,4*]

1. Modern Research Center for Traditional Chinese Medicine, Beijing University of Chinese Medicine, Beijing 100029, China;

2. School of Traditional Chinese Medicine, Beijing University of Chinese Medicine, Beijing 100029, China;

3. School of Life Sciences, Beijing University of Chinese Medicine, Beijing 100029, China;

4. State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China;

a. These two authors contributed equally to this article.

[*]Corresponding author

Yong Wang, Beijing University of Chinese Medicine, No. 11, North 3rd Ring East Road, Chaoyang District, Beijing, 100029, China. Tel.: +86 10 6428 6180; E-mail: doctor_wangyong@163.com

Chun Li, Beijing University of Chinese Medicine, No. 11, North 3rd Ring East Road, Chaoyang District, Beijing, 100029, China. Tel.: +86 10 6428 6180; E-mail: lichun19850204@163.com

Pengfei Tu, Peking University Health Science Center, Xueyuan Rd. 38, Haidian District, Beijing, 100191, China. Tel.: +86 10 8280 2750; E-mail: pengfeitu@163.com

Abstract

Ethnopharmacological relevance

Cistanche tubulosa (Schenk) R. Wight has been used frequently in traditional folk medicine for treatment of male sexual dysfunction (MSD). Phenylethanol glycosides, the main components of *C. tubulosa*, possess a variety of pharmacological activities due to their multiple properties. However, the underlying mechanism by which phenylethanol glycosides from *C. tubulosa* (CPhGs) regulates testicular steroids has not been elucidated to date.

Aim of the study

This study is to determine whether CPhGs promotes the reproductive functions of mice through CYP450-3β-HSD pathway of testosterone synthesis.

Materials and methods

The major compositions of *C. tubulosa* (CPhGs) were quantified by high performance liquid chromatography (HPLC). The model of reproductive injury in mice were induced by injection of hydrocortisone (HCT). Different doses of CPhGs (72, 145 and 289 mg/kg) and testosterone propionate (TP, positive control drug) were administrated intragastrically for 14 d. The reproductive functions (erectile incubation period, capture and ejaculation incubation period, number of captures and ejaculations) and organ weights (testicle, epididymis, seminal vesicle and penis) were then determined. The levels of luteinizing hormone and testosterone in serum were quantified by radioimmunoassay. The key enzymes in testosterone synthesis pathways such as steroidogenic acute regulatory protein (StAR), cytochrome P450

cholesterol side chain cleavage enzyme (P450scc/CYP11A1) and 3β-hydroxysteroid dehydrogenase (3β-HSD) in the testis were assessed by immunofluorescence (IF) staining or/and western blot (WB) analysis.

Results

The results illustrated that the low dose of CPhGs (72 mg/kg) had no significant protective effect against the reproductive injury caused by HCT, while the moderate dose of CPhGs (145 mg/kg) improved the damaged reproductive ability and the declined levels of luteinizing hormone and testosterone in the model mice ($P<0.001$, $P<0.05$, respectively). In particular, high dose of CPhGs (289 mg/kg) was most effective in improving HCT-induced changes in body weight ($P<0.01$), reducing the incubation period of the erectile ($P<0.001$), capture ($P<0.05$) and ejaculation ($P<0.01$), and increasing the number of captures and ejaculations ($P<0.01$, $P<0.05$, respectively). The weights of testcle, epididymis, seminal vesicle and penis ($P<0.001$, $P<0.01$, $P<0.01$, $P<0.001$, respectively) were improved by high dose of CPhGs. The levels of testosterone and its upstream luteinizing hormone were up-regulated by high dose of CPhGs ($P<0.001$). Meanwhile, the expressions of the key steroidogenic enzymes including CYP11A1 and 3β-HSD were significantly up-regulated after CPhGs treatment ($P<0.001$), demonstrated that CPhGs exerted the effect through enhancing testosterone biosynthesis via CYP450-3β-HSD pathway.

Conclusions

CPhGs could significantly protect against HCT-induced deleterious reproductive dysfunction and testis injury. The protective effects were exerted by up-regulating synthesis of testosterone via the CYP450-3β-HSD pathway in Leydig cells.

Keywords

Cistanche tubulosa; testosterone; StAR; CYP11A1; 3β-HSD

1. Introduction

Male sexual dysfunction (MSD) is one of the most common health issues, with an estimated prevalence rate of 4.2% worldwide (Hotaling et al., 2015). A variety of factors, including age, smoking, obesity, diabetes and cardiovascular disease et al., can eventually lead to MSD. The pathological changes will severely affect the physical and mental health of individuals, including sexual psychological response, physiological structure, neuroendocrine and vascular function. Evidence demonstrated that reduced level of serum testosterone is the primary cause of MSD and testosterone deficiency dominated the whole process of MSD (Zhu et al., 2016). Improving synthesis of testosterone can alleviate weakened reproductive capacity and improve reproductive function. Therefore, testosterone synthesis is believed to be the most attractive therapeutic target in the treatment of MSD.

The biosynthesis of testosterone takes place in the Leydig cells of testis. Cholesterol in circulation is the major precursor for testosterone synthesis. Steroidogenic acute regulatory protein (StAR) mediates the transport of cholesterol from cytoplasm into mitochondria. Cholesterol is then converted to pregnenolone through cytochrome P450 cholesterol side chain cleavage enzyme (P450scc/CYP11A1). Afterwards, pregnenolone is turned to dehydroepiandrosterone which is eventually converted to testosterone in a series of enzymatic reactions mediated by critical enzymes including 3β-hydroxysteroid dehydrogenase (3β-HSD) (Fig. 1) (Kumar et al., 2008).

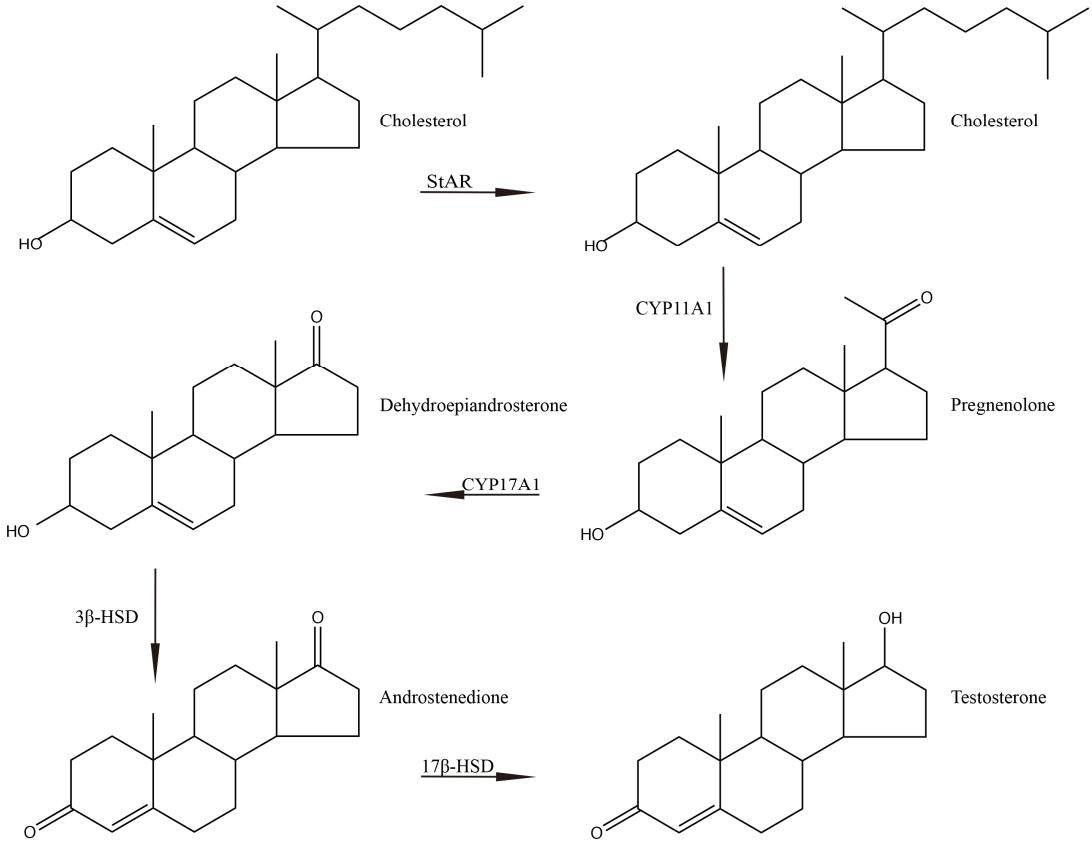


Fig. 1 Major biosynthesis pathway of testosterone in testicle. Notes: Steroidogenic acute regulatory protein (StAR); cytochrome P450 cholesterol side chain cleavage enzyme (P450scc/CYP11A1); cytochrome P450 family 17 subfamily A member 1 (CYP17A1); 3β-hydroxysteroid dehydrogenase (3β-HSD); 17β-hydroxysteroid dehydrogenase (17β-HSD).

Cistanches Herba (CH) is a genus of medicinal plant that has been used in China and other eastern Asian countries since the fifteenth century for the treatment of impotence, seminal emission, infertility, and chronic constipation (Wang et al., 2012). *Cistanche tubulosa* (Schenk) R. Wight (*C. tubulosa*) and *Cistanche deserticola* Y. C. Ma (*C. deserticola*) have similar chemical compounds and pharmacological activities, and they have been documented in the Chinese Pharmacopoeia (Editorial Committee of Chinese Pharmacopoeia, 2015). The main medicinal ingredients in CH are phenylethanol glycosides, and the content in *C. tubulosa* is higher than that of *C.*

deserticola (Song et al., 2016; Liu et al., 2018). *C. tubulosa* is considered as an alternative to *C. deserticola*, which is on the verge of extinction due to decades of over-exploitation.

Evidence showed that *C. tubulosa* could effectively protect against the reproductive system damage of male mice caused by tripterygium glycosides. Moreover, alcohol extract of *C. tubulosa* could increase the expressions of key enzymes, including CYP11A1 and cytochrome P450 family 17 subfamily A member 1 (CYP17A1), in the synthesis of testosterone in normal male rats (Wang et al., 2016). In addition, the preliminary research of our group indicated that phenylethanol glycosides from *C. tubulosa* (CPhGs) promoted the release of sex hormones and improved the testicular pathological status of reproductive injury model animals by regulating function of hypothalamus-pituitary-gonadal (HPG) axis (Wang et al., 2018).

Therefore, the aimed of this study was to investigate the effects of CPhGs on the reproductive dysfunction and testosterone levels in the model of hydrocortisone (HCT)-induced reproductive dysfunction of male mice. The regulative effects of CPhGs on key enzymes involved in synthesis of testosterone were explored by *in vivo* studies.

2. Materials and methods

2.1 Preparation of CPhGs

The *C. tubulosa* was provided and indentified by Prof. Peng-fei Tu (Peking University, Beijing, China). The *C. tubulosa* was collected from the cultivation base

of Peking University in Yutian County of Xinjiang Autonomous Region. CPhGs extract was prepared by Tianjin Bei Da Cong Rong Biotechnology Co. Ltd. The dried slices of *C. tubulosa* (50 kg) were pulverized and filtered through a 20-mesh sieve. Then was extracted twice by water and the extracts were combined. The extracts were subjected to D101 macroporous resin to obtain 70% ethanol elution, and then lyophilized to dryness. The powder was stored at 4°C for further studies.

2.2 High performance liquid chromatography (HPLC) analysis of CPhGs

In accordance with the Chinese Pharmacopoeia (2015), the quality standard of *C. tubulosa* was tested by using echinacoside and acteoside. The CPhGs powder sample (1 g) was accurately weighed, 50% methanol (10 mL) was added, and then sonicated for 30 min. After standing, the supernatant fluid was filtered through a 0.22 μm microporous membrane to obtain the filtrate. Echinacoside and acteoside were dissolved in appropriate dimethyl sulfoxide (DMSO) to prepare stock solutions (10 mg/mL). Accurately absorbed the standard stock liquid and diluted it with 50% methanol solution. The calibration standards were prepared by adding known volumes of echinacoside and acteoside (4, 8, 20, 40, 100 and 200 μg/mL). Then, the supernatant (1 μL) was injected into the HPLC System (LC-20AD series, Shimadzu, Japan) which was equipped with an Acquity HSS T3 C18 column (2.1 mm × 100 mm, 1.8 μm; Waters). The mobilephase was prepared with a gradient of 0.1% formic acid (component A) and acetonitrile (component B). The linear gradient was as follows: 10% B over 0-3 min, 10%-15% B over 3-5 min, 15%-30% B over 5-22 min, 30%-60% B over 22-24 min and then returned to 10% B at 24 min immediately. The

flow rate was 0.3 mL/min. Detection was performed at 330 nm and the peak area was used as the analytical measurement for the evaluation.

The standards echinacoside and acteoside were purchased from Yuanye Biotech Co., Ltd. (Shanghai, China). HPLC solvents were supplied by Thermo-Fisher (Pittsburgh, PA). DMSO was provided by Merck (Darmstadt, Germany). Ultrapure water was prepared by the Milli-Q Integral water purification system (Millipore, Bedford, MA). And other chemicals were available from Beijing Chemical Co. Ltd. (Beijing, China).

2.3 Animals

Sixty male Institute of Cancer Research mice weighing 31.47 ± 0.85 g were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China), and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health (NIH Publication No. 85-23, revised 1996). The animals were maitained at a controlled room temperature (25 ± 2°C) and humidity (55 ± 5%), and were automatically subjected to a 12 h light/dark cycle in cages and allowed to freely access water and food.

2.4 Reproductive injury modeling method

The dose preparation for reproductive injury model was optimized based on the previous reports, intragastric administration of HCT 50 mg/kg was adopted for its remarkable effect (Qiu et al, 2008; Qin et al., 2017). Animals in the model group, TP

group, and low, moderate and high doses groups were continuously treated with HCT at 50 mg/kg for 14 d; the control group was given the same amount of normal saline.

2.5 Treatment of animals

After 1 week acclimation, the mice were randomly distributed into six groups of ten animals each (N=10). All the treatment groups were given gavage for 14 d according to the following experimental design. (1) control group (treated with normal saline), (2) model group: HCT group (treated with HCT 50 mg/kg), (3) testosterone propionate (TP) group: positive control group (treated with HCT 50 mg/kg, TP 2 mg/kg) (4) CPhGs-l, (5) CPhGs-m, and (6) CPhGs-h groups (treated with HCT 50 mg/kg, CPhGs 72, 145 and 289 mg/kg of CPhGs, respectively).

The doses of CPhGs were selected according to the content of the extract of *C. tubulosa* and refer to the clinic doses of 5, 10 and 20 g daily. 50 kg crude drug of *C. tubulosa* was isolated and extracted, and the yield rate of phenylethanol glycosides was 8.76%, accounting for 4380 g. The drug-administered groups were given 72, 145 and 289 mg/kg aqueous solution of CPhGs according to the 0.83, 1.66 and 3.3 g/kg crude drug doses of *C. tubulosa*. Materials were given to mice by gavage for 14 d.

Within 24 h of the last administration, the blood samples of mice were collected from the abdominal aorta and stood for 1 h, then centrifuged at 3000 rpm for 15 min. The serum was stored at -40°C until further use to analyze hormone levels. The testis, epididymides, seminal vesicles and penises of mice were stripped. Then, the collected tissue samples were weighed.

Unilateral testis were fixed in paraformaldehyde at room temperature for hematoxylin-eosin (HE) and immunofluorescence (IF) staining, and other testis were frozen in liquid nitrogen and stored at -80°C for further research.

2.6 Determination of reproductive functions

2.6.1 Erectile incubation period test

After the last administration, the penises of mice were electrically stimulated with a current of 4 mA and a voltage of 25 V. The erection incubation periods from the start of the stimulus to the penile erection was recorded.

2.6.2 Mating ability test

Mating experiments were performed after the last administration. Adult female mice underwent bilateral ovariectomy and recovered for 2 weeks. 48 h before the mating experiment were subcutaneous injected with 20 μg estradiol benzoate, and 4 h before mating, they were injected with 500 μg progesterone to synchronize their oestrus time with the mating experiment (Lin et al., 2016).

A male mouse was placed in one cage for 5 min at the time of mating to adapt to the environment, and then 2 female mice were placed in each cage. Record the capture and ejaculation incubation period of male mice within 30 min (the time of male mice captured the female and ejaculation for the first time), the number of captures and ejaculations (Makarova et al., 2007).

2.7 Determination of serum hormone levels

The levels of luteinizing hormone and testosterone were quantified directly from prepared serum using radioimmunoassay (RIA) kits (Beijing Sino-UK institute of biological technology, Beijing, China) according to the instruction.

2.8 Histopathological examination

Unilateral testicle tissues were fixed in 4% paraformaldehyde for 24 h, then were embedded in paraffin and cut into sections (5 μm) (Amin et al., 2008). Hematoxylin-eosin (HE) was used for staining and the slides were examined under a light microscope.

2.9 IF staining

The testicular sections were rounded with appropriated antibody (anti-3β-HSD 1:1000; Santa Cruz Biotechnology, CA, USA) at 4°C for 12 h, then washed three times with Tris-Buffered Saline and Tween 20 (TBS-T) and animated with secondary antibody (Alexa 568-conjugated goat-anti-mice, 1:500; Bioss, Beijing, China) for 1 h at room temperature. After washing with TBS-T, the testicular sections were stained with DAPI for 5 min and covered with anti-fading fixative (Beyotime, Beijing, China). A digital camera (Leica, EVOS f1, Germany) was used to capture fluorescent images.

2.10 Western blot (WB) analysis

StAR, CYP11A1, and 3β-HSD expressions were assessed by WB. Total proteins of testcle tissues were lysed by RIPA lysate (50:1) and quantified by the bicinchoninic acid (BCA) method. Each lane was loaded with 50 μg of protein and electrophoresis at 100 V for 1.5 h, separated by sodium dodecyl sulfate–polyacrylamide gel

electrophoresis (SDS-PAGE) using an 10% dissociation gel. Afterwards, separated proteins were then transferred to poly vinylidene fluoride (PVDF) membranes at 300 mA for 1.5 h, and blocked with 5% skim milk for 1 h at room temperature, further incubated with anti-StAR (1:200; Abcam, Cambridge, UK), anti-CYP11A1(1:500; Abcam, Cambridge, UK) and anti-3β-HSD (1:1000; Santa Cruz Biotechnology, CA, USA) in TBS-T at 4°C overnight, followed by the appropriate secondary antibodies (1:8000; Sangene, Tianjin, China) at room temperature for 2 h. Chemiluminescent detection was performed using Bio-Rad ChemiDocTM MP Imaging System (Bio-Rad Laboratories Hercules, CA, USA) and the expression of StAR, CYP11A1, and 3β-HSD proteins were normalized by GAPDH, the grayscale analysis was performed by Image J software (National Institutes of Health, Bethesda, MA, USA).

2.11 Statistical analysis

All data were analyzed using SPSS software (version 19.0, SPSS Institute Inc., Chicago, IL). Differences between groups were analyzed with one-way repeated measures analysis of variance (ANOVA). The results were expressed as mean ± standard deviation (SD) using GraphPad Prism software (version 7.0, San Diego, CA, USA). Differences with a *P* value less than 0.05 were considered as statistically significant.

3. Results

3.1 Quantitative determination of the echinacoside and acteoside contents in CPhGs

The contents of echinacoside and acteoside in CPhGs were quantitated by using HPLC (Fig. 2). The retention times for echinacoside and acteoside were 11.5 min and 15.1 min, respectively at a maximum ultraviolet (UV) absorbance of 330 nm. The standard curves of the echinacoside and acteoside concentrations were y = 13869x - 991.14 (R^2 = 1.0000) and y = 4056.9x - 3224.9 (R^2 = 0.9998), respectively, where y was the peak area and x was the concentration of echinacoside or acteoside. The echinacoside and acteoside contents of CPhGs were 310 mg/g and 40 mg/g, respectively.

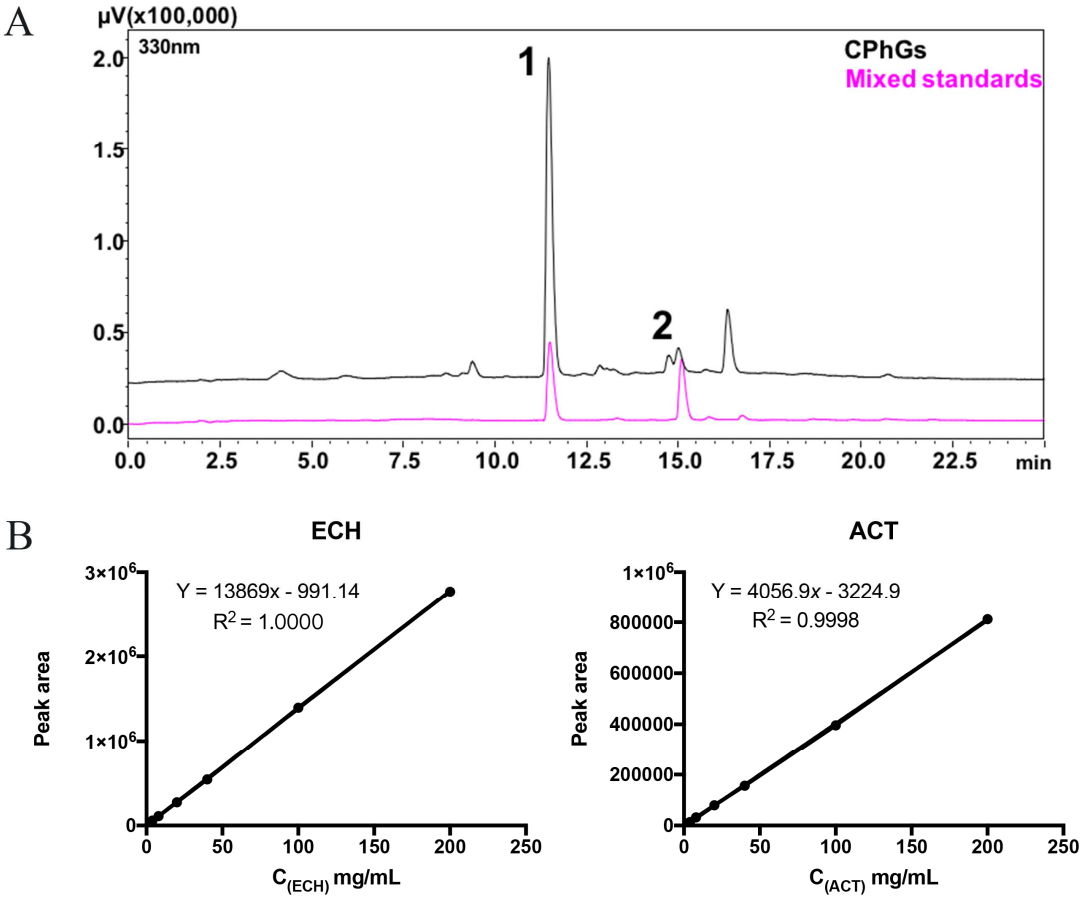


Fig. 2 Quantitative determination of the ECH and ACT contents in CPhGs. A: The HPLC analysis of CPhGs and two phenylethyl alcohol glycosides ECH and ACT. B: Standard curves of ECH and

ACT. Notes: peak 1, ECH; peak 2, ACT. Phenylethanol glycosides from C. tubulosa (CPhGs); echinacoside (ECH); acteoside (ACT).

3.2 CPhGs-h improved HCT-induced weight loss of mice

After being intervened with HCT, mice in model group showed a decrease in responsiveness and activity. Meanwhile, the weight decreased by 12.46% (3.94 g) in model group. After treatment with CPhGs for 14 d, the weight of mice in high dose group was significantly higher than that of the model group (P<0.01). In the TP treatment group, the mice gained some weight at 14 d (P<0.001). These data indicated that high dose of CPhGs could improve body weight loss caused by HCT (Fig. 3).

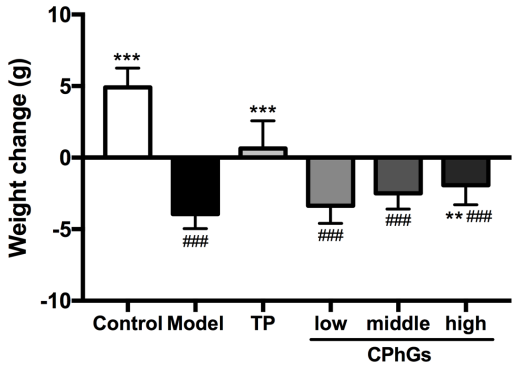


Fig. 3 Effects of CPhGs on the body weight loss induced by HCT. The high dose of CPhGs could improve the body weight loss induced by HCT (*P<0.01*). Notes: Values were expressed as means ± SD. **P<0.01, $^{***}P$<0.001 versus model; $^{###}P$<0.001 versus control. N = 10.

3.3 CPhGs-h improved reproductive functions of mice

3.3.1 CPhGs-m and CPhGs-h shortened erectile incubation period in mice

Compared with the control group, the erectile incubation period of mice in the model group was significantly prolonged (P<0.001). The administration with TP and CPhGs (CPhGs-m and CPhGs-h) significantly shortened the erectile incubation

period compared with the model group (P<0.001, P<0.01, P<0.001, respectively). CPhGs had a similar effect on improving the erectile function of mice with TP (Fig. 4A).

3.3.2 CPhGs-m and CPhGs-h improved mating ability of mice

Mating experiment showed that the capture incubation period of mice was increased and the number of captures within 30 min was reduced in the model group, as compared with control group (P<0.001). The ejaculation incubation period was delayed (P<0.001) and the number of ejaculations were reduced in mice treated with HCT compared with control group (P<0.001).

The capture incubation period of mice in the TP, CPhGs-m and CPhGs-h groups were reduced compared with model group (P<0.01, P<0.05, P<0.05, respectively). Meanwhile, TP and high dose of CPhGs could increase the number of captures within 30 min compared with the model group (P<0.001, P<0.01, respectively). Moreover, TP and high dose of CPhGs could also significantly shorten the ejaculation incubation period (P<0.001, P<0.01, respectively) and increase ejaculation times over 30 min (P<0.01, P<0.05, respectively). These data suggested that TP and CPhGs could improve reproductive functions damaged by HCT (Fig. 4B).

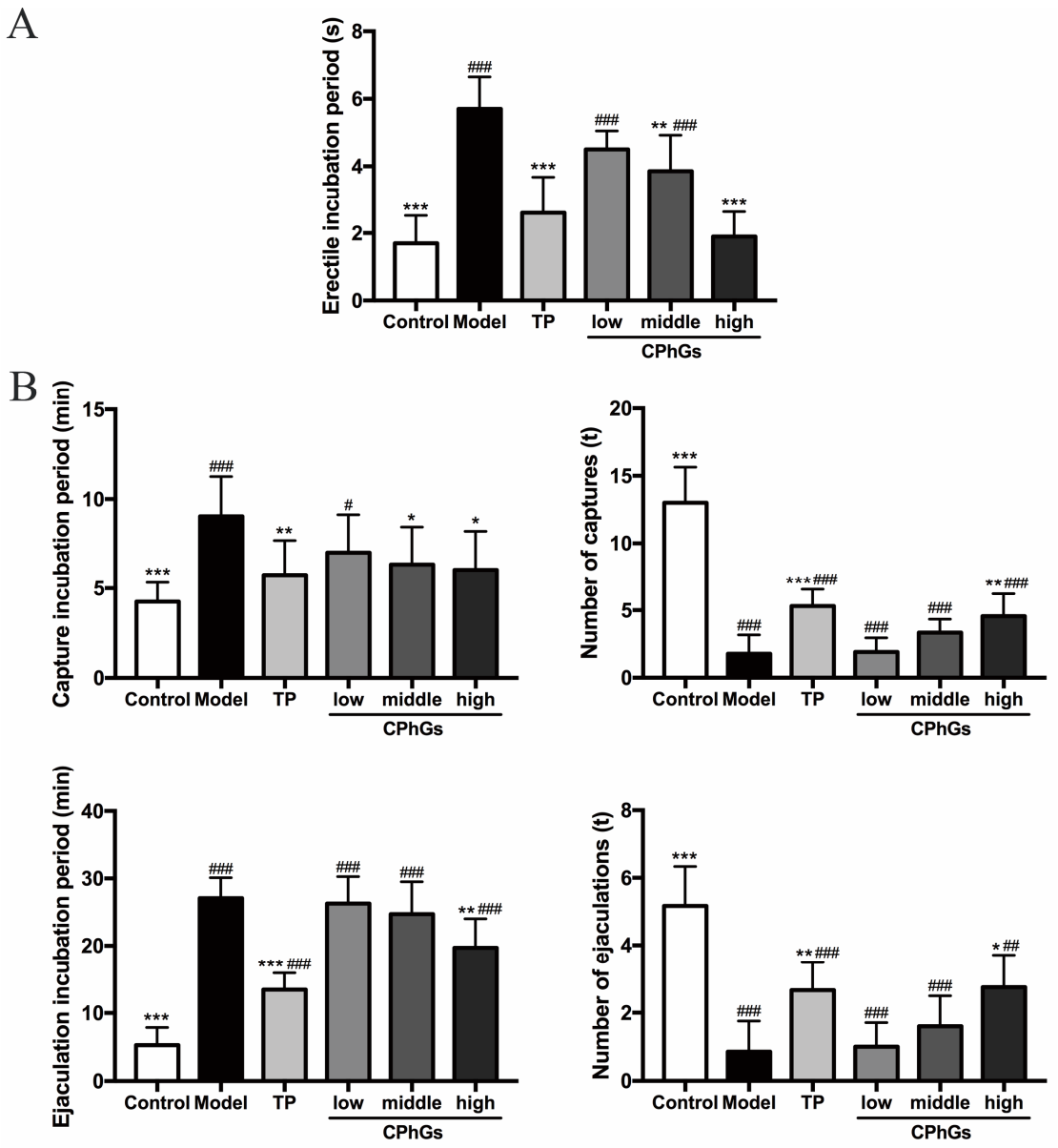


Fig. 4 Effects of CPhGs on improving reproductive functions. A: Moderate and high doses of CPhGs could shorten erectile incubation period in mice; B: High dose of CPhGs reduced the incubation period and increased the number of captures and ejaculations. Notes: Values were expressed as means ± SD. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus model; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ versus control, N = 10.

3.4 CPhGs-m and CPhGs-h regulated hormone levels of mice

The sex hormones play important roles in regulating the development of animal reproductive system. To determine the steroid effects of CPhGs, the levels of luteinizing hormone and testosterone were detected by radioimmunoassay. The serum levels of luteinizing hormone and testosterone in the model group were significantly reduced compared with those of the control group (P<0.01). After treatment with moderate (P<0.001, P<0.05, respectively) and high doses (P<0.001) of CPhGs, the levels of the two hormones were significantly up-regulated, suggesting that CPhGs had an androgen-like effect. Notably, the level of testosterone in the TP treatment group was significantly higher than that of the control group (P<0.001) (Fig. 5).

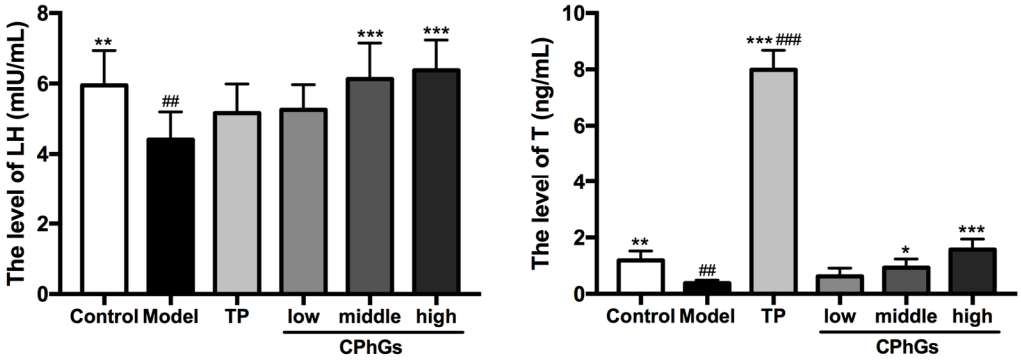


Fig. 5 Effects of CPhGs on regulating the levels of luteinizing hormone and testosterone in serum. The levels of luteinizing hormone and testosterone were increased in CPhGs-m and CPhGs-h groups compared with model group. Notes: luteinizing hormone (LH); testosterone(T). Values were expressed as means ± SD. $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 versus model; $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 versus control. N = 10.

3.5 CPhGs-m and CPhGs-h regulated organ weights of mice

Variances of male gonadal organ weights between model and treatment groups were significantly different. Compared with the control group, the organ weights of testicle, epididymis, seminal vesicle and penis in the model group were significantly reduced (P<0.001). Compared with the model group, TP had no effect on organ weights, while moderate dose of CPhGs regulated organ weights other than the seminal vesicles (P<0.001, P<0.01, P<0.01, respectively). Intervention of high dose of CPhGs significantly adjusted those organ weights back to control group levels (P<0.001, P<0.01, P<0.01, P<0.001, respectively) (Table 1).

Table 1. Organ weights of testicle, epididymis, seminal vesicle and penis.

Organ weight (g)	Control	Model	TP	CPhGs-l	CPhGs-m	CPhGs-h
Testicle	$0.234 \pm 0.021^{***}$	$0.175 \pm 0.018^{\#\#\#}$	$0.184 \pm 0.008^{\#\#\#}$	$0.193 \pm 0.009^{\#\#\#}$	$0.211 \pm 0.017^{***\#}$	$0.225 \pm 0.023^{***}$
Epididymis	$0.105 \pm 0.013^{***}$	$0.062 \pm 0.010^{\#\#\#}$	$0.064 \pm 0.014^{\#\#\#}$	$0.070 \pm 0.015^{\#\#\#}$	$0.074 \pm 0.016^{\#\#\#}$	$0.083 \pm 0.009^{**\#\#}$
Seminal vesicle	$0.265 \pm 0.028^{***}$	$0.161 \pm 0.023^{\#\#\#}$	$0.190 \pm 0.034^{\#\#\#}$	$0.157 \pm 0.031^{\#\#\#}$	$0.210 \pm 0.025^{**}$	$0.212 \pm 0.030^{**}$
Penis	$0.067 \pm 0.011^{***}$	$0.040 \pm 0.008^{\#\#\#}$	$0.045 \pm 0.007^{\#\#\#}$	$0.048 \pm 0.008^{\#\#\#}$	$0.051 \pm 0.009^{**\#\#}$	$0.052 \pm 0.006^{***}$

Notes: Values were expressed as means ± SD. $^{**}P$<0.01, $^{***}P$<0.001 versus model; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 versus control. N = 10.

3.6 CPhGs-m and CPhGs-h improved testicular histopathology of mice

Normal histological structures of testis were observed in control group. Testicular sections of HCT-treated mice showed remarkable degenerative changes. Disorganization and necrosis of the seminiferous tubules could be detected. In addition, degeneration and irregular arrangement of spermatids, large gaps in the lumen and interstitium of the seminiferous tubules, decreased number of Leydig cells

were also seen in HCT-treated mice. Administrations of moderate and high doses of CPhGs significantly prevented HCT-induced testicular injury. Normal histological pattern, normal spermatogenic series, and Leydig cells formation were observed in CPhGs-treated mice (Fig. 6). The positive control drug TP had no significant protective effect against pathological changes of testis.

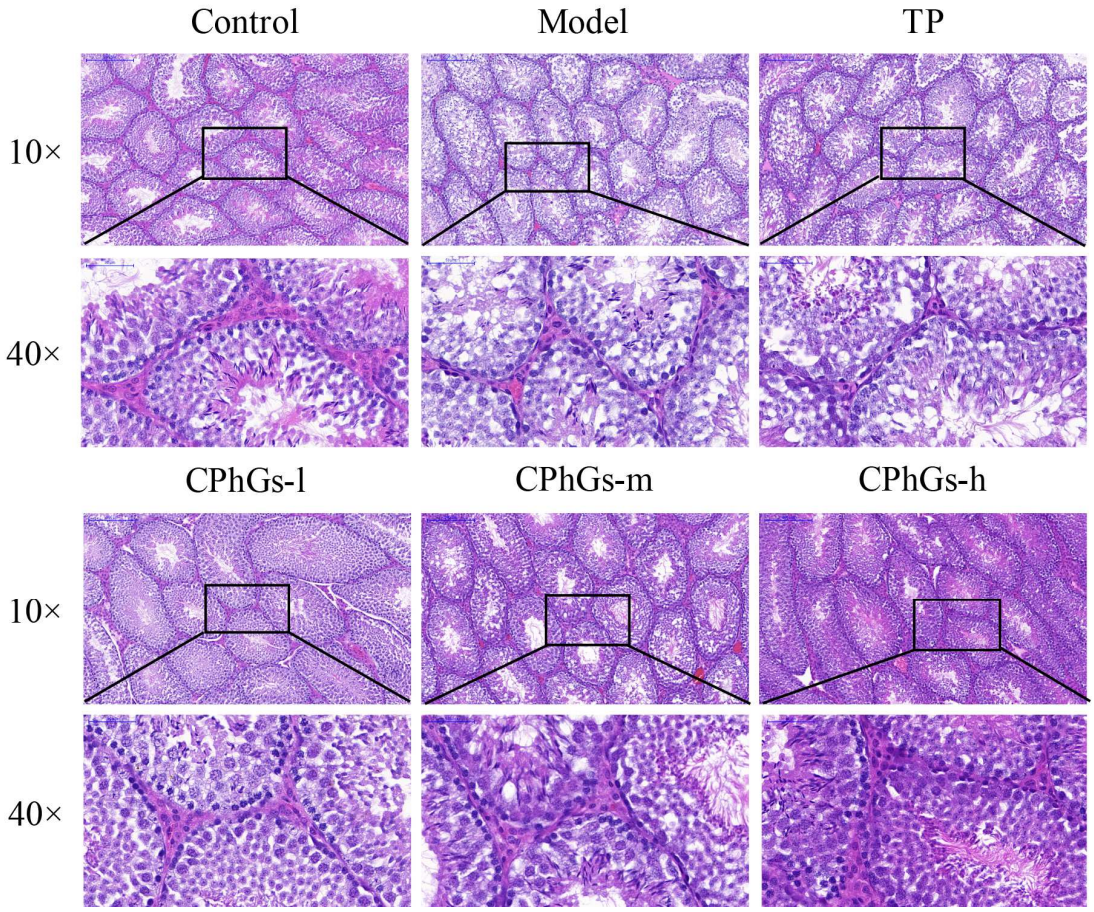
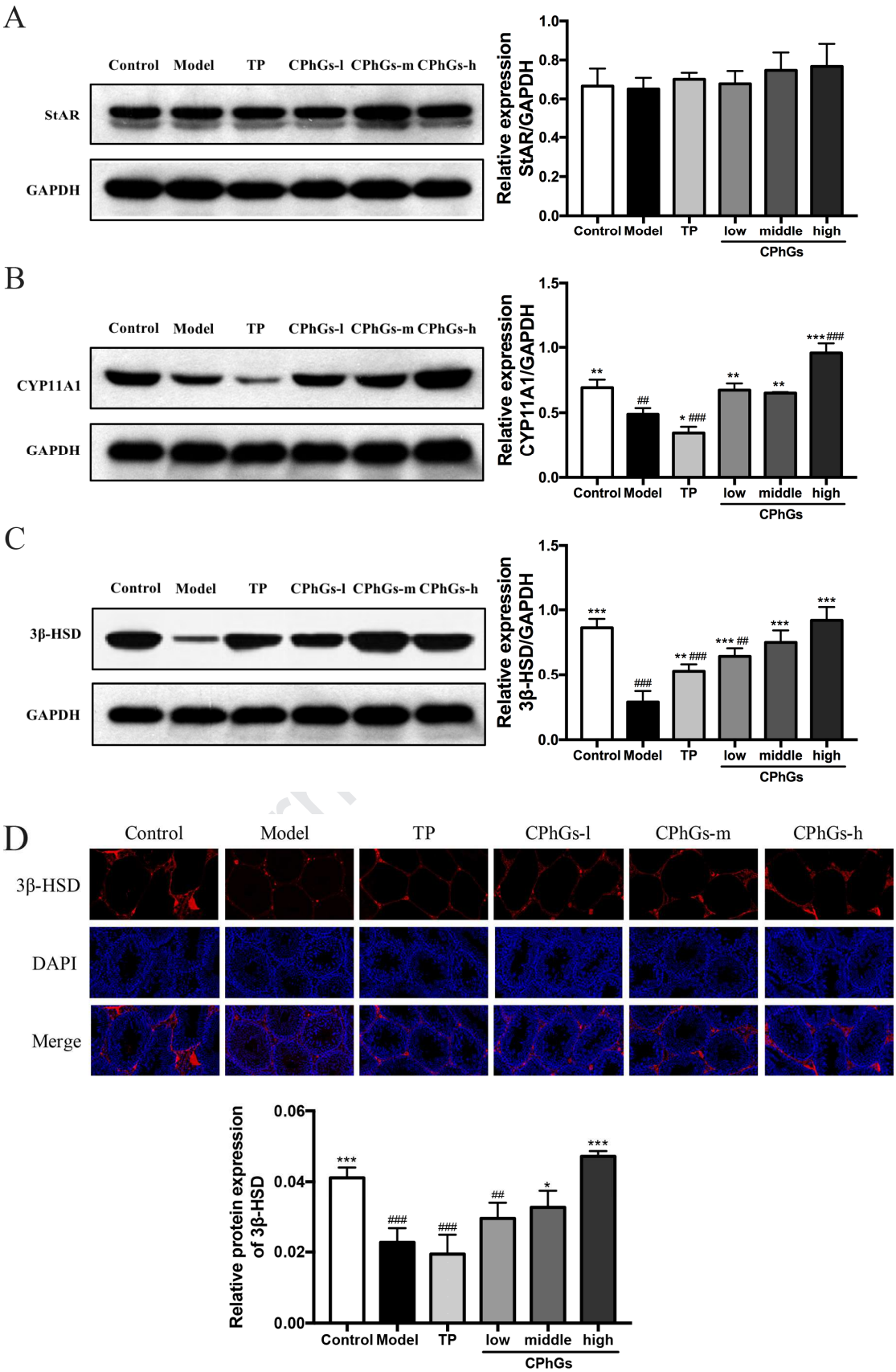


Fig. 6 The testicular sections of mice in each group were stained with HE after the experiments. Moderate and high doses of CPhGs significantly prevented HCT-induced testicular injury. Notes: magnifications = 10× and 40×; HE: hematoxylin-eosin.

3.7 CPhGs up-regulated expressions of CYP11A1 and 3β-HSD in the testis of mice

StAR, CYP11A1, and 3β-HSD are involved in the biosynthesis of testosterone in Leydig cells. Expressions of StAR were similar among different groups of mice (Fig. 7A). The treatment of HCT significantly inhibited the expressions of key steroidogenic enzymes including CYP11A1 and 3β-HSD (P<0.01, P<0.001, respectively) in the testis, whereas intervention with CPhGs up-regulated expesssions of CYP11A1 and 3β-HSD (P<0.01, Fig. 7B and 7C). IF was then further applied to locate and quantify the expression of 3β-HSD. Consistent with WB results, the level of 3β-HSD was lower in model group compared with that in control group, whereas the expression of 3β-HSD increased after high dose of CPhGs treatment (P<0.001, Fig. 7D). Interesting, the positive control drug TP inhibited expression of CYP11A1, probably due to negative feedback effect (Fig. 7B). The results indicated that CPhGs could promote expressions of upstreaming regulators in testosterone synthesis pathway.


A
Control Model TP CPhGs-l CPhGs-m CPhGs-h
StAR
GAPDH
Relative expression StAR/GAPDH
1.0
0.8
0.6
0.4
0.2
0.0
Control Model TP low middle high
CPhGs
B
Control Model TP CPhGs-l CPhGs-m CPhGs-h
CYP11A1
GAPDH
Relative expression CYP11A1/GAPDH
1.5
1.0
0.5
0.0
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##
* ###
**
**
***###
Control Model TP low middle high
CPhGs
C
Control Model TP CPhGs-l CPhGs-m CPhGs-h
3β-HSD
GAPDH
Relative expression 3β-HSD/GAPDH
1.5
1.0
0.5
0.0
***
###
** ###
*** ##
***
***
Control Model TP low middle high
CPhGs
D
Control Model TP CPhGs-l CPhGs-m CPhGs-h
3β-HSD
DAPI
Merge
Relative protein expression of 3β-HSD
0.06
0.04
0.02
0.00
***
###
###
##
*
***
Control Model TP low middle high
CPhGs

Fig. 7 Protein expressions of StAR (A), CYP11A1 (B) and 3β-HSD (C, D) were evaluated by WB or/and IF. Notes: A, B and C: Western blot analysis of StAR, CYP11A1 and 3β-HSD in testis. The right panels are histograms of protein expressions. D: Immunofluorescence analysis of 3β-HSD. Red fluorescence represents 3β-HSD, and DAPI stained for nuclei. Values were expressed as means ± SD. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus model; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ versus control. N = 3.

4. Discussion

MSD is one of the common diseases that affect male health and living standard. Decrease in synthesis of testosterone is the major cause of MSD (Helo et al., 2019). Therefore, promoting the production of testosterone and maintaining normal sexual physiology are the key treatment strategies. The synthesis of artificial testosterone provides a feasible approach for the treatment of MSD. However, the application is limited due to multiple side effects (Rao et al., 2015). Traditional Chinese medicine is an alternative option in the effective treatment of MSD due to the low rate of side effects (Edouard et al., 2014). Among the natural herbs, CH is considered as one of the firstline choices for MSD in China (Liu et al., 2015). Studies showed that CH could improve male sexual behavior and development of male reproductive system through an androgen-like effect (Song et al., 2003). It was included in the China Pharmacopoeia in 2005 Year due to its reliable effect. Studies had shown that the *C. tubulosa* had similar chemical constituents and pharmacological activities to *C. deserticola* (Song et al., 2016). *C. tubulosa* contains a variety of active components, including CPhGs, iridoids, polysaccharides, etc. As one of many active components in

C. tubulosa, CPhGs were shown to have antioxidant, anti-fatigue, neuroprotective, and anti-inflammory effects in both *in vivo* and *in vitro* studies (Ayupbek et al., 2012). The content of CPhGs is higher in *C. tubulosa* than that of *C. deserticola.* In recent years, it has been reported that CPhGs could improve reproductive function (Jiang et al., 2016). However, the detailed mechanism remains unclear. In this study, we explored whether CPhGs could improve male reproductive function and ameliorate MSD through promoting synthesis of testosterone.

HCT was used to induce a reproductive injury mice model. The reproductive functions were evaluated by capture and ejaculation incubation periods, number of captures and ejaculations, and erectile incubation period. Organ weights of the testicle, epididymis, seminal vesicle and penis, and the morphology of the testis were also observed. Accroding to our results, high dose of CPhGs could improve weight loss and erectile time that was compromise by HCT. Meanwhile, capture and ejaculation incubation period was shortened, and the frequency of captures and ejaculations were increased by CPhGs treatment. In addition, high doses of CPhGs could relieve HCT-induced atrophy of various organs (testicle, epididymis, seminal vesicle and penis). These behavioral experiments showed that CPhGs could ameliorate HCT-induced injury of reproductive functions.

To further explore the mechanism of CPhGs in improving reproductive function, we investigated the effects of CPhGs on production of testosterone in testis. Steroidogenesis is a tightly controlled process for the development of male reproductive system. Metabolism of steroid is dependent on transport protein StAR

and steroidogenic enzymes, including CYP11A1 and 3β-HSD (Van, 2013). StAR is an essential regulator of steroid hormone biosynthesis. It transports cholesterol into the mitochondria and present cholesterol to CYP11A1 (Miller, 2007). CYP11A1 is the key factor in converting cholesterol into pregnenolone and is abundant in the mitochondria of Leydig cells. 3β-HSD is another key enzyme that is involved in the conversion of pregnenolone into progesterone, which will be further converted to testosterone (Payne and Hales, 2004). 3β-HSD is specifically expressed in Leydig cells of testis, and its activity is the biomarker of testosterone synthesis in Leydig cells (Lai et al., 2014). In addition, 3β-HSD is regulated by luteinizing hormone and the negative feedback of downstream production of sex hormones (LaVoie and King, 2009; Hou and Hao, 2018; Steckelbroeck et al., 2004). The target of multiple drugs and endogenous substances is Leydig cells or 3β-HSD (Park et al., 2013). Reactions catalyzed by StAR, CYP11A1 and 3β-HSD are the rate-limiting steps in the synthesis of steroid hormones. Previous studies have shown that *C. tubulosa* and its alcoholic extracts could increase the expressions of proteins in the testosterone synthesis pathway of normal animals and thereby increase testosterone levels. However, there has been no systematic research conducted on reproductive injury model up till now. Our results showed that CPhGs could protect testicular morphology, improve circulating luteinizing hormone level and maintain testosterone level. The key enzymes, including CYP11A1 and 3β-HSD, in testosterone synthesis were up-regulated by CPhGs treatment. However, the level of StAR was not regulated by CPhGs. Previous study showed that *C. tubulosa* and echinacoside, a major component

of CPhGs, could reverse bisphenol A-induced abnormality in testicular structure and normalize serum testosterone. Treatment with *C. tubulosa* could increase expressions of the key steroidogenic enzymes including StAR, CYP11A1 and 3β-HSD, whereas echinacoside had no effect on StAR (Jiang et al., 2016), which was consistent with our findings. These data suggest that expression of StAR is regulated by other components of *C. tubulosa*, rather than CPhGs, while the major targets of CPhGs are CYP11A1 and 3β-HSD. Further studies are warranted to explore which components of *C. tubulosa* could act on StAR.

Our results demonstrated that CPhGs promoted the synthesis of testosterone by activating CYP11A1-3β-HSD pathway in Leydig cells. As a positive control drug, TP could improve the mating and erectile abilities of mice with reproductive injury. However, it didn't show protective effect on the testis. The serum level of testosterone in TP group exceeded the normal levels, which in turn inhibited expressions of enzymes involved in testosterone synthesis by a negative feedback loop. Although supplementing synthetic androgens to treat MSD can increase the testosterone content in the short term, the exact formulation, dosage and duration of treatment remain to be clarified and the safety profile also remains unclear. Traditional Chinese medicine is an effective alternative therapeutic drug for treating MSD.

5. Conclusions

Our results showed that CPhGs could ameliorate HCT-induced damage of reproductive function and protect mice testis from injury. Further experiments

demonstarted that CPhGs regulated synthesis of testosterone via CYP450-3β-HSD pathway. Our study provides experimental evidence that CPhGs extract is an alternative choice for clinical treatment of MSD.

Acknowledgements

This work was supported by the National Key Research and Development Plan [grant number 2017YFC1702400].

Conflict of interest statement

The authors declare that there is no conflict of interest.

Author contributions

Pengfei TU and Chun LI conceived and designed study; Qixin WANG and Jianteng DONG conducted majority of experiments; Wenji LU, Hao HE and Xiaoqian SUN contributed to hormone analysis and immunofluorescence staining; Qingqing SONG, Ke ZHANG contributed to HPLC analysis of CPhGs; Qixin WANG wrote the manuscript; Yong JIANG and Yong WANG edited the manuscript.

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