Chestnut polysaccharides benefit spermatogenesis through improvement in the expression of important genes

Shuai Yu1,2, Yong Zhao3, Fa-Li Zhang3, Ya-Qi Li4, Wei Shen3, Zhong-Yi Sun1,2

1Urology Department, Peking University Shenzhen Hospital, Shenzhen 518036, China
2Center for Reproductive Medicine, Peking University Shenzhen Hospital, Shenzhen 518036, China
3College of Life Sciences, Qingdao Agricultural University, Qingdao 266109, China
4Urology Department, Zaozhuang Hospital of Zaozhuang Mining Group, Zaozhuang 277100, China

Correspondence to: Zhong-Yi Sun; email: sunzhy199481@hotmail.com

Keywords: chestnut polysaccharides, spermatogenesis, gene expression, hormone

Received: December 17, 2019   Accepted: March 30, 2020   Published: June 21, 2020

ABSTRACT

Recently there has been a continuing worldwide decrease in the quality of human spermatozoa, especially in spermatozoa motility and concentration. Many factors are involved in this decline, and great efforts have been made to rescue spermatogenesis; however, there has been little progress in the improvement of sperm quality. Chestnuts are used in traditional Chinese medicine; their major active components are chestnut polysaccharides (CPs). CPs have many biological activities but their effects on spermatogenesis are unknown. The current investigation was designed to explore the impact of CPs on spermatogenesis and the underlying mechanisms. We demonstrated that CPs significantly increased sperm motility and concentration (4-fold and 12-fold, respectively), and improved seminiferous tubule development by increasing the number of germ cells after busulfan treatment. CPs dramatically rescued the expression of important genes and proteins (STRA8, DAZL, SYCP1, SYCP3, TNP1 etc.) in spermatogenesis. Furthermore, CPs increased the levels of hormone synthesis proteins such as CYP17A1 and HSD17B1. All the data suggested that CPs improved the testicular microenvironment to rescue spermatogenesis. With CPs being natural products, they may be an attractive alternative for treating infertile patients in the future. At the same time, the deep underlying mechanisms of their action need to be explored.

INTRODUCTION

During the past three decades, a growing number of people have been suffering from reproductive health issues leading to infertility. Many factors are involved in infertility, but in the male, a decrease in the quality of spermatozoa is a key factor. Many studies have revealed that environmental pollution and ionizing radiation played vital roles in the deterioration of spermatozoa quality (spermatozoa motility and concentration) [1–4]. Therefore, a feasible approach to protect fertilization capacity or to reverse any damage is necessary.

Recently, it has been found that polysaccharides have many biological functions. They pose anti-oxidative and anti-inflammatory functions through promoting the expression of antioxidant enzymes and decreasing the expression levels of inflammation factors [5–8], immunobiological activity [9, 10], and anti-cancer functions [11, 12]. Chestnuts are found throughout China and are used in traditional Chinese medicine [13]; they contain many nutrients that have several health benefits. Chestnut polysaccharides (CPs) include many monosaccharides such as glucose, rhamnose, arabinose, galactose, xylose, mannose, and fructose. CPs have been shown to have anticancer activity [14, 15], antifatigue effects [16], and antioxidative activity [17–21] via inhibiting tyrosinase and cancer cell proliferation; they also improve the ability of endurance and scavenge free radicals. Although CPs have many
biological functions, their effects on the recovery of spermatogenesis are unknown.

Spermatogenesis takes place in the seminiferous tubules of the testis; it includes spermatocyte proliferation, spermatogonial differentiation into spermatocytes, spermatid production, and sperm maturation. Many factors are involved in regulating spermatogenesis such as genes, hormones, and epigenetic regulators [22–24]. The most important genes include VASA, DAZL, and DMCI. VASA is a germ cell marker important for germ cell proliferation and differentiation, and VASA mutation results in the cessation of germ cell differentiation [25]. DAZL serves as a gateway in oogenesis and spermatogenesis, and the abnormal expression of DAZL will affect the initiation of gametogenesis [26]. DMCI plays an important role in spermatogenesis, and its mutation leads to obstacles in male sterility [27].

Hormones such as testosterone and estrogen play essential roles in regulating spermatogenesis [28]. Many proteins such as cytochrome P450, cholesterol side-chain cleavage enzyme (CYP11A1), hydroxy-Δ-5-steroid dehydrogenase 3β-steroid Δ-isomerase 1 (HSD3β1), cytochrome P450 17α-hydroxylase/C17, and 20-lyase (CYP17A1) [29, 30] are involved in the synthesis of testosterone and estrogen.

Although CPs have been shown to be beneficial for human health, the effects on spermatogenesis and the underlying mechanisms are not understood. The aim of this study was to explore the means of CPs improve spermatogenesis and the underlying mechanisms.

**RESULTS**

**CPs increased sperm motility and sperm concentration**

CPs alone did not change murine sperm motility (Figure 1A), however, sperm concentration was increased significantly (Figure 1B). Busulfan dramatically disrupted spermatogenesis by decreasing sperm motility and concentration almost to a level of infertility (Figure 1A–1C). However, busulfan plus CPs significantly increased sperm motility and concentration, especially in the B+CPs 0.10 mg/kg group (Figure 1A, 1B). Busulfan impaired spermatogenesis through decreasing the number of spermatogenetic cells and disrupting the structure of seminiferous tubules, as revealed by testicular histopathology (Figure 1D). CPs alone did not change the structure of the seminiferous tubules; however, busulfan plus CPs dramatically improved seminiferous tubules through an increase in the number of germ cells, especially in the B+CPs 0.10 mg/kg group (Figure 1D). Testicular histopathology confirmed the data for sperm motility and concentration. We then set out to explore how CPs improved spermatogenesis. The concentration of 0.10 mg/kg CPs produced a profound improvement, therefore this dose was used for further investigations. Body weights and organ indexes are shown in Table 1.

**CPs improved the expression of important genes involved in spermatogenesis in mouse testes**

First, testicular tissue transcriptomes were determined after busulfan and/or CPs treatments to search for gene expression patterns. Principal components analysis (PCA) showed that the busulfan and control groups were well separated, which suggested that the busulfan treatment produced profound effects on gene expression (Figure 2A). The B+CPs 0.10 mg/kg group overlapped with the control group, which suggested that the CP 0.10 mg/kg group recovered the gene expression that was changed by busulfan (Figure 2A). In total, 52 459 genes were found in the testicular tissues in the current investigation. A total of 15 738 genes were differentially expressed in the Control-vs-B group including 10 136 genes down-regulated and 5602 genes up-regulated. In addition, 13 796 genes were differentially expressed in the B-vs-B+CPs 0.10 mg/kg group including 4398 genes down-regulated and 9398 genes up-regulated (Figure 2B). The functions of these differentially expressed genes (DEGs) were displayed by GO analysis. In the comparison of the Control-vs-B group, the genes decreased by busulfan were enriched during spermatogenesis, germ cell development, and other sperm function related groups; the genes that were increased were in other functional groups (non-sperm related groups). But, in the comparison of the B-vs-B+CPs 0.10 mg/kg group, the genes increased by treatment B+CPs 0.10 were enriched during spermatogenesis, germ cell development, and other sperm function related groups, while the decreased genes were enriched in other functional groups (non-sperm related groups). The data suggested that treatment B+CPs 0.10 reversed the disruption of spermatogenesis caused by busulfan (Figure 2C).

Then we compared the DEGs in Control-vs-B and B-vs-B+CPs 0.10. It was interesting that most of the decreased DEGs in Control-vs-B were increased in B-vs-B+CPs 0.10; while most of the increased DEGs in Control-vs-B were decreased in B-vs-B+CPs 0.10 (Figure 3A). Spermatogenesis is a complex process involving many genes, such as VASA, CREM, SYCP3, DAZL, REC8, PGK2, DMCI, SYCP1, STRA8, and ZFP42. Most of the DEGs that were decreased in Control-vs-B while being increased in B-vs-B+CPs 0.10, were involved in spermatogenesis or reproduction, including STRA8, DAZL, SYCP1, SYCP3, TNP1, etc. (Figure 3B–3D and Supplementary Figure 1).
Figure 1. Chestnut polysaccharides (CPs) improved sperm quality. (A) Sperm motility (% of total cells; grade A + grade B). X-axis represents the treatment groups; Y-axis represents sperm motility. Data are represented as mean ± SEM, *P < 0.05, **P < 0.01. (B) Sperm concentration. X-axis represents the treatment groups; Y-axis represents sperm concentration (million/ml). Data are represented as mean ± SEM, *P < 0.05, **P < 0.01. (C) Photos of sperm quality. (D) Histopathology photos of mouse testes.
Table 1. Mouse body parameters.

|                  | Control  | CP 0.01µg/kg | CP 0.10µg/kg | CP 1.00µg/kg | B         | B+ CP 0.01µg/kg | B+ CP 0.10µg/kg | B+ CP 1.00µg/kg |
|------------------|----------|--------------|--------------|--------------|-----------|----------------|----------------|----------------|
| Body weight (g)  | 36.27±1.45 | 37.49±0.92   | 36.59±1.16   | 36.88±0.72   | 33.80±1.04 | 26.13±1.51**   | 30.72±1.03     | 31.54±1.00     |
| Kidney index     | 1.65±0.052 | 1.67±0.04    | 1.63±0.04    | 1.68±0.03    | 1.83±0.06  | 1.50±0.05*      | 1.67±0.04      | 1.72±0.04      |
| Spleen index     | 0.49±0.06  | 0.66±0.15*   | 0.39±0.03    | 0.44±0.05    | 0.36±0.02  | 0.61±0.08       | 0.39±0.02      | 0.38±0.01      |
| Liver index      | 6.06±0.13  | 6.30±0.20    | 6.00±0.11    | 5.76±0.14    | 6.34±0.27  | 5.62±0.09       | 5.57±0.12'     | 5.73±0.13      |

Data is presented as mean ± SEM. * indicate a significant difference compared with B group (P < 0.05), ** indicate a significant difference compared with B group (P < 0.01). Index (organ weight/body weight).

Note: B=busulfan; CPs=Chestnut polysaccharides.

Quantitative RT-PCR (qRT-PCR) was used to verify the RNA-seq data. The expression of eight DEGs was confirmed and the results revealed a similar trend for RNA-seq and qRT-PCR (Figure 4).

Furthermore, the protein levels of important genes involved in spermatogenesis were determined in the current investigation. Many of these proteins formed a network to regulate spermatogenesis (Figure 5A).

![Figure 2. Overview of the RNA-seq data for mouse testes. (A) Principal components analysis (PCA). (B) The heatmap of differentially expressed genes (DEGs). (C) GO enrichment analysis of up-regulated and down-regulated genes in biological processes in the Control-vs-B and B-vs-B+CPs 0.10 groups, respectively.](image-url)
Busulfan alone decreased the protein levels of DAZL, PGK2, CREM, and VASA, while B+CPs 0.10 increased these protein levels as revealed by Western blot analysis (Figure 5B, 5C). Immunohistochemistry (IHC) confirmed the protein changes during spermatogenesis. The number of positive cells staining for VASA and DAZL were decreased by busulfan while they were increased by B+CPs 0.10 as seen by IHC (Figure 5D–5F) which confirmed the Western blot analysis data. The data in this section suggested that CPs rescued mouse spermatogenesis through an improvement in the levels of relevant genes and proteins.

**CPs increased the level of proteins involved in hormone production**

Testosterone and estrogen play vital roles in spermatogenesis and many proteins (or enzymes) are involved in the production of these hormones. We determined these proteins by IHC and found that the levels of CYP17A1, and HSD17β1 were decreased by busulfan while elevated by B+CPs 0.10 (Figure 6A–6C). The data here suggested that CPs 0.10 may improve the local hormones to enhance spermatogenesis.

**DISCUSSION**

Chestnuts have been used as a traditional food and medicine for millennia in China due to their health advantages [31]. CPs, key substances in chestnut, have been investigated extensively; they are known to inhibit cancer cell proliferation, enhance endurance performance, and to reduce ROS levels [14–21]. However, the effects of CPs on the recovery of spermatogenesis are not understood. Therefore, in the present study we aimed to investigate the rescuing effects of CPs on spermatogenesis after busulfan treatment. We found that busulfan disrupted spermatogenesis by

---

**Figure 3. Differentially expressed genes (DEGs) involved in spermatogenesis.** (A) The Venn diagram of up-regulated and down-regulated genes in the intersection of the Control-vs-B and B-vs-B+CPs 0.10 groups. (B) The representative genes in spermatogenesis. (C) The heatmap of 20 representative genes in spermatogenesis. (D) GO enrichment analysis of the DEGs in biological processes.
decreasing the motility and concentration of mouse sperm, through reducing the expression of genes and proteins involved in spermatogenesis, which was similar to the findings of previous studies [32–35]. On the other hand, CPs increased the motility and concentration of mouse sperm, especially at a treatment level of 0.10 mg/kg.

Subsequently, we explored the underlying mechanisms of CPs in rescuing mouse spermatogenesis. Gene

Figure 4. The expression of important genes in spermatogenesis by RNA-seq, and confirmed by q-RT-PCR. (A) DAZL; (B) PRM1; (C) ZFP42; (D) REC8; (E) STRA8; (F) SYCP1; (G) SYCP3; and (H) TNP1. The results are presented as mean ± SEM, **P < 0.01.
Figure 5. The expression of important proteins in spermatogenesis. (A) The network of important genes/proteins in spermatogenesis. (B) The protein levels of VASA, DAZL, PGK2, and CREM using Western blot analysis. (C) The relative abundance of VASA, DAZL, PGK2, and CREM in different groups, ns is stand for no difference, *P<0.05, **P < 0.01. (D) The immunofluorescence staining images of VASA and DAZL. (E) The quantitative data for VASA in immunofluorescence staining. The results are presented as mean ± SEM, **P < 0.01. (F) The quantitative data for DAZL in immunofluorescence staining. The results are presented as mean ± SEM, **P < 0.01.
expression within testicular samples was determined by RNA-seq analysis. It was interesting to notice that 9043 DEGs were decreased by busulfan while increased by CPs, and 4248 DEGs were increased by busulfan while decreased by CPs. GO enrichment analysis revealed that these 9043 DEGs, that were decreased by busulfan while being increased by CPs, were enriched in spermatogenesis related functional pathways. But the 4248 DEGs, those were increased by busulfan while decreased by CPs, were enriched in other functional pathways (non-sperm related groups). The data indicated that CPs may regulate gene expression machinery to improve spermatogenesis.

Moreover, we explored the effect of CPs on hormone production pathways in mouse testes, because of the critical role that hormones play in spermatogenesis [28, 36, 37]. CPs increased the protein levels of CYP17A1 and HSD17β1 which were decreased by busulfan.

CYP17A1 and HSD17β1 are important for the synthesis of hormones such as testosterone and estrogen, which are essential for spermatogenesis. The data suggested that CPs benefit the testicular microenvironment to improve spermatogenesis.

In conclusion, the results of this study suggested that CPs improved spermatogenesis by increasing sperm motility and concentration. The underlying mechanisms involved CPs regulation of gene expression machinery to increase the expression levels of genes and proteins involved in spermatogenesis and to improve the testicular microenvironment to rescue spermatogenesis. A decrease in the quality of spermatozoa is a key factor that caused infertility. Therefore, looking for a feasible approach to protect fertilization capacity or to reverse any damage in testes is necessary. CPs being as natural products, they may be an attractive alternative method for treating infertile patients in the future.

Figure 6. Effects of CPs on hormone synthesis exposed to busulfan. (A) The representative images of HSD17β1 and CYP17 in the Control, CPs 0.10, B, and B+CPs 0.10 groups. (B) The fluorescence intensity analysis of CYP17 in different groups (Control: 19.28 ± 0.9276, CPs 0.10: 18.97 ± 0.5916, B: 11.8 ± 0.4849, B+CPs 0.10: 22.8 ± 0.8389) (C) The fluorescence intensity analysis of HSD17β1 in different groups (Control: 16.38 ± 0.3876, CPs 0.10: 15.88 ± 0.537, B: 9.892 ± 0.2834, B+CPs 0.10: 20.42 ± 0.6048). The results are presented as mean ± SEM. **p < 0.01.
**MATERIALS AND METHODS**

**Mouse experimental environment**

Male ICR mice were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). The mice were fed in constant temperature (22-23°C) rooms with a 12 h light: 12 h dark photoperiod and had free access to food (chow diet) and water throughout the period of study. All procedures used in this study were in accordance with the guidance of the Ethics Committee of Qingdao Agricultural University.

**Mouse treatments and sample collection**

Busulfan was used as a model compound to create an infertile animal model as it has been frequently used in recent published articles [38, 39]. CPs were brought from Wo Te Lai Si biotechnology co., LTD (Lan Zhou, China). We set out to examine the remedial efficacy of CPs on busulfan-exposed murine spermatogenesis and to examine the underlying mechanisms. Male mice under treatment were exposed to busulfan at a concentration of 40 mg/kg and CPs treatment via oral gavage. The concentrations of CPs used in this investigation were 0.01-1.00 mg/kg. The different concentrations of CPs solutions were freshly prepared every day in ultrapure water. There were 8 treatments (10 mice/treatment): (1) vehicle control (ultrapure water); (2) CPs 0.01 mg/kg (CPs 0.01); (3) CPs 0.10 mg/kg (CPs 0.10); (4) CPs 1.00 mg/kg (CPs 1.00); (5) busulfan (B); (6) busulfan + CPs 0.01 mg/kg (B+CPs 0.01); (7) busulfan + CPs 0.10 mg/kg (B+CPs 0.10); (8) busulfan + CPs 1.00 mg/kg (B+CPs 1.00). The volume of fluid used for the oral gavage was 0.10 ml/mouse/day. In this study we chose 3-week-old mice, the gavage took place every morning and lasted for 5 weeks. After 5 weeks, the mice were humanely slaughtered and we collected tissue samples for analysis such as testis, blood, and sperm.

**Evaluation of sperm motility and concentration**

Sperm motility was analyzed during sample collection. Sperm were collected from the epididymis then cultured in DMEM/F12 (Gibco, 8119172, U.S.A.) with 10% FBS (Gibco, 10099-141, USA) medium and incubated at 37.5°C on a heated stage for 5 min, counting boards were prepared in advance. Then samples were placed in a counting chamber [35] and sperm motility and concentration were analyzed using an SCA sperm class analyzer. We used WHO standards for the analysis of sperm motility: sperm progressing at above 22 μm/s were defined as grade A; the curvilinear velocity of sperm under 22 μm/s and greater than 5 μm/s were defined as grade B; the curvilinear velocity of sperm under 5 μm/s were defined as grade C and immotile sperm were defined as grade D [40-42]. Concentration was evaluated during motility and sperm were diluted with medium during the process of evaluation [40, 41].

**RNA extraction and RNA-seq and bioinformatics analysis**

Total RNA was extracted from collected testis samples using a kit (Thermo Fisher 12183018A, USA) and according to the manual instructions [43]. Sequencing was conducted using an Illumina HiSeq2000 system (Novogene Co. Ltd., Beijing, China). FastQC (v0.11.8) and Fastp (v0.19.5) were adopted to assess the quality of the raw data and to remove low quality data, adapter, and poly-N sequences, respectively. Clean data were used in subsequent analyses [44]. DEGs were analyzed using the DESeq2 package. R Bioconductor/cluster Profiler package and Gene Ontology (GO) were used to analyze DEG functional profiles [45]. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was used to predict protein interaction network [46].

**Quantitative real-time PCR**

Total RNA was reverse transcribed using the TransScript One-Step gDNA Removal Kit and cDNA Synthesis Kit (TransGen, AT311-03, Beijing, China). To evaluate the changes in mRNA level, we conducted quantitative real-time PCR (qRT-PCR) using transcribed cDNA. qRT-PCR was conducted using a Light Cycler real-time PCR instrument (LC480, Roche, Basel, Switzerland) using Light Cycler SYBR Green I Master Mix according to the manufacturer’s instructions. A reaction system volume of 24 μl was used in this study containing 2 μl cDNA, 12 μl of SYBR green master mix, 1.2 μl of primer mix including forward and reverse primer genes, and 8.8 μl of RNase-free water. The PCR conditions of a previous study were followed [47]. We quantified actin as an internal reference when analyzing gene expression. Relative expression abundance was calculated using the method given in a previous study [48]. Gene primer sequences are listed in Supplementary Table 1.

**H&E and immunofluorescence**

Collected testes were fixed in 4% paraformaldehyde and kept in a refrigerator at 4°C overnight, then subsequently stored in different concentrations of dehydrating solutions. The dehydrated testicular samples were then embedded in paraffin and the resulting paraffin blocks were sectioned at 5 μm thickness following standard histological procedures. Sections were stained with H&E, following the procedure of a previous study [49, 50]. Testicular sections were also used for IHC; the
sections were prepared and subjected to xylene and ethanol solution followed by antigen retrieval. Sections were then blocked with blocking buffer [3% bovine serum albumin (BSA, Solarbio, A8020, Beijing, China), 10% normal goat serum in TBS buffer] at room temperature for 30 min. Each section was incubated with primary antibodies (Supplementary Table 2) and secondary antibodies (Beyotime, A0516, Nantong, China) then sections were imaged under an Olympus fluorescence microscope (Olympus, BX51, Tokyo, Japan) [51].

Western blot analysis

Testicular proteins were extracted using the process described below. Samples were submerged in cold RIPA buffer and a crusher was used to treat the samples twice at a cold temperature. The protein lysates were centrifuged at 10000 rcf/min for 10 min then the supernatant was collected and added to SDS-loading buffer, and boiled for 5 min in water till the protein was completely denatured. Protein samples were mixed evenly before loading onto 10% SDS polyacrylamide electrophoresis gels and processed at 100V for 1.5 h; the specific time depended on the electrophoresis situation. The proteins on the gels were transferred to a polyvinylidene fluoride membrane. Subsequently, membranes were blocked with skimmed milk or 5% bovine serum albumin at a cold temperature for at least an hour, followed by 3 washes with Tris-buffered saline, 0.1% Tween 20 (TBST). We then used primary antibodies to incubate membranes in the refrigerator overnight at 4°C. On the second day, membranes were washed 3 times with TBST and incubated with the HRP-labeled secondary antibody (Beyotime, A0208, Nantong, China) for 1 h at room temperature. After 3 washes with TBST, the membranes were imaged [51]. The images were analysed using the AIC. Alpha View software.

Statistical analysis

All experiments were repeated at least 3 times and results were expressed as the mean ± SEM. SPSS software one-way analysis of variance (ANOVA) following by LSD multiple comparison test was used for data analysis and we defined $P < 0.05$ as a significant difference, while $P < 0.01$ was a highly significant difference.

AUTHOR CONTRIBUTIONS

ZS, WS, and YZ designed the study. SY, FZ, and YL performed the animal studies, finished all the experiments, analyzed the data, and prepared the draft figures and tables. ZS, WS and YZ prepared the manuscript. All authors have reviewed the final manuscript.

CONFLICTS OF INTEREST

None of the authors have any conflicts of interest.

FUNDING

This work was supported by the Shenzhen Sanning Project (SZSM201612066) and Taishan Scholar Construction Foundation of Shandong Province (ts20190946).

REFERENCES

1. De Iuliis GN, Newey RJ, King BV, Aitken RJ. Mobile phone radiation induces reactive oxygen species production and DNA damage in human spermatozoa in vitro. PloS One. 2009; 4:e6446. https://doi.org/10.1371/journal.pone.0006446 PMID:19649291

2. Agarwal A, Deepinder F, Sharma RK, Ranga G, Li J. Effect of cell phone usage on semen analysis in men attending infertility clinic: an observational study. Fertil Steril. 2008; 89:124–28. https://doi.org/10.1016/j.fertnstert.2007.01.166 PMID:17482179

3. Gorpinchenko I, Nikitin O, Banyra O, Shulyak A. The influence of direct mobile phone radiation on sperm quality. Cent European J Urol. 2014; 67:65–71. https://doi.org/10.5173/ceju.2014.01.art14 PMID:24982785

4. Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ Jr, Jégou B, Jensen TK, Jouannet P, Keiding N, Leffers H, McLachlan JA, Meyer O, et al. Male reproductive health and environmental xenoestrogens. Environ Health Perspect. 1996 (Suppl 4); 104:741–803. https://doi.org/10.1289/ehp.96104s4741 PMID:8880001

5. Han J, Guo D, Sun XY, Wang JM, Ouyang JM, Gui BS. Repair effects of astragalus polysaccharides with different molecular weights on oxidatively damaged HK-2 cells. Sci Rep. 2019; 9:9871. https://doi.org/10.1038/s41598-019-46264-y PMID:31285477

6. Xu Z, Wang H, Wang B, Fu L, Yuan M, Liu J, Zhou L, Ding C. Characterization and antioxidant activities of polysaccharides from the leaves of lilium lancifolium thunb. Int J Biol Macromol. 2016; 92:148–55. https://doi.org/10.1016/j.ijbiomac.2016.07.028 PMID:27397720
7. Duan GL, Yu XB. Isolation, purification, characterization, and antioxidant activity of low-molecular-weight polysaccharides from sparrisia latifolia. Int J Biol Macromol. 2019; 137:1112–20. https://doi.org/10.1016/j.ijbiomac.2019.06.177 PMID:31271800

8. Gao J, Zhang T, Jin ZY, Xu XM, Wang JH, Zha XQ, Chen HQ. Structural characterisation, physicochemical properties and antioxidant activity of polysaccharide from Lilium lancifolium Thunb. Food Chem. 2015; 169:430–8. https://doi.org/10.1016/j.foodchem.2014.08.016 PMID:25236248

9. Jiang Y, Qi X, Gao K, Liu W, Li N, Cheng N, Ding G, Huang W, Wang Z, Xiao W. Relationship between molecular weight, monosaccharide composition and immunobiologic activity of astragalus polysaccharides. Glycocon J. 2016; 33:755–61. https://doi.org/10.1007/s10719-016-9669-z PMID:27129881

10. Peng Q, Li M, Xue F, Liu H. Structure and immunobiological activity of a new polysaccharide from bletilla striata. Carbohydr Polym. 2014; 107:119–23. https://doi.org/10.1016/j.carbpol.2014.02.042 PMID:24702926

11. Zhao YM, Wang J, Wu ZG, Yang JM, Li W, Shen LX. Extraction, purification and anti-proliferative activities of polysaccharides from lentinus edodes. Int J Biol Macromol. 2016; 93:136–44. https://doi.org/10.1016/j.ijbiomac.2016.05.100 PMID:27246376

12. Sohretoglu D, Huang S. Ganoderma lucidum polysaccharides as an anti-cancer agent. Anticance Agents Med Chem. 2018; 18:667–74. https://doi.org/10.2174/1871520617666171113121246 PMID:29141563

13. Liu HX, Jiang WG, Song CW, Peng L, Fang NB, Yu SG. The effect of Chinese Chestnut on diabetes model mice. J Mathematical Med. 2012; 25:57–61.

14. Tang M, Hou F, Wu Y, Liu Y, Ouyang J. Purification, characterization and tyrosinase inhibition activity of polysaccharides from chestnut (castanea mollissima bl.) kernel. Int J Biol Macromol. 2019; 131:309–14. https://doi.org/10.1016/j.ijbiomac.2019.03.065 PMID:30872058

15. Barbat A, Gloaguen V, Moine C, Sainte-Catherine O, Kraemer M, Rogniaux H, Ropartz D, Krausz P. Structural characterization and cytotoxic properties of a 4-O-methylglucuronoxylan from castanea sativa. Z. Evidence of a structure-activity relationship. J Nat Prod. 2008; 71:1404–09.

https://doi.org/10.1021/np800207g PMID:18646856

16. Li QY, Yang YJ, Lin F, Peng J, Dai CG, Duan YF. Purification, structural analysis and antifatigue assay of polysaccharide from Castanea mollissima blume. J Food Sci Biotechnol. 2013; 32:767–72.

17. Li RF, Diao HJ, Peng YS, Chang XD. Study on the antioxidant activity of polysaccharide from Chestnut. Food Res Dev. 2011; 32:21–25.

18. Wang X, Kong Y, Liu B, Wang J. Isolation, purification and antioxidant activities of polysaccharides from the leaves of Castanea mollissima Bl. J Liaoning Uni. 2011; 38:54–59.

19. He LL, Wang X, Liu B, Wang J, Lan LY. Study on isolation, purification and antioxidant activities of polysaccharides from seeds of Castanea mollissima BL. Food Machinery. 2010; 26:72–75.

20. Shao TT, Zhang HH, Duan YQ, Sun GB, Sun XB, Zhang R. Extraction on chestnut polysaccharide by subcritical water and analysis on free radical scavenging capacity. Food Sci Technol. 2012; 12:156–160.

21. Chen G, Kan J. Ultrasound-assisted extraction, characterization, and antioxidant activity in vitro and in vivo of polysaccharides from chestnut rose (Rosa roxburghii tratt) fruit. J Food Sci Technol. 2018; 55:1083–92. https://doi.org/10.1007/s13197-017-3023-8 PMID:29487451

22. Neto FT, Bach PV, Najari BB, Li PS, Goldstein M. Spermatogenesis in humans and its affecting factors. Semin Cell Dev Biol. 2016; 59:10–26. https://doi.org/10.1016/j.semcdb.2016.04.009 PMID:27143445

23. Schlatt S, Ehmcke J. Regulation of spermatogenesis: an evolutionary biologist’s perspective. Semin Cell Dev Biol. 2014; 29:2–16. https://doi.org/10.1016/j.semcdb.2014.03.007 PMID:24685618

24. Bai W, Yang W, Wang W, Wang Y, Liu C, Jiang Q, Hua J, Liao M. GED: a manually curated comprehensive resource for epigenetic modification of gametogenesis. Brief Bioinform. 2017; 18:98–104. https://doi.org/10.1093/bib/bbw007 PMID:26851225

25. Tanaka SS, Toyooka Y, Akasu R, Katoh-Fukui Y, Nakahara Y, Suzuki R, Yokoyama M, Noce T. The mouse homolog of drosophila vasa is required for the development of male germ cells. Genes Dev. 2000; 14:841–53. PMID:10766740
26. Gill ME, Hu YC, Lin Y, Page DC. Licensing of gametogenesis, dependent on RNA binding protein DAZL, as a gateway to sexual differentiation of fetal germ cells. Proc Natl Acad Sci USA. 2011; 108:7443–48. https://doi.org/10.1073/pnas.1104501108 PMID: 21504946

27. Chen J, Cui X, Jia S, Luo D, Cao M, Zhang Y, Hu H, Huang K, Zhu Z, Hu W. Disruption of dmc1 produces abnormal sperm in medaka (oryzias latipes). Sci Rep. 2016; 6:30912. https://doi.org/10.1038/srep30912 PMID: 27480068

28. Walker WH. Testosterone signaling and the regulation of spermatogenesis. Spermatogenesis. 2011; 1:116–20. https://doi.org/10.4161/spmg.1.2.16956 PMID: 22319659

29. Ye L, Li X, Li L, Chen H, Ge RS. Insights into the development of the adult Leydig cell lineage from stem Leydig cells. Front Physiol. 2017; 8:430. https://doi.org/10.3389/fphys.2017.00430 PMID: 28701961

30. Payne AH, Hales DB. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev. 2004; 25:947–70. https://doi.org/10.1210/er.2003-0030 PMID: 15583024

31. Iwamoto T, Hiraku Y, Oikawa S, Mizutani H, Kojima M, Kawanishi S. DNA intrastrand cross-link at the 5′-GA-3′ sequence formed by busulfan and its role in the cytotoxic effect. Cancer Sci. 2004; 95:454–58. https://doi.org/10.1111/j.1349-7006.2004.tb03231.x PMID: 15132775

32. Vasiliausha SR, Beltrame FL, de Santi F, Cerri PS, Caneguiu BH, Sasso-Cerri E. Seminiferous epithelium damage after short period of busulphan treatment in adult rats and vitamin B12 efficacy in the recovery of spermatogonial germ cells. Int J Exp Pathol. 2016; 97:317–28. https://doi.org/10.1111/iep.12195 PMID: 27578607

33. Jung SW, Kim HJ, Lee BH, Choi SH, Kim HS, Choi YK, Kim JY, Kim ES, Hwang SH, Lim KY, Kim HC, Jang M, Park SK, et al. Effects of korean red ginseng extract on busulfan-induced dysfunction of the male reproductive system. J Ginseng Res. 2015; 39:243–49. https://doi.org/10.1016/j.jgr.2015.01.002 PMID: 26199556

34. Nasimi P, Vahdati A, Tabandeh MR, Khatamsaz S. Cytoprotective and anti-apoptotic effects of satureja khuzestanica essential oil against busulfan-mediated sperm damage and seminiferous tubules destruction in adult male mice. Andrologia. 2016; 48:74–81. https://doi.org/10.1111/and.12421 PMID: 26011020

35. Panahi S, Abdollahifar MA, Aliaighaei A, Nazarian H, Paktinat S, Abdi S, Farahani RM. Application of stereological methods for unbiased estimation of sperm morphology in the mice induced by busulfan. Anat Cell Biol. 2017; 50:301–05. https://doi.org/10.5115/acb.2017.50.4.301 PMID: 29354302

36. Walker WH. Non-classical actions of testosterone and spermatogenesis. Philos Trans R Soc Lond B Biol Sci. 2010; 365:1557–69. https://doi.org/10.1098/rstb.2009.0258 PMID: 20403869

37. Alves MG, Rato L, Carvalho RA, Moreira PL, Socorro S, Oliveira PF. Hormonal control of Sertoli cell metabolism regulates spermatogenesis. Cell Mol Life Sci. 2013; 70:777–93. https://doi.org/10.1007/s00018-012-1079-1 PMID: 23011766

38. Panahi M, Keshavarz S, Rahamanifar F, Tamadon A, Mehrabani D, Karimaghaj S, Sepehrimanesh M, Aqqabaha H. Busulfan induced azoospermia: stereological evaluation of testes in rat. Vet Res Forum. 2015; 6:273–78. PMID: 26973761

39. Choi YJ, Ok DW, Kwon DN, Chung JI, Kim HC, Yeo SM, Kim T, Seo HG, Kim JH. Murine male germ cell apoptosis induced by busulfan treatment correlates with loss of c-kit-expression in a fas/FasL- and p53-independent manner. FEBS Lett. 2004; 575:41–51. https://doi.org/10.1016/j.febslet.2004.08.034 PMID: 15388331

40. Zhao Y, Zhang WD, Liu XQ, Zhang PF, Hao YN, Li L, Chen L, Shen W, Tang XF, Min LJ, Meng QS, Wang SK, Yi B, Zhang HF. Hydrogen sulfide and/or ammonia reduces spermatogenesis through AMPK/ATK related pathways. Sci Rep. 2016; 6:37884. https://doi.org/10.1038/srep37884 PMID: 27883089

41. Zhang W, Zhao Y, Zhang P, Hao Y, Yu S, Min L, Li L, Ma D, Chen L, Yi B, Tang X, Meng Q, Liu L, et al. Decrease in male mouse fertility by hydrogen sulfide and/or ammonia can be inherited. Chemosphere. 2018; 194:147–57. https://doi.org/10.1016/j.chemosphere.2017.11.164 PMID: 29202267

42. Lu JC, Huang YF, Lü NQ. [WHO laboratory manual for the examination and processing of human semen: its applicability to andrology laboratories in China]. Zhonghua Nan Ke Xue. 2010; 16:867–71. PMID: 21243747
43. Chen S, Zhou Y, Chen Y, Gu J. Fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics. 2018; 34:i884–90. https://doi.org/10.1093/bioinformatics/bty560 PMID:30420386

44. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS. 2012; 16:284–87. https://doi.org/10.1089/omi.2011.0118 PMID:22455463

45. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 2003; 13:2498–504. https://doi.org/10.1101/gr.1239303 PMID:14597658

46. Zhang T, Zhou Y, Li L, Zhao Y, De Felici M, Reiter RJ, Shen W. Melatonin protects prepuberal testis from deleterious effects of bisphenol a or diethylhexyl phthalate by preserving H3K9 methylation. J Pineal Res. 2018; 65:e12497. https://doi.org/10.1111/jpi.12497 PMID:29655234

47. Wang YF, Sun XF, Han ZL, Li L, Ge W, Zhao Y, De Felici M, Shen W, Cheng SF. Protective effects of melatonin againstnicotine-induced disorder of mouse early folliculogenesis. Aging (Albany NY). 2018; 10:463–80. https://doi.org/10.18632/aging.101405 PMID:29615536

48. Zhang LJ, Chen B, Feng XL, Ma HG, Sun LL, Feng YM, Liang GJ, Cheng SF, Li L, Shen W. Exposure to brefeldin a promotes initiation of meiosis in murine female germ cells. Reprod Fertil Dev. 2015; 27:294–303. https://doi.org/10.1071/RD13281 PMID:24209976

49. Zhang P, Chao H, Sun X, Li L, Shi Q, Shen W. Murine folliculogenesis in vitro is stage-specifically regulated by insulin via the akt signaling pathway. Histochem Cell Biol. 2010; 134:75–82. https://doi.org/10.1007/s00418-010-0708-8 PMID:20495820

50. Liu H, Zhu R, Liu C, Ma R, Wang L, Chen B, Li L, Niu J, Zhao D, Mo F, Fu M, Brömme D, Zhang D, Gao S. Evaluation of decalcification techniques for rat femurs using HE and immunohistochemical staining. Biomed Res Int. 2017; 2017:9050754. https://doi.org/10.1155/2017/9050754 PMID:28246608

51. Wang Y, Zhao Y, Yu S, Feng Y, Zhang H, Kou X, Chu M, Cui L, Li L, Zhang P, Shen W, Min L. Regulation of steroid hormones and energy status with cysteamine and its effect on spermatogenesis. Toxicol Appl Pharmacol. 2016; 313:149–58. https://doi.org/10.1016/j.taap.2016.10.025 PMID:27815134
Supplementary Figure 1. DEGs involved in spermatogenesis. (A) The Venn diagram of up-regulated and down-regulated genes in the intersection of the Control-vs-B and B-vs-B+CPs 0.10 groups. (B) The representative genes in spermatogenesis. (C) GO enrichment analysis of the DEGs in molecular function and cellular components in the intersection of the Control-vs-B and B-vs-B+CPs 0.10 groups.
### Supplementary Tables

#### Supplementary Table 1. Primers used for quantitative RT-PCR.

| Genes | GenBank   | Forward primer sequences       | Reverse primer sequences       |
|-------|-----------|--------------------------------|--------------------------------|
| β-actin | NM_007393.5 | ACCTCAGTACACCTGATGA            | CCACATAGCACAGCTTCTCTT          |
| DAZL  | NC_000003.12 | TCCTGACTTGTGGGTGCTG          | CCACCTCGAGGTCTTACC            |
| PRMI  | NC_000082.6  | AGCAAAACGAGGAGCATG            | CTTGCTATATTCTGTGCATCTAG       |
| ZFP42 | NC_000074.6  | TCCATCGCATAGTCCTCAACAG        | TAACTGATTCTCTGCCATG           |
| REC8  | NC_000080.6  | GGTAAGACCTCGAGGAAA           | GCGGAGAGATAGAGGGAGAA         |
| STRA8 | NC_000072.6  | GTTTCTGCGTGTCCACAAG         | CACCCGAGCTCAAGCTTC            |
| SYCP1 | NC_000069.6  | CTCAAGGAAAACCTGTGCTAGAT      | TGCAACAGGAAGTTCTCAGA         |
| SYCP3 | NC_000076.6  | AGCAGAGAGCTTGGTGCGGG         | TCCGGTGAGCTGTCGCTGTC         |
| TNP1  | NC_000067.6  | AGCCGCAAGCTAAGACTCA         | CTCTCTGACGCCCTTGTGA          |

#### Supplementary Table 2. Information on the antibodies used in this paper.

| Antibodies (IF/WB) | Vendor | Dilution |
|--------------------|--------|----------|
| HSD17β1            | Bioss (bs-6603R) | 1:150    |
| CYP17A1            | Bioss (bs-6695R) | 1:150    |
| PGK2 (WB)          | Sangon (D221803) | 1:500    |
| CREM (WB)          | Sangon (D152356 ) | 1:500    |
| VASA (IF/WB)       | Abcam(ab13840)  | 1:150/1:1000 |
| DAZL (IF/WB)       | Abcam (ab2920)  | 1:150/1:1000 |
| β-ACTIN (WB)       | Abcam (ab3280)  | 1:1000  |