Chestnut Polysaccharides Rescue Impaired Spermatogenesis by Adjusting Gut Microbiota

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Research

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Abstract

Background: Our previous study confirmed the beneficial effects of chestnut polysaccharides on the spermatogenesis process, but the exact mechanism is not clear. Several studies have demonstrated the importance of a balanced gut microbiota in maintaining normal reproductive function. In this study, we investigated the biological functions of chestnut polysaccharides from the perspective of gut microbiota function, expecting to elaborate the specific mechanism of chestnut polysaccharides beneficial to spermatogenesis.

Results: Compared with the Vehicle group, germ cell quantity, intestinal structure and tight junction of intestinal tissue were altered in the Busulfan-treated mice, and the intestinal microbiota was also disturbed at several levels, including phylum and genus. The *Firmicutes* was predominant bacteria in all group followed by *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Tenericutes*, *Cyanobacteria* and *unidentified-Bacteria*, however, the composition of those gut microbiota changes caused by different treatment. Busulfan disturbed the homeostasis of the intestinal microenvironment. Chestnut polysaccharides could rescue this aberrance and improve spermatogenesis via steroid hormone synthesis process.

Conclusions: Chestnut polysaccharides can change gut structure and tight junctions to rescue Busulfan-impaired spermatogenesis by altering the composition of different groups of gut microbes, a rescue process that is most likely related to hormone synthesis processes. It will provide a new insight for treating the male-related infertility.

Introduction

Polysaccharides are complex compounds including different carbohydrates with various bioactivities and have attracted more concerns in recent years [1, 2]. Numerous studies have revealed that polysaccharides have the ability to regulate the immunity of intestinal mucosal, alter the composition of gut microbiota, inhibit cancer cell proliferation and improve spermatogenesis [3–10]. Polysaccharides can work as health care products because of these biological functions in regulating body health. Chestnut polysaccharides (CPs) are bioactive substances with multiple nutrients extracted from the chestnut fruits and possesses several health benefits. CPs are compounds containing many monosaccharides in different proportions including glucose, galactose, arabinose, mannose, xylose, rhamnose, and fructose [11, 12].

The link between gut microorganism and the reproductive system has attracted the attention of scientists in recent years. Studies have demonstrated that changes in the gut microbiota were related to body health maintenance because the microbiota formed a network regulating the immune system homeostasis, the metabolome, and the reproductive system [13–22]. So, bacteria derived from the gut can help us to understand the health status of an individual. It was revealed that gut microbiota could act as protectors or invaders in the female reproductive system [23, 24]. There are many studies about the roles of gut microbe disturbance in inducing the polycystic ovary syndrome (PCOS) pathogenesis.

Researchers have found that the gut bacteria of healthy women differed from those of patients with PCOS [25–27], including changes in bacterial composition or global diversity (α -diversity or β -diversity) [25, 28–32]. The aberrant behavior of gut microbes also disrupted normal physiological process in causing the reproductive problem in the host such as gestational diabetes mellitus (GDM) and early preeclampsia (PE) [24, 33, 34]. The researchers also found that gut microbiota was not only involved in inducing of reproductive problems, but also protected against reproductive problems in PCOS mice by transplanting microbes from healthy bodies [23]. The study of gut microbiota on male reproductive system was also conducted by researchers. Ding et al. revealed the mechanism of gut microbiota facilitating male reproduction, and that gut microbiota altered the expression of important genes in the testis [35]. Recent studies also suggested that the gut microbiota changes could rescue male infertility following administration of alginate oligosaccharides (AOS). The authors explored the sperm quality and spermatogenesis in AOS-dosed animals after fecal microbiota transplantation into Busulfan-treated mice and found that healthy-body microbiota transplantation could improve spermatogenesis by altering bacterial species [36, 37].

Studies have shown that CPs also played the profound role in inhibiting tyrosinase, oxidative activity and benefiting the spermatogenesis of mice which exposed to busulfan by improving the expression of important genes [11, 12, 38], but whether the rescue process is caused by the gut microbiota is unknown. 16S rRNA sequencing is a method to detect microbiota landscape, and bioinformatic analysis can indicate changes in microbiota and predict functional pathways of microbiota. In present study, 16S rRNA sequencing technology was used to do comprehensive research on the combined effects of CPs and gut microbes in promoting spermatogenesis *in vivo* (Figure S1). The aim of this research was to investigate the mechanism of improving spermatogenesis via CPs and its relevance at the gut microbial level.

Results

CPs treatment improved spermatogenesis

Being consistent with our previous study, we found that CPs treatment improved spermatogenesis, increased the number of germ cells, reduced vacuoles in seminiferous tubules and increased sperm quality in Busulfan-exposed mice by HE staining and the makers of germ cell (Ctrl, CPs, Bus and B + CPs) (Fig. 1).

CPs treatment changed the intestinal structure and tight junction

From the HE staining of intestine, we found that intestinal structure changed under different treatment (Fig. 2a). By IHC and RT-qPCR experiments, we examined the tight junction makers (Claudin 1, Zo-1, Occludin, and Desmoglein 2). The expression of these proteins decreased after exposed to Busulfan, and CPs could reverse this trend and increase the expression of these representative proteins (Fig. 2a, 2b). The results of RT-qPCR (*Cldn1*, *Cldn3*, *Cldn5*, and *Pmp22*) also confirmed the trend that busulfan

decreased tight junction in intestine and CPs could reverse this trend increasing those representative genes (Fig. 2c).

CPs treatment altered the gut microbiota composition

Since intestinal structure and tight junctions are associated with the intestinal microbes closely, which is highly correlated with the microenvironment, we examined changes intestinal bacteria in four groups that are closely related to the intestine. We detected the microbes in intestine. Three-dimensional principal coordinate analysis (PCoA) and random forest classification revealed that the four groups of samples were well separated from each group (Fig. 3a; Supplementary Fig. 3b). The richness of bacteria species in different groups was displayed (Supplementary Fig. 2a, 3a). The ecological diversity of the microbial community was determined using the α -diversity index including Observed, Chao1, Shannon, and Simpson. The Shannon, Chao1, and Simpson indices showed lower species richness and evenness in Bus group than in the other groups, a phenomenon suggests that the Busulfan treatment disturbed the balance of the gut microbiota (Fig. 3b).

Phylum and genus information was used to illustrate the differences in the levels of abundant taxa between treatments, indicating differences in composition between treatments. Individuals of bacterial families are represented by different colors according to their relative abundance, the phylum and genus level details are shown below (Fig. 3c; Supplementary Fig. 2b). The relative abundance of OUT is showed in Fig. 3d. From the figure, it can be seen that busulfan treatment changed the pattern of intestinal bacterial diversity and that the intestinal bacterial diversity declined after busulfan treatment. The *Firmicutes* was the dominant phylum, followed by *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Tenericutes*, *Cyanobacteria*, and *unidentified-Bacteria*. However, the busulfan treatment group had lower content of *Proteobacteria*, *Bacteroidetes* and *Tenericutes* and higher content of *Firmicutes* than the other groups (Fig. 3d; Supplementary Fig. 3c). At the genus level, the heatmap of the genus showed differences among the different treatment groups (Fig. 4).

LEfSe was used to compared the effect of CPs on the mice intestinal microbiota and to evaluate the bacterial composition between different treatment groups at different taxonomic levels (LDA value of 4) (Fig. 5a-5d). The gut microbiota composition changes in intestine were great. There were many taxa different in Ctrl, Bus, B + CPs group. The abundance of *Lactobacillaceae*, *Anaeroplasmatales* were higher than Ctrl, B + CPs group, and *Pseudomonadales* and *Mycoplasmatales* existing in Ctrl and B + CPs group were higher than Bus group.

CPs treatment improved spermatogenesis via steroid hormone synthesis process

Function analysis showed that mouse microbiota modifications affected many functional pathways such as Cellular Processes, Diseases, and Organismal Systems (Fig. 6a). Secondary KEGG pathway analysis was shown, with crossover between the Ctrl vs Bus group and Bus vs B + CPs group (Fig. 6b-6d;

Supplementary Fig. 4a, 4b). There was a greater alteration in steroid hormone biosynthesis function in the Bus group compared to the B + CPs group at third KEGG pathway analysis. This is similar to our previous study, where CPs restored Busulfan-impaired spermatogenesis by increasing the levels of proteins associated with hormone production. Afterwards, we detected hormone synthesis-related proteins, including CYP17A1 and HSD17 β 1 in the testis by IHC. From the results, it was shown that the expression of CYP17A1 and HSD17 β 1 decreased in Busulfan-exposed group, while CPs alleviated this trend and increased the protein expression (Fig. 7a, 7b), from the results we could conclude that gut microbiota participated in regulating spermatogenesis via hormone synthesis process.

Discussion

In this study, we used Busulfan to create a sterility model and high-throughput 16S rRNA sequencing method to study the effects of the gut microbiota of mice treated with Busulfan and CPs. The results showed that although *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* were present in all groups, the composition of different microbe was different. Busulfan treatment disrupted the homeostasis of gut microbiota, and resulting in significant changes in mouse intestinal microbiota composition at the phylum and genus levels. This phenomenon is consistent with previous report: significant changes of the bacterial composition affected the microbiota function on the organism [39].

Accumulating studies have revealed that bacterial composition changes are related to perturbations in the gut microbiota and that these changes could serve as indicators of many diseases, such as weight problems [40–42], cardiovascular disease [43], nervous system disease [44] and insulin resistance [45], for example, bacterial ratios could affect obesity that associated with the *Bacteroidetes* reduction and the *Firmicutes* proportion increase [45, 46], this conclusion is similar to our study in which changes in bacteria affected mouse health. In contrast, mice treated with the combination of Busulfan and CPs had bacterial abundance similar to the bacterial abundance in control group, which further proved the rescue effect of CPs on homeostasis of the intestinal microenvironment. The *Bacteroidetes* and *Proteobacteria* proportion both decreased in the Busulfan-treated group, but the *Bacteroidetes* and *Proteobacteria* proportion increased in Busulfan-exposed plus CPs-treated mice compared to the Busulfan-treated group. At the bacteria genus level, the predominant genus in all treated groups was *Lactobacillus*, which is consistent with previous study [47]. These results further validated that CPs could work as rescue agents to save the reproductive function by adjusting the balance of intestinal microbiota in mice with destroyed-intestinal microbiota.

Studies have revealed that the alteration of gut microbiota composition not only disturbed reproductive barrier but only regulated the steroid hormone production in men or women [48]. Abnormal behavior in hormone production could lead to obstruction of spermatogenesis according to previous studies [49–51]. In our previous study, Busulfan altered spermatogenesis and reduced sperm quality, including sperm viability and concentration, and CPs treatment reversed Busulfan-induced disturbances and improved sperm quality through the hormone synthesis pathway [38]. Coincidentally or not, we detected proteins related to the hormone synthesis pathway in mouse testes in the present study. The results demonstrated

that steroid hormone synthesis protein levels were elevated in group B + CPs compared to group Bus. It is in high agreement with the findings of our previous study, the present study further confirmed the rescue of impaired spermatogenesis through steroid synthesis pathway after alteration of gut microbiota by chestnut polysaccharide through the third level functional analysis.

Conclusions

CPs can change gut structure and tight junctions to rescue Busulfan-impaired spermatogenesis by altering the composition of different groups of gut microbes, a rescue process that is most likely related to hormone synthesis processes.

Materials And Methods

Breeding environment of mice

Male CD1 mice were purchased from company in Beijing. The mice were kept in a house with a 12-hour cycle of light and constant temperature (22–23°C), and could eat food and water freely throughout the study period. All procedures used in this study were approved by the Institutional Animal Care and Ethics Committee of Qingdao Agricultural University [52].

Treatment of mouse

Busulfan was used to establish an animal model of male sterility [53–59]. The main aim of this research was to investigate the changes of gut bacteria in Busulfan-exposed and Busulfan-exposed plus CPs-treated mice. In this study, some male mice were treated with Busulfan in 40 mg/kg concentration. The concentration of CPs was referred to our previous study (38), which was done by gavage exposure on mice. The dose of CPs used in this experiment was 0 mg/kg and 0.10 mg/kg, respectively. The fresh CPs solutions were prepared daily. Treatment groups were shown below (10 mice/group): (1) Vehicle control (Ctrl); (2) Chestnut polysaccharides (CPs); (3) Busulfan (Bus); (4) Busulfan + CPs 0.10 mg/kg (B + CPs). The gavage volume was 0.10 ml/mouse/day. Gavage was performed every day lasting five weeks, starting at three weeks of age, a procedure similar to our previous study [38].

Collection of samples

After five weeks of treatment, mice were slaughtered in humane patterns, and the tissues and gut bacteria were collected for further analysis. Intestine and testis tissue were placed in paraformaldehyde solution for fixation. Gut bacteria were quickly collected from gut and stored at -80°C.

16S rRNA sequencing

We conducted 16S rRNA sequencing (V3-V4 region). The amplified V3-V4 region gene were mixed and purified by GeneJET Gel Extraction Kit (Thermo Scientific, United States), generated the sequencing libraries. Then using the NovaSeq PE250 platform (Novogene, Beijing, China) to sequence. Using FLASH (V1.2.71) to merge paired-end reads. QIIME (V1.7.02) software was used to control the quality of the Tags

and to remove all chimeras at the same time. Classification used the Greengenes database 3, and sequences (similarity $\geq 97\%$) were assigned to the same operation altaxonomic units (OTUs).

16S rRNA sequencing analysis

QIIME (Version 1.7.0) was used to calculate alpha diversity index and Unifrac distance. Vegan (Version 2.5.4) and ade4 package (Version 1.7.13) were used to obtain PCoA (principal coordinate analysis). Differences in abundance were determined by the Linear discriminate analysis effect size (LEfSe), the threshold of LDA score was 4.0. Tax4Fun (Version 0.3.1) was used to function prediction. Extract the 16S rRNA gene sequence from the KEGG database of the prokaryotic genome, then compare it to the SILVA SSU Ref NR database (BLAST bitscore > 1500) using the BLASTN algorithm and to create a correlation matrix, mapping the prokaryotic functional information annotated by UProC and PAUDA methods to the SILVA database to realize the SILVA database functional annotation [60–62].

H&E and IHC staining

Collected testicular and intestinal tissues were placed in fixation solution overnight at 4°C refrigerator, embedded procedure was according to a standard process, and then serially sectioned to 5 μm thickness following standard procedures. After sections prepared and they were used for Hematoxylin eosin (H&E) and immunohistochemistry (IHC) staining, as in previous studies [63]. For IHC, slices were rehydrated in different concentrations of alcohol solution, and then antigens were retrieved, blocked, and antibodies were added to slices and incubated overnight at 4°C. Images of the sections were obtained using an Olympus fluorescence microscope (BX51, Japan).

RNA extraction and real-time fluorescence quantitative PCR

Total RNA was extracted from intestine tissue using the SPARKscript II RT Plus Kit (HAOSAIL, AG0304-A, Qingdao, China) according to the manufacturer's instructions. Using the TransScript One-Step gDNA Removal Kit and cDNA Synthesis Kit (TransGen Biotech, AT311, Beijing, China) to perform reverse transcription. Gene expression was detected by Light Cycler real-time PCR instrument (Roche LC96; Basel, Switzerland). *GAPDH* was used as a standard normalization. $2^{-\Delta\Delta CT}$ was used to calculated relative transcript abundance according to described in the SYBR Green user manual (Takara, RR820A, Japan). Primer sequences used in study were listed in Table S1.

Statistical analysis

Experiments in this study were performed in triplicates and results expressed as mean $\pm$ SD. Data were statistically tested by Student's t test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using SPSS software. Comparisons were considered significant at $*P < 0.05$, $**P < 0.01$.

Abbreviations

IHC

Immunochemistry; RT-qPCR:Real-time fluorescence quantitative PCR; CPs:Chestnut polysaccharides; PCOS:polycystic ovary syndrome; GDM:gestational diabetes mellitus; PE:early preeclampsia; AOS:alginate oligosaccharides; OTUs:operation altaxonomic units; PCoA:principal coordinate analysis; LEfSe:Linear discriminate analysis effect size.

Declarations

Ethics approval and consent to participate

All animal experiments were approved by Animal Care and Use Committee of Qingdao Agricultural University.

Consent for publication

Not applicable.

Availability of data and material

The microbiota raw sequencing data generated in this study has been uploaded to the Genome Sequence Archive (GSA) with the accession number CRA004367 that are publicly accessible at <https://ngdc.cncb.ac.cn/gsa>[64, 65].

Competing interests

The authors declare no completing interests.

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Authors' contributions

YZS and WS designed the study methods, SY wrote the manuscript, YT analyzed the data. BQH, YJS and YZ collected samples. YQL and WY prepared the figure.

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Figures

Figure 1

Figure 1

Chestnut polysaccharides (CPs) increased germ cell quantity. Histopathology photos of HE staining and MVH, DAZL staining of mouse testes.

Figure 2

Figure 2

CPs Effects on expression of intestine tight junction makers in mice. (A) The representative images of HE staining, claudin1, Zo-1, cccludin1 and Desmoglein 2 in the Ctrl, CPs, Bus, and B+CPs groups. (B) Analysis of fluorescence intensity of claudin1 (Ctrl: 29.82 ± 7.52 , CPs: 24.94 ± 3.11 , Bus: 12.59 ± 2.74 , B+CPs: 23.34 ± 8.57), Zo-1 (Ctrl: 49.73 ± 5.03 , CPs: 48.20 ± 9.91 , Bus: 35.77 ± 6.23 , B+CPs: 54.19 ± 8.93), occludin1 (Ctrl: 27.16 ± 7.45 , CPs: 32.24 ± 5.33 , Bus: 16.51 ± 3.20 , B+CPs: 31.22 ± 9.96) and

Desmoglein2 (Ctrl: 22.64 ± 2.14, CPs: 25.76 ± 6.47, Bus: 20.70 ± 4.47, B+CPs: 25.57 ± 4.36) in different groups. (C) The expression of *cln1*, *cln3*, *cln5*, *pmp22* by q-RT-PCR. The results are presented as mean ± SD. **P < 0.01.

Figure 3

Figure 3

The changes of gut microbiota in different treatment (A) The three-dimensional PCoA of unweighted UniFrac distances for Ctrl vs Bus group, Bus vs B+CPs 0.1 group. (B) alpha diversity indexes of gut

microbiota, observed, Shannon, Simpson, Chao1. (C) The relative abundance of microbiota at phylum level. (D) The table and heatmap of relative richness of microbiota at phylum level.

Figure 4

Figure 4

The heatmap of microbiota at genus level.

Figure 5

Figure 5

Differences in bacterial abundance between different groups: (A) LDA distribution of Ctrl group vs CPs group with LDA score threshold of 4.0. (B) Cladogram of Ctrl group vs CPs group. Linear discriminant analysis effect size (LEfSe) was used to determine differences in microbiota abundance; one color represents the same microbial taxon, and dot size reflects the relative abundance at the same microbial taxonomic level; (C) LDA distribution of Bus group vs B+CPs 0.1 group with LDA score threshold of 4.0.

(D) Cladogram of Bus group vs B+CPs group. Linear discriminant analysis effect size (LEfSe) was used to determine differences in microbiota abundance; one color represents the same microbial taxon, and dot size reflects the relative abundance at the same microbial taxonomic level.

Figure 6

Figure 6

The analysis of function pathway. (A) The representative KEGG pathways at the first classification level. (B) The heat map of annotated KEGG pathways at the second classification level. (C) The Venn diagram in Ctrl group vs CPs group and Bus group vs B+CPs group. (D) Functional pathways at the third classification level in Bus group vs B+CPs group.

Figure 7

Figure 7

The changes of hormone synthesis proteins in testes. (A) The images of HSD17 β 1 and CYP17 in the Ctrl, CPs, Bus, and B+CPs groups. (B) Analysis of the fluorescence intensity of HSD17 β 1 in different groups (Ctrl: 22.77 ± 3.71 , CPs: 19.99 ± 2.59 , Bus: 13.85 ± 1.89 , B+CPs: 22.60 ± 3.12) (C) Analysis of the fluorescence intensity of CYP17 in different groups (Ctrl: 21.53 ± 4.33 , CPs: 21.98 ± 3.52 , Bus: 17.88 ± 5.22 , B+CPs: 28.95 ± 4.47). The results are presented as mean $\pm$ SD. **P < 0.01.

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