Research Paper

Epimedium polysaccharides impacts the reproductive function of pubertal male mice

Accepted 19th December, 2017

ABSTRACT

This study was conducted to investigate the effects of epimedium polysaccharides (EPS) on reproductive function of pubertal male mice. Healthy pubertal male Kunming mice were randomly divided into 5 groups: the control group receiving 0.1 ml of 0.9% physiological saline solution and 4 treatment groups receiving different doses of EPS at 2, 4, 6 and 8 mg per day. After 20 consecutive days, the testes together with epididymides from euthanized mice were removed and weighed. Blood samples were collected for biochemical and steroid hormone analyses and total RNA from testes was extracted for real-time PCR; epididymides were cut to harvest spermatozoa and two testes randomly used for histological analysis. EPS had no effect on body weight, but at higher dosages it had a raising effect on organ coefficients of the testes or epididymis. EPS significantly increased epididymal sperm count, elevated superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities, but decreased malondialdehyde (MDA) levels. EPS at 6 mg significantly increased testosterone levels. These results demonstrate that EPS within an appreciable dose range does improve development of pubertal male reproductive organs, increase epididymal sperm count and improve testicular antioxidant capacity.

Key words: Epimedium polysaccharides, testosterone, sperm count, antioxidant capacity, mice.

INTRODUCTION

Epimedium is a widely used traditional herbal medicine, belonging to the family Berberidaceae (Cheng et al., 2013). Epimedium and its active compounds have been demonstrated to possess far-reaching pharmacological actions in vivo and/or in vitro experiments. It is known to improve cardiovascular and cerebrovascular functions, and modulate immunological function. It is also reported to have anti-osteoporosis, antioxidation, anti-tumor and anti-aging effects (Ma et al., 2011; Makarova et al., 2007). It has been used for treatment of “coldness” to improve female health, strengthen bones and tendons and to relieve pain in back bone and knees, treat coronary arterial diseases and male erectile dysfunction (Chen and Chiu, 2006; Yap et al., 2007).

Flavonoids and polysaccharides are the main bioactive components of epimedium. In the last decades, there have been several reports on flavonoids in epimedium, but only a small number of these studies have focused on epimedium polysaccharides (EPS) (Zhang et al., 2014; Gao et al., 2012). Flavonoids extracted from herba epimedii can improve the antioxidant capacity as proven by several researchers. To prevent oxidative stress, both enzymatic and non-enzymatic antioxidant systems are present in the testes (Long et al., 2017).

Antioxidants are substances that can prevent or diminish oxidation of biomolecules by their direct or indirect action. Superoxide dismutase (SOD) serves an important antioxidant defense against oxidative stress, which in cells rapidly converts superoxide anion (O2•−) to less dangerous hydrogen peroxide (H2O2). Three forms of SOD; namely, cytoplasmic superoxide dismutase (SOD1), mitochondrial superoxide dismutase (SOD2), and extracellular superoxide dismutase (SOD3) are present in mammals. Gluthione
peroxidase (GPx) is one of the key antioxidative enzymes against oxidative damage (Kheradmand et al., 2009). GPx1 is the most abundant selenoperoxidase and is a key antioxidant enzyme in many cell types (Xia et al., 2010; Kim et al., 2015). Malondialdehyde (MDA) is frequently used as an index of tissue oxidative stress, which results from the free radical damage to membrane components of cells (Hong et al., 2010). Few studies have investigated EPS pharmacological activities on male reproductive function, such as spermatogenesis and testosterone production or its underlying mechanisms.

Puberty is the final stage of maturation of the hypothalamus-pituitary-gonad axis and is characterized by changes in circulating gonadotropins and increased levels of sex steroids (Long et al., 2017). In the present study, we treated pubertal mice with different doses of EPS and analyzed the effects on male reproductive function as well as, underlying mechanisms by using histological examinations, sperm density count and real-time PCR.

MATERIALS AND METHODS

Drugs and chemicals

EPS was kindly provided by Dr. Deyun Wang of Traditional Chinese Medicine Laboratory, Nanjing Agricultural University (NJAU). EPS was extracted by water decoction and ethanol precipitation. Seriatim was purified using trichloroacetic acid method to eliminate protein, permeating through macroporous adsorption resin (ADS-7) to eliminate protein and pigment and DEAE-Sephadex A-25 to separate from other carbohydrates. All dialysis in the process of the isolation was substituted for ultrafiltration using 1 kDa ultrafiltration membrane. Finally, the liquor was freeze dried. The carbohydrate concentration (%) of total EPS was 20.59 compared with D-glucose (Lu et al., 2008). EPS may be composed of 1, 4-linked-d-GalpA, 1,3,4-linked-d-GalpA, 1,6-linked-d-Galp and terminal [-Rhap residues in a molar ratio of 11.0:1.0:1.0:1.0 by chemical and spectroscopic analysis (Wu et al., 2016).

The SYBR® PrimeScript® RT-PCR Kit (Perfect Real Time) was purchased from TaKaRaBiotech (Liaoning, China). SOD, GPx and MDA assay kits were obtained from Jiancheng Bioengineering Institute (Nanjing, China). Radioimmunoassay (RIA) kits for determining testosterone (T) were purchased from Beijing North Institute of Biological Technology (Beijing, China).

Animals

Sixty healthy male Kunming mice (4-weeks-old) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (permit number SCXK-Hu 2014-0002) and used in this experiment. The mice were purchased 1 week prior to the study, for the purposes of acclimatization. All mice were routinely raised in a clean area with normal room temperature and fed with standard mouse feed and ordinary water ad libitum.

The mice were randomly assigned to 5 groups; 12 mice per group. EPS (dissolved in 0.1 ml distilled water) was administered at a dose of 2, 4, 6 and 8 mg per mouse according to the groups using oral gavage for 20 consecutive days, whereas the control group received 0.1 ml of 0.9% sodium chloride solution (physiological saline). All procedures and protocols involving animals were approved by the Animal Research Committee of NJAU and according to the Guide for the Care and Use of Laboratory Animals.

Collection of testes and epididymides

At the end of the treatment period, the mice were sacrificed by cervical dislocation. The testes and epididymides were quickly removed and weighed. Two testes from each group used for histopathological examination were randomly kept in Bouin's fixatives to preserve normal morphology and facilitate further processing into paraffin blocks. Other testes for assays of biochemical analysis and real-time PCR were snap-frozen in liquid nitrogen and stored at -80°C until further processing. The epididymides were used for sperm density analysis.

Epididymal sperm density count

The left epididymis was used for sperm count. The epididymides were cut into small pieces after which epididymal spermatozoa were collected and immersed in 1 mL of human tubal fluid (HTF) in a petri dish (Yan et al., 2009; Goodson et al., 2012). This was incubated in 5% CO2 in air for 10 min at 37°C and homogenization-resistant sperm counted using a haemocytometer. The number of cells in at least 2 of the corner larger squares (1 mm2) was routinely counted (Oliveira et al., 2009).

Histopathological examination

The right testis was fixed in Bouin’s solution for 24 h and processed by standard histological methods. Specimens were dehydrated in xylene and embedded in paraffin wax. 5 μm thick slices were cut from each sample into a glass slide and stained with haematoxylin-eosin (H and E) routinely. Changes in testicular morphology and structure were observed under the microscope.

Detection of serum testosterone

Blood samples were collected from the eye 8 h after fasting,
and the serum separated using centrifugation at 2,000 × g for 10 min. The serum was stored at −20°C for further analysis. The serum concentrations of testosterone were measured using a RIA kits. All operations followed the protocols provided in the kits.

**Determination of SOD, GPx activity and MDA level**

The testicular tissues stored at −80°C were homogenized at 4°C after adding pre-cooled 0.9% saline in the ratio of 1:9. When testicular tissues were disrupted, the homogenate was centrifuged at 3,000 × g for 10 min at 4°C. The supernatant was used for the assay of SOD, GPx, and MDA according to the manufacturer’s instructions.

**Quantitative real-time PCR (qRT-PCR) analysis**

The expression levels of 3β-Hydroxysteroid dehydrogenase (3β-HSD), 17β-Hydroxysteroid dehydrogenase (17β-HSD), cytoplasmic superoxide dismutase (SOD1), mitochondrial superoxide dismutase (SOD2), extracellular superoxide dismutase (SOD3) and glutathione peroxidase 1 (GPx1) mRNA in the testicular tissues were assessed by qRT-PCR. Primer synthesis was performed at Shanghai Invitrogen Biotech Co., Ltd (Table 1). First, total RNA was extracted from the testicular tissue, thereafter, RNA was isolated by Trizol reagent using kits from Invitrogen and according to manufacturer’s instructions. The quality and quantity of the RNA preps were determined by gel electrophoresis and the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., DE, USA). Each real-time PCR reaction was carried out in triplicate in a 20-μl reaction mixture (2 μl cDNA, 6.8 μl H2O, 10 μl SYBR® Premix Ex Taq™, and 0.6 μl of each 10 μM forward and reverse primers). The PCR program was 30 s at 95°C followed by 40 cycles of 5 s at 95°C, 31 s at 60°C. A melting curve was generated at the end of every run to ensure product uniformity (95°C for 15 s, 60°C for 15s and 95°C for 15s). The relative expression of target genes was calculated using 2^-ΔΔCt. PCR with β-actin chosen as an internal control carried out in the same tubes as the genes.

**Statistical analysis**

All data were collated and expressed as mean ± standard deviation (SD) and statistically analyzed using one-way analysis of variance using SPSS Statistics 20 (IBM Corporation, Armonk, NY, USA). Differences between experimental groups were considered significant at p<0.05 and extreme at p<0.01.

**RESULTS AND DISCUSSION**

**Effect of EPS on body growth, testicular and epididymal weights**

Table 2 shows the body, testicular and epididymal weights of the EPS and control groups. Before the experiment, body weights were not significantly different among the control group and EPS groups (data not shown). At the end of the treatment period, body weights of control and EPS-treated mice were not significant in any group (p>0.05). The absolute weights of the testes in all EPS-treated groups were significantly higher compared with control group (p<0.05). The absolute weights of epididymides of 6 mg treated groups were significantly higher compared with the controls (p<0.05). The relative weights of testes and epididymides in 4, 6 and 8 mg treated groups were significantly higher compared with the controls (p<0.05).

**Effects of EPS on testicular morphology**

The diameter of the lumen of the seminiferous tubules in EPS groups was bigger than the control, and spermatozoa were observed in the lumen of seminiferous tubules. The basement membrane of the seminiferous tubules was continuous and complete; germ cells had normal morphology and regular arrangement. These findings suggest that the

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Lengths of amplicons (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>AAATCGTGGGTCAGCATCAA</td>
<td>ATGCCACAGGATCTCCATACC</td>
<td>202</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>GCACATTTACCTCCGTTGCAAA</td>
<td>GATTTGACTGAGTCGTGTTT</td>
<td>182</td>
</tr>
<tr>
<td>17β-HSD</td>
<td>GATGGGAGCTGAATCCTGTG</td>
<td>CTGGTTGCTGATTGTGGTTTT</td>
<td>150</td>
</tr>
<tr>
<td>StAR</td>
<td>ATGGGAGCTGGGAACCCAAA</td>
<td>AACCTCTGGCTTGTTGACAG</td>
<td>154</td>
</tr>
<tr>
<td>SOD1</td>
<td>CGGATAGGAAGAGGACATGGTT</td>
<td>AATCCCAATCACTCCACAGAG</td>
<td>230</td>
</tr>
<tr>
<td>SOD2</td>
<td>GAAGGTAAATGGCTCTGCTG</td>
<td>TTGCGCTACTGAAAGGGTT</td>
<td>245</td>
</tr>
<tr>
<td>SOD3</td>
<td>CTGCTGCTGCTGCTACAATAAC</td>
<td>AGCTGTCGTGCTAGGTTGAA</td>
<td>247</td>
</tr>
<tr>
<td>GPx1</td>
<td>AGTCACCCGCTATGGCTCTTC</td>
<td>TCTGGTGTCGGAATCGATT</td>
<td>217</td>
</tr>
</tbody>
</table>

*Table 1: Primer sequences used in qRT-PCR.*
4 doses of EPS did not adversely affect the testis histopathologically.

EPS-induced changes in epididymal sperm density

Table 2 showed that EPS treatments caused a significant increase in sperm counts; epididymal sperm density was significantly higher in all EPS groups compared with the control (p < 0.05). Epididymal sperm density in 6 mg EPS treatment group was the highest.

Effects of EPS on serum concentrations of testosterone

In this study, mice in EPS at 2, 4, and 6 mg treatment groups showed higher serum testosterone concentrations than control group; EPS at 6 mg treatment was significantly higher compared with controls (p < 0.05; Table 3).

Effects of EPS on the contents of MDA and activities of SOD, GPx in mouse testis

The activities of SOD and GPx in the 4 and 6 mg groups were significantly higher than that of the control group (p < 0.01). The concentration of MDA in EPS at 6 mg treatment group were significantly lower than that of the control group (p < 0.01; Table 3).

Effects of EPS on mRNA expression level of steroidogenic and antioxidant genes in mouse testis

The mRNA expressions of 3β-HSD, 17β-HSD in testicular tissue in EPS at 2, 4 and 6 mg groups were increased significantly compared with the control (p < 0.05), and expression of StAR mRNA was significantly increased in all EPS groups when they were also compared with the control (p < 0.05; Figure 1A).

The mRNA expressions of SOD1, SOD2, SOD3 and GPx1 in testicular tissue of EPS at 4, 6, and 8 mg groups were increased significantly as compared with the control (p < 0.01; Figure 1B).

EPS seems to have no obvious effects on pubertal Kunming male mice growth; this outcome is quite similar to previous reports (Xue et al., 2012; Yang et al., 2014). We investigated the effects of EPS on the reproductive function of pubertal male mice. EPS significantly increased testicular and epididymal weights, epididymal sperm density, and serum testosterone concentrations. Although epididymal sperm quality and quantity sometimes represent a delayed functional alteration of testes and epididymis due to the long spermatogenetic process, relative data analysis is always helpful.

Hokkraft and Braun (2004) reported that testosterone plays a leading role in both morphological development and reproductive function of the testis. In the present experiment, it is confirmed that EPS has the ability to increase serum testosterone, revealing the possible mechanism of EPS improved reproductive function.

Leydig cells in mammalian males are the main source of the androgenic hormone, testosterone, which is essential for male sexual differentiation, maintenance of spermatogenesis and expression of male secondary sex characteristics (Dong and Hardy, 2004; Payne and Youngblood, 1995). There were higher testosterone levels in the serum of 6 mg EPS-treated mice than the control group. The acute and chronic stimulation of Leydig cells by the pituitary hormone LH is responsible for the biosynthesis of testosterone. Both the acute and the chronic effects of LH are mediated by increases in cAMP (Payne, 1990). Results suggested that EPS increases cAMP levels, but the pathway by which it acts still needs to be explored.

This study revealed the possible mechanism of EPS in improving testosterone production. The mRNA expression levels of testicular key androgenic enzymes like 3β-HSD and 17β-HSD were increased following EPS exposure. StAR transport of cholesterol across the mitochondrial membrane is generally considered the rate-limiting step in steroidogenesis (Das et al., 2012), and expression of StAR mRNA was significantly increased following EPS administration.

There exist an elaborate array of antioxidant enzymes and

<table>
<thead>
<tr>
<th>Items</th>
<th>0</th>
<th>2 mg</th>
<th>4 mg</th>
<th>6 mg</th>
<th>8 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of Body Weight (g)</td>
<td>39.24±1.53</td>
<td>39.07±1.95</td>
<td>39.17±2.07</td>
<td>38.78±1.99</td>
<td>39.27±2.03</td>
</tr>
<tr>
<td>Testicular weight(g)</td>
<td>0.195±0.013</td>
<td>0.211±0.016&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.224±0.017&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.243±0.023&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.214±0.016&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Testicular index (mg/g)</td>
<td>5.02±0.381</td>
<td>5.14±0.397</td>
<td>5.56±0.411&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.83±0.398&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.52±0.423&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epididymal weight (g)</td>
<td>0.063±0.004</td>
<td>0.065±0.003</td>
<td>0.067±0.005</td>
<td>0.073±0.004&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.066±0.004</td>
</tr>
<tr>
<td>Epididymal index (mg/g)</td>
<td>1.56±0.134</td>
<td>1.65±0.136</td>
<td>1.78±0.142&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.77±0.139&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.73±0.138&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sperm density (&lt;i&gt;x&lt;/i&gt;10&lt;sup&gt;6&lt;/sup&gt;/ml)</td>
<td>0.98±0.09</td>
<td>1.48±0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.62±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.90±0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.68±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All data was expressed in mean ± SD; compared with the control group, <sup>a</sup>P < 0.05; <sup>b</sup>P < 0.01. Testicular/epididymal index means testicular/epididymal weight (mg)/body weight (g).
free radical scavengers in the testes, and they ensure that the twin spermatogenic and steroidogenic functions of this organ are not impacted by oxidative stress (Aitken and Roman, 2008). The results of this study demonstrate that EPS elevates the SOD, GPx activities, which are accompanied by corresponding decreases in MDA levels in the testis of pubertal mice. In order to further study on the SOD and GPx changes in testicular tissue, SOD1, SOD2, SOD3 and GPx1 mRNA levels were detected by qRT-PCR in this study. The mRNA expressions of SOD1, SOD2, SOD3, and GPx1 in testicular tissue in EPS at 4 and 6 mg groups were increased significantly compared with the control.

In conclusion, EPS within a certain dose range can improve development of pubertal male reproductive organs and increase epididymal sperm counts; meanwhile, EPS elevates the SOD and GPx activities, which are accompanied by corresponding decreases in MDA levels. These findings demonstrate that EPS is beneficial for the reproductive system of pubertal male mice and it has antioxidative properties.

ACKNOWLEDGEMENTS

This work was granted by the Research Fund for Doctoral Program of the Ministry of Education of China (20130097110020) and the PAPD of Jiangsu Province. We sincerely thank Dr. Gary Anderson from the Department of Animal Science, University of California, Davis for his comments and kind help with revision in English.

REFERENCES


Table 3: The SOD, GPx activities and MDA level in testicular tissues, and serum testosterone levels in mice.

<table>
<thead>
<tr>
<th>Variable</th>
<th>0</th>
<th>2 mg</th>
<th>4 mg</th>
<th>6 mg</th>
<th>8 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone (ng/ml)</td>
<td>0.280±0.035</td>
<td>0.280±0.047</td>
<td>0.289±0.045</td>
<td>0.338±0.031a</td>
<td>0.271±0.030</td>
</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td>1348.3±55.3</td>
<td>1377.4±38.3</td>
<td>1403.6±39.9b</td>
<td>1412.3±44.5b</td>
<td>1359.6±39.7</td>
</tr>
<tr>
<td>GPx (U/mg protein)</td>
<td>16.32±0.28</td>
<td>16.66±0.37</td>
<td>18.27±0.53b</td>
<td>18.71±0.59b</td>
<td>16.79±0.32</td>
</tr>
<tr>
<td>MDA (nmol/g protein)</td>
<td>0.88±0.029</td>
<td>0.907±0.041</td>
<td>0.809±0.036</td>
<td>0.748±0.033b</td>
<td>0.803±0.038</td>
</tr>
</tbody>
</table>

SOD: superoxide dismutase; GPx: glutathione peroxidase; MDA: malondialdehyde. All data was expressed in mean±SD; compared with the control group, *p<0.05; **p<0.01.

Figure 1: Expressions of 3β-HSD, 17β-HSD, StAR, SOD1, SOD2, SOD3 and GPx1 mRNA in the testes of mice treated with different doses of EPS. The mRNA levels were normalized to β-actin mRNA. The mRNA levels of the control group were set as 1. Values are in mean ± SD. *p<0.05, **p=0.01 vs. control group.


Cite this article as:

Submit your manuscript at http://www.academiapublishing.org/ajpp