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Male Health

# Effects of velvet antler polypeptide on sexual behavior and testosterone synthesis in aging male mice

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Twenty-four-month-old male C57BL/6 mice with low serum testosterone levels were used as a late-onset hypogonadism (LOH) animal model for examining the effects of velvet antler polypeptide (VAP) on sexual function and testosterone synthesis. These mice received VAP for 5 consecutive weeks by daily gavage at doses of 100, 200, or 300 mg kg<sup>-1</sup> body weight per day ( $n = 10$  mice per dose). Control animals ( $n = 10$ ) received the same weight-based volume of vehicle. Sexual behavior and testosterone levels in serum and interstitial tissue of testis were measured after the last administration of VAP. Furthermore, to investigate the mechanisms of how VAP affects sexual behavior and testosterone synthesis *in vivo*, the expression of steroidogenic acute regulatory protein (StAR), cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc), and 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ HSD) in Leydig cells was also measured by immunofluorescence staining and quantitative real-time PCR. As a result, VAP produced a significant improvement in the sexual function of these aging male mice. Serum testosterone level and intratesticular testosterone (ITT) concentration also increased in the VAP-treated groups. The expression of StAR, P450scc, and 3 $\beta$ -HSD was also found to be enhanced in the VAP-treated groups compared with the control group. Our results suggested that VAP was effective in improving sexual function in aging male mice. The effect of velvet antler on sexual function was due to the increased expression of several rate-limiting enzymes of testosterone synthesis (StAR, P450scc, and 3 $\beta$ -HSD) and the following promotion of testosterone synthesis *in vivo*.

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**Keywords:** aged; male hypogonadism; mouse; sexual behavior; testosterone synthesis; velvet antler polypeptide

## INTRODUCTION

The number of individuals over 65 years old has increased more than 10-fold in the past century, and according to current demographic trends, it will continue to increase.<sup>1</sup> The rapid growth of the aging male population is concomitant with an increased prevalence of age-related diseases such as heart disease, hypertension, cancer, osteoporosis, benign prostatic hyperplasia, and diabetes. Late-onset hypogonadism (LOH) is also one of the most common age-related diseases. According to the recommendations by Lunenfeld *et al.*, LOH is defined as a clinical and biochemical syndrome associated with advancing age and characterized by hypogonadal symptoms (poor morning erection, low sexual desire, and erectile dysfunction) and a decline in serum testosterone levels (total testosterone below 11 nmol l<sup>-1</sup> and free testosterone below 220 pmol l<sup>-1</sup>).<sup>2,3</sup> There are many other symptoms related to LOH including fat mass increase, muscle mass loss, fatigue, insomnia, bone mineral density decrease and osteoporosis, poor concentration, forgetfulness, and depression.<sup>4,5</sup> Severe LOH is even considered to be associated with substantially higher risks of

all-cause and cardiovascular mortality.<sup>6</sup> So, LOH affects the aging males' quality of life and health greatly.

In recent years, testosterone replacement treatment (TRT) has been widely administered to men with LOH and is reported to be an effective therapy to relieve main symptoms and improve quality of life.<sup>7–9</sup> However, some side-effects of exogenous testosterone cannot be ignored, such as excessive libido and aggression, erythropoiesis, hepatic tumors, intrahepatic cholestasis, hepatotoxicity, liver failure, and sleep apnea.<sup>1</sup> TRT is not recommended in men with breast cancer, prostatic cancer, and lower urinary tract symptoms caused by an enlarged prostate.<sup>10</sup> Males who desire to maintain fertility are also advised against the use of TRT because exogenous testosterone can suppress the hypothalamic-pituitary-gonadal axis (HPG axis) and result in infertility.<sup>10,11</sup> Developing a method that reaps the benefits of TRT while avoiding adverse effects would be a much better alternative.

As a typical animal drug, velvet antler (VA) has been used in traditional Chinese medicines for more than 2000 years. Several countries such as China, Japan, and Korea have designated VA from sika

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deer and red deer as appropriate for medicinal use.<sup>12</sup> VA is commonly used to enhance sexual functioning or delay the aging process in these countries, and its positive effects have been found.<sup>13–16</sup> However, there have been few reports discussing the underlying mechanisms of VA. Several studies have suggested that velvet antler polypeptide (VAP) is the main active component in VA.<sup>17–19</sup> The aim of this study was to assess whether VAP could enhance the level of testosterone *in vivo* and improve the sexual function of old male mice. Moreover, we tested the expression of several rate-limiting enzymes of testosterone synthesis, including steroidogenic acute regulatory protein (StAR), cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc), and 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) to make further efforts to elucidate VAP's mechanism of action in this process.

## MATERIALS AND METHODS

### Ethics statement

Animal and experiment protocols were approved by the Sun Yat-sen University Institutional Animal Care and Use Committee. All animals received humane care, and all efforts were taken to minimize suffering. After the experiments, we performed euthanasia on the animals by CO<sub>2</sub> inhalation.

### Animals

All of the male C57BL/6 mice were purchased from the Animal Center of Sun Yat-sen University (Guangzhou, China) and were maintained on a 12-h-day/12-h-night schedule (lights on from 19:00 to 07:00 h) at constant temperature (22  $\pm$  1°C) and humidity (60%). These mice were approximately 24 months of age and were housed solely in cages. Female mice were about 3 months old and were housed five per cage.

The sera of each male mouse were collected from the retrobulbar space to allow serum testosterone quantification. Only those with the serum testosterone concentration below 8 ng ml<sup>-1</sup> were accepted and randomly assigned to four different groups. Each group contained 10 male mice.

The female mice were used as stimulus females in behavioral testing and were resected with double ovaries. One month after ovariectomy, each female mouse was pretreated with estradiol benzoate (50  $\mu$ g, 48 h before testing, dissolved in 50  $\mu$ l of peanut oil) and progesterone (500  $\mu$ g, 5 h before testing, dissolved in 50  $\mu$ l of peanut oil) reach a state of estrous.

### Drugs

VA from *Cervus nippon* Temminch was harvested in the deer breeding base affiliated with Bei Jing Jiu Lu Deer Industry Breeding Co., Ltd., Beijing, China. VAP was isolated from those VA by the School of Chemistry and Chemical Engineering, South China University of Technology. VAP was a light, yellow powder with a molecular weight of 3.2 kDa. The main ingredient of VAP has been depicted previously.<sup>12</sup> After being dissolved in distilled water, VAP was administered orally for five consecutive weeks. One group was treated with starch as the control group, and the other three groups were treated with VAP at different dosages every day (100, 200, and 300 mg kg<sup>-1</sup> day<sup>-1</sup>).

### Tests of sexual behavior

Sexual behavior assays have been previously described.<sup>20</sup> The following measure targets were recorded and scored: the latency of mount, intromission and ejaculation; the frequency of mount and intromission; copulatory efficacy (calculated as intromission frequency divided by mount frequency + intromission frequency). Tests were conducted in a clean testing cage (40 cm  $\times$  26 cm  $\times$  21 cm) in a quiet environment. Male

mice were placed individually into the testing cage for preadaptation. After 15 min, a pretreated female mouse was placed into the same testing cage. If 10 min passed without an intromission, this female mouse was replaced with another pretreated female mouse. If the male mouse failed to achieve intromission with the second female mouse again within the next 5 min, a third pretreated female mouse was introduced for a final 15 min period. Failure to achieve intromission within 15 min and ejaculation within 45 min from the onset of the test resulted in termination of the test, and the maximum latency value of 45 min was assigned for that behavior. All of the sexual behavior tests were operated by an observer who did not know which group these male mice belonged to.

### Weighting of body and reproductive organs

Twenty-four hours after receiving the last feeding, each of the male mice was first weighed. Then, all of the animals were anesthetized with ether, and blood samples were obtained from the vena cava. Serum was separated for measurement of the testosterone. Immediately after blood samples were collected, bilateral testes, epididymides, and seminal vesicles were obtained and weighed.

### Testosterone assays

Serum testosterone was determined by the enzyme-linked immunosorbent assay (ELISA) kit (R and D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Intratesticular testosterone (ITT) concentrations were assayed as previously described.<sup>21</sup> Briefly, testicular tissues (50 mg) obtained from each mouse were homogenized by sonication (2 s  $\times$  20 s) in phosphate buffer solution (PBS) and then centrifuged at 10 000 g for 10 min. Testosterone concentrations in the supernatants were also determined employing the ELISA kit (R and D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The outcomes were expressed as ng g<sup>-1</sup> tissue.

### Immunofluorescence staining

For immunofluorescence staining, testes fixed with 4% paraformaldehyde were cryo-embedded in an optimal cutting temperature medium (Sakura Finetek, Torrance, CA, USA) and sectioned at a thickness of 4  $\mu$ m. The frozen testis sections were blocked by incubation in 5% normal serum and 0.1% Triton X-100 (Hyclone, Logan, Utah, USA) in PBS for 1 h at room temperature, followed by incubation with primary antibody overnight at 4°C. The next day, the sections were incubated with secondary antibody for 30 min followed by DAPI (blue) staining of nuclei. The primary and secondary antibodies used in this process are listed in **Table 1**. PBS replaced the primary antibody as negative control. All the images were captured using an LSM710 confocal microscope (Zeiss, Jena, Germany) and were analyzed using the Image J software (University Health Network, USA).

**Table 1: Primary and secondary antibodies used for immunofluorescence analysis**

Antibodies	Dilution	Distributor/source (catalog number)
Mouse polyclonal to STAR (D-2)	1:200	Santa Cruz, Heidelberg, Germany (sc-166821)
Goat polyclonal to 3 $\beta$ -HSD (D-18)	1:100	Santa Cruz, Heidelberg, Germany (sc-30820)
P450scc Enzyme rabbit polyclonal IgG	1:200	Millipore, Darmstadt, Germany (AB11244)
DAPI	1:1	Millipore, Darmstadt, Germany (S7113)
Goat anti-mouse IgG Alexa 488	1:1000	Invitrogen, Carlsbad, CA, USA (A11001)
Goat anti-rabbit IgG Alexa 488	1:1000	Invitrogen, Carlsbad, CA, USA (A11008)
Donkey anti-goat IgG Alexa 488	1:1000	Invitrogen, Carlsbad, CA, USA (A11055)

DAPI: 4',6-diamidino-2-phenylindole

The method of cell counting was similar to that employed in an earlier study.<sup>20</sup> Two sections per testis, five testes per group, were analyzed. The number of positive cells was counted in random interstitial spaces (space enclosed by three or four contorted seminiferous tubules). The numbers of positive cells per group were averaged for statistical analysis. All counts were performed using an Olympus microscope (Olympus Corporation, Tokyo, Japan, magnification:  $\times 400$ ).

### Quantitative real-time PCR

Total RNA from the testes was extracted using an RNeasy mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Reverse transcription reactions were performed using murine leukemia virus reverse transcriptase and oligo-dT primers (Fermentas, Lithuania). Real-time PCR was performed using the Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan) following the manufacturer's instructions. A Light Cycler 480 Detection System (Roche, Basel, Switzerland) was used to detect signals.  $\beta$ -actin mRNA was selected as an internal control. The primer sequences are listed as follows: 5-GGGCATACTCAACAACCAG-3 (upper primer) and 5-ACCTCCAGTCGGAACACC-3 (lower primer) for StAR, 5-AGTATCCGTGATGTGGGG-3 (upper primer) and 5-CATACAGTGTGCCTTTTCT-3 (lower primer) for P450 $\text{csc}$ , 5-TGTGCCAGCCTTCATCTAC-3 (upper primer) and 5-CTTCTCGGCCATCCTTTT-3 (lower primer) for 3 $\beta$ -HSD, and 5-TCGTGCGTGACATTAACGAG-3 (upper primer) and 5-ATTGCCTATCGTGATGACCT-3 (lower primer) for  $\beta$ -actin.

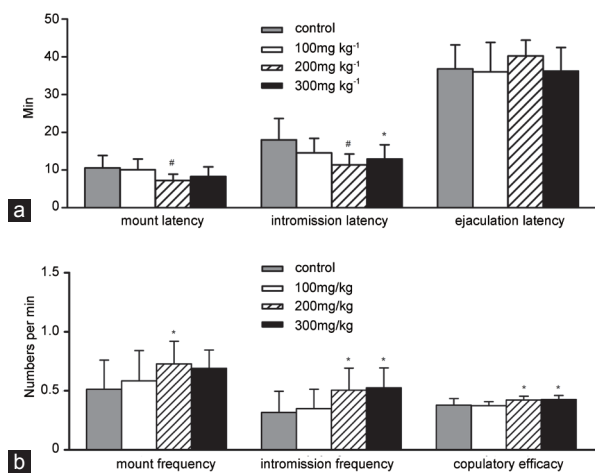
### Statistical analysis

One-way ANOVA for multiple group comparisons was used to assess the significance of differences. Results were presented as mean  $\pm$  s.e.m. In all cases,  $P < 0.05$  was considered statistically significant. All analytic results were performed using the GraphPad Software package (GraphPad Software, La Jolla, CA, USA).

## RESULTS

### Outcomes of sexual behavior

Sexual behavior changes after the administration of VAP are shown in **Figure 1**. The mount latency was shortened at the dose of 200 mg kg<sup>-1</sup> ( $P < 0.01$ ) and the intromission latency was shortened at the



**Figure 1:** Measures of sexual behavior of the control group and VAP-treated groups. All values are mean  $\pm$  s.e.m., \* $P < 0.01$ , \* $P < 0.05$  compared with control group. (a) Mean latency in minutes of mount, intromission and ejaculation. (b) Mean frequency per minute of mount, intromission and mean copulatory efficacy per minute.

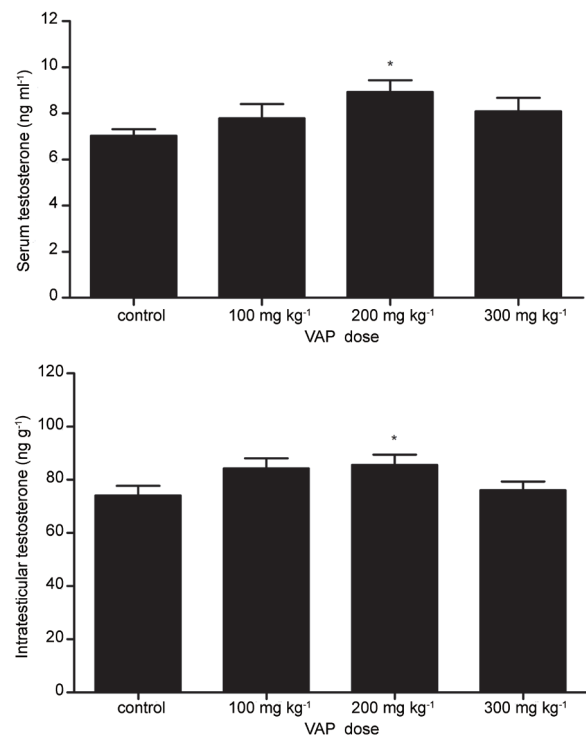
dose of 200 mg kg<sup>-1</sup> ( $P < 0.01$ ) and 300 mg kg<sup>-1</sup> ( $P < 0.05$ ). No differences of ejaculation latency were seen between the control and experimental groups ( $P > 0.05$ ). Comparison of the frequency of mount showed that although all of the three experimental groups had an upward trend, only the change at 200 mg kg<sup>-1</sup> was statistically higher than the control ( $P < 0.05$ ). Similarly, there was an increase in intromission frequency both in the 200 mg kg<sup>-1</sup> group and 300 mg kg<sup>-1</sup> group compared with the control group ( $P < 0.05$ ). VAP improved copulatory efficacy in groups treated with the dosage of 200 mg kg<sup>-1</sup> ( $P < 0.05$ ) and 300 mg kg<sup>-1</sup> ( $P < 0.05$ ), but no statistical difference was observed in group at the dosage of 100 mg kg<sup>-1</sup> ( $P > 0.05$ ).

### Effect of VAP on the weight of body and reproductive organs

As shown in **Table 2**, the control mice and experimental mice did not exhibit differences in body weight after the treatment of VAP. There was an increase in the testis weight of the 100 mg kg<sup>-1</sup> group compared to that of the control group ( $P < 0.05$ ). There was also an increase in the relative testis weight in the groups at the dose of 200 mg kg<sup>-1</sup> ( $P < 0.05$ ) and 300 mg kg<sup>-1</sup> ( $P < 0.05$ ). The epididymis weights of the 200 mg kg<sup>-1</sup> group and 300 mg kg<sup>-1</sup> group were higher than the control group ( $P < 0.05$ ). However, no statistical difference of the relative epididymis weight was observed between the control group and the experimental groups. Both the weight and the relative weight of seminal vesicle in groups at the dose of 200 mg kg<sup>-1</sup> and 300 mg kg<sup>-1</sup>, respectively were higher than the control group ( $P < 0.05$ ).

### Changes in serum testosterone and ITT

As shown in **Figure 2**, the serum testosterone levels of these mice at a dose of 200 mg kg<sup>-1</sup> increased obviously after treatment with VAP ( $P < 0.05$ ). Similarly, the ITT of the 200 mg kg<sup>-1</sup> group was also statistically higher than the control group ( $P < 0.05$ ). No changes were seen in other experimental groups compared with the control group.



**Figure 2:** Effects of VAP on the levels of serum testosterone and intratesticular testosterone. All values are mean  $\pm$  s.e.m., \* $P < 0.05$  compared with control group.

**Immunofluorescence staining results**

The effects of VAP at different dosages on the rate-limiting enzymes of testosterone synthesis are shown in **Figure 3**. The number of StAR-positive Leydig cells in groups at the dosage of 200 mg kg<sup>-1</sup> and 300 mg kg<sup>-1</sup> was markedly larger than that of the control group ( $P < 0.01$  and  $P < 0.05$ , respectively). Similarly, treatment with VAP at the dosage of 200 mg kg<sup>-1</sup> and 300 mg kg<sup>-1</sup> also increased the number of P450scc-positive Leydig cells ( $P < 0.05$  both). Only VAP at the dosage of 200 mg kg<sup>-1</sup> improved the expression of 3 $\beta$ -HSD in Leydig cells ( $P < 0.05$ ).

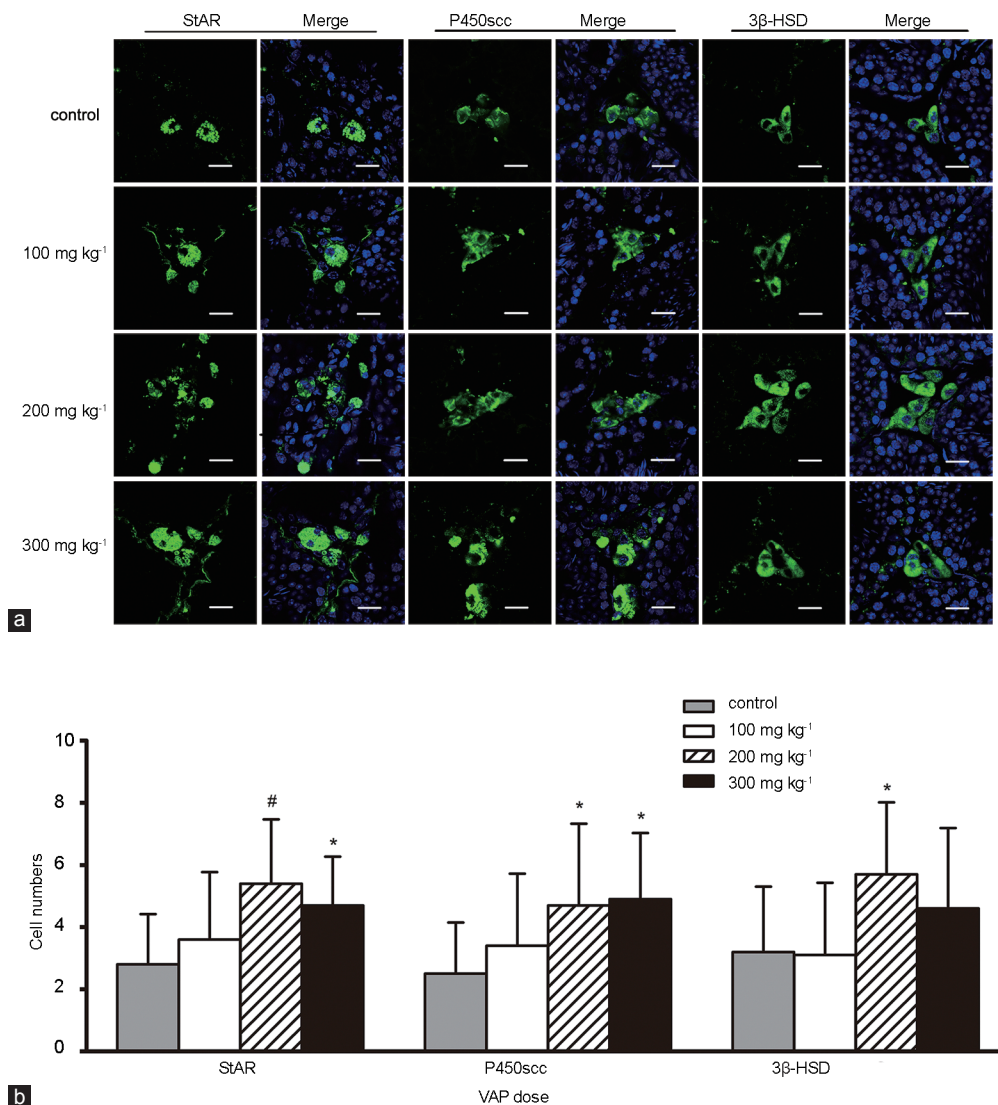
**Quantitative real-time PCR results**

The effects of VAP administration on the expression levels of genes related to testosterone synthesis were determined by quantitative real-time PCR (**Figure 4**). The mRNA levels of StAR were improved at the dose of 200 mg kg<sup>-1</sup> ( $P < 0.05$ ). The expression of P450scc was dramatically increased in the 300 mg kg<sup>-1</sup> group compared with control group ( $P < 0.05$ ). Similarly, the mRNA expression of 3 $\beta$ -HSD was also notably up-regulated in testes from mice dosed with 200 mg kg<sup>-1</sup> and 300 mg kg<sup>-1</sup> VAP ( $P < 0.05$  and  $P < 0.01$  respectively).

**Table 2: Changes in body weight and reproductive organs**

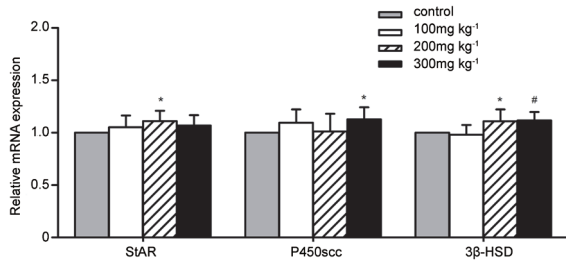
Group	n	BW (g)	TW (g)	Relative TW (g per 100 g)	EW (g)	Relative EW (g per 100 g)	SW (g)	Relative SW (g per 100 g)
Control	10	29.500±2.550	0.206±0.034	0.695±0.068	0.071±0.012	0.242±0.041	0.204±0.048	0.690±0.133
100 mg kg <sup>-1</sup>	10	30.800±3.155	0.238±0.042*	0.770±0.090*	0.077±0.014	0.253±0.050	0.228±0.049	0.737±0.133
200 mg kg <sup>-1</sup>	10	30.300±3.268	0.237±0.028	0.785±0.081*	0.082±0.010*	0.274±0.049	0.254±0.039*	0.843±0.140*
300 mg kg <sup>-1</sup>	10	30.500±2.759	0.226±0.031	0.742±0.083	0.083±0.011*	0.275±0.053	0.259±0.044*	0.848±0.133*

Data are presented as mean±s.e.m. \* $P < 0.05$ : statistical significance compared with control group; BW: body weight; TW: testis weight; Relative TW: TW/BW × 100; EW: epididymis weight; Relative EW: EW/BW × 100; SW: weight of seminal vesicle; Relative SW: SW/BW × 100; s.e.m: standard error of mean



**Figure 3:** The StAR, P450scc, and 3 $\beta$ HSD-positive Leydig cells in interstitial spaces of all groups. **(a)** The number of StAR, P450scc, and 3 $\beta$ -HSD-positive cells ( $n = 10$  per group) was determined,  $\times 400$ . Scale bar, 20  $\mu$ m. **(b)** The number of StAR, P450scc and 3 $\beta$ -HSD-positive cells per interstitial space. Results are shown as mean  $\pm$  s.e.m., # $P < 0.01$ , \* $P < 0.05$  compared with control group.





**Figure 4:** Quantitative real-time PCR analysis of StAR, P450scc, and 3 $\beta$ -HSD gene expression in testes of all groups. Data are expressed as the mean  $\pm$  s.e.m., # $P < 0.01$ , \* $P < 0.05$  compared with control group.

## DISCUSSION

In the present study, we selected naturally aging male mice as an animal model of LOH to test the effects of VAP on LOH. Our results show that the administration of VAP could improve the sexual function, enhance the testosterone synthesis *in vivo* and increase the expression of StAR, P450scc, and 3 $\beta$ -HSD in Leydig cells of aging male mice.

Testicular testosterone production in men is reported to decrease by 1%–2% per year after the age of 40 years.<sup>22</sup> Some aging men develop mild symptomatic testosterone deficiency, such as sexual dysfunction, muscle weakness, osteoporosis, obesity, hot flashes, insomnia, fatigue, poor concentration, and depression.<sup>22</sup> Several names, including male menopause, partial androgen deficiency of the aging male (PADAM), and andropause, have been used to describe these testosterone deficiency symptoms depicted above. LOH was first used by Morales and Lunenfeld in 2002<sup>23</sup> and was thought to be able to describe the nature of the syndrome best. In 2005, LOH was officially adopted by the International Society of Andrology (ISA), the International Society for the Study of the Aging Male (ISSAM), and the European Association of Urology (EAU).<sup>24</sup> Wang *et al.* defined LOH as “a clinical and biochemical syndrome associated with advancing age and characterized by symptoms and a deficiency in serum testosterone levels (below the young healthy adult male reference range).”<sup>25</sup> According to the European Male Ageing Study (EMAS), sexual symptoms (erectile dysfunction, lessened sexual thoughts, and weakened morning erections) are essential to the diagnosis of LOH.<sup>22</sup> Improving the sexual function of patients with LOH is very important. Since testosterone production is insufficient in these men, testosterone supplementation is considered an effective method to ameliorate sexual function. A recent meta-analysis has shown that TRT plays positive effects on male sexual function in hypogonadal subjects.<sup>26</sup> But, as mentioned above, it is necessary to explore a new approach to the treatment of LOH because of the non-negligible side-effects of TRT.

In China, Japan, and other regions of Southeast Asia, some traditional medicines are often the first choice for treating LOH in order to avoid the adverse effects of TRT.<sup>27</sup> VA has been used in the East for thousands of years to prevent or treat diseases. According to modern medical research, VA has been proven to have various pharmacological effects, such as enhancing the immune system and physical strength, anti-inflammatory effects, antioxidant properties, antilipid peroxidation properties, and improving sexual function.<sup>28–32</sup> Zhang *et al.*<sup>33</sup> treated osteoporotic rats caused by bilateral ovariectomy with VAP and detected their have anti-osteoporotic effects. Several reports suggested that VA reduces fatigue.<sup>34,35</sup> Shao *et al.* investigated the therapeutic effects of VA on cardiac function in rats with heart failure following myocardial infarction and found it may be beneficial for the treatment of heart failure.<sup>36</sup> VA also showed anti-aging effects and

could improve the behavior and immune function of aging mice.<sup>15,16</sup> As mentioned above, these symptoms were also seen in LOH and were considered to be related to a deficiency in serum testosterone levels.

In the present study, we observed the beneficial effects of repeated oral administration of VAP on the sexual behavior of old male mice. To the best of our knowledge, this research is the first time this research topic was conducted on a naturally aging animal model. We selected 24-month-old male mice as an LOH animal model for examining the effects of VAP on sexual functions and testosterone levels *in vivo*. In order to investigate whether the administration of VAP can have positive effect on the sexual behavior of aging male mice, we tested several relevant items described above. We found that VAP could increase the number of mounts and intromissions before ejaculation and shorten the latency of them. No obvious difference of ejaculation latency in VAP treated groups was seen compared with the control group. Also, copulatory efficacy was also improved by VAP. According to previous studies, mount latency and intromission latency are inversely proportional to sexual motivation, while ejaculation latency, intromission frequency, and copulatory efficacy are indicative of potency.<sup>37,38</sup> That is, shorter latency of mount and intromission implies higher sexual desire, while longer ejaculate latency, higher intromission frequency and copulatory efficacy mean increased potency. All of these results suggest that VAP may have a favorable effect on sexual function in aging male mice. This is in accordance with the fundamentals of traditional Chinese medicine.

After tests of sexual behavior, the weight of reproductive organs and testosterone levels were measured. We found that testis weight and relative testis weight increased after the treatment of VAP. This phenomenon indicated that the function of testis might have been strengthened. This was further indicated by the fact that the levels of serum testosterone at the dosage of 200 mg kg<sup>-1</sup> increased after the VAP treatment compared with the control group. The weights of epididymis and seminal vesicle also rose after the administration of VAP, and this might be attributed to the increase of testosterone synthesis. ITT was also improved at the dosage of 200 mg kg<sup>-1</sup> compared to that in the control group. The improvement of testis weight and ITT also indicated that VAP might raise the testosterone *in vivo* by acting on interstitial tissue of testis.

The first rate-limiting step in the biosynthesis of testosterone in testis is the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane. This process depends on the action of StAR.<sup>39,40</sup> After that, cholesterol is converted into pregnenolone by the effect of the P450scc enzyme residing in the inner mitochondrial membrane.<sup>41</sup> Then, pregnenolone is metabolized to androgens modulated by 3 $\beta$ -HSD.<sup>42</sup> Our *in vivo* results had demonstrated that the administration of VAP could improve the concentration of testosterone, both in serum and in testis. We observed the expression of these rate-limiting enzymes mentioned above by use of immunofluorescence staining. We found that, accompanied with the increase of testosterone concentration, the expression of StAR, P450scc, and 3 $\beta$ -HSD had been enhanced after the use of VAP. To further explore the changes of these rate-limiting enzymes, we also monitored the mRNA levels of StAR, P450scc, and 3 $\beta$ -HSD in the testis tissue by use of quantitative RT-PCR analyses. In agreement of the immunofluorescence staining results, the expression level of StAR, P450scc, and 3 $\beta$ -HSD genes was also increased. Several articles have reported that some traditional medicines can improve sexual behavior by increasing serum testosterone concentrations.<sup>43–47</sup> However, seldom studies have revealed the mechanism of improving testosterone secretion by traditional medicines. One research claimed

that *Zingiber officinale* and *Pentadiplandra brazzeana* could increase serum testosterone levels and ITT in rats by elevating testicular cholesterol, the starting material for androgen biosynthesis.<sup>48</sup> Our previous study indicates that saikokaryukotsuboreito, an herbal medicine, could improve the serum testosterone concentrations by activating the expression of StAR.<sup>20</sup> In the present study, the data indicate that VAP could improve the testosterone biosynthesis by increasing the expression of three rate-limiting enzymes of testosterone synthesis (StAR, P450scc, and 3 $\beta$ -HSD). In addition, as previously stated, we also found that VAP could raise ITT, which was thought to be most critical to spermatogenesis.<sup>49,50</sup> Some previously reported studies on traditional medicines have also observed the improvement of ITT.<sup>48,51</sup> We hypothesize that some traditional medicines could stimulate an increase in the body natural endogenous testosterone levels, which might lead to promotions of spermatogenesis and fertility. However, it should be studied by further experiments.

It has been suggested that cardiovascular risk was associated with TRT.<sup>52</sup> For cardiovascular safety, the US Food and Drug Administration (FDA) has made strict provisions in the production and sales of testosterone products and have ruled against many of them.<sup>53,54</sup> As previously mentioned, VA is found to be beneficial for the treatment of heart failure.<sup>36</sup> According to our data, all of the animals exhibited perfect state of health, and no side-effects were seen during the experiment, which means that VAP is safe. Taken together, we think that VAP can improve endogenous testosterone and benefit heart diseases. Hence, VAP might be a good choice for LOH patients with cardiovascular diseases.

According to our results, no obvious dose-dependent effects were seen, and the effect at the dose of 200 mg kg<sup>-1</sup> seemed most noticeable. This phenomenon indicated that VAP might have an optimal dose for treatment. There are some limitations to our study. Previous studies have shown that the synthesis of testosterone is modulated by the HPG axis. Further studies are required to investigate whether VAP administration has an effect on the release of gonadotropin-releasing hormone, luteinizing hormone, and follicle-stimulating hormone. In addition, the number of samples in the present study is relatively small, which may influence the power of statistical tests.

## CONCLUSION

Our results indicate that VAP has a physiological role in the synthesis of testosterone by enhancing the expression of StAR, P450scc, and 3 $\beta$ -HSD in the testis of aging male mice. The administration of VAP might be a safe therapy method of treating LOH, and further clinical trials and safety assessments in humans still need to be performed.

## AUTHOR CONTRIBUTIONS

ZJZ designed and performed the experiments, analyzed the data, drafted the manuscript and supervised the team. HFT helped design the study, conduct the experiments and drafted the manuscript. YT and WJX helped conduct the experiments. SYJ and YG helped analyzing the data. CHD conceived the idea of this research. All authors read and approved the final version of the manuscript.

## COMPETING INTERESTS

The authors declared that they had no competing interests.

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## REFERENCES

- Mäkinen JI, Huhtaniemi I. Androgen replacement therapy in late-onset hypogonadism: current concepts and controversies – A mini-review. *Gerontology* 2011; 57: 193–202.
- Lunenfeld B, Saad F, Hoels CE. ISA, ISSAM and EAU recommendations for the investigation, treatment and monitoring of late-onset hypogonadism in males: scientific background and rationale. *Aging Male* 2005; 8: 59–74.
- Wu FC, Tajar A, Beynon JM, Pye TR, Phil M, *et al.* Identification of late-onset hypogonadism in middle-aged and elderly men. *N Engl J Med* 2010; 363: 123–35.
- Okada K, Yamaguchi K, Chiba K, Miyake H, Fujisawa M. Comprehensive evaluation of androgen replacement therapy in aging Japanese men with late-onset hypogonadism. *Aging Male* 2014; 17: 72–5.
- Yassin DJ, Doros G, Hammerer PG, Yassin AA. Long-term testosterone treatment in elderly men with hypogonadism and erectile dysfunction reduces obesity parameters and improves metabolic syndrome and health-related quality of life. *J Sex Med* 2014; 11: 1567–76.
- Pye SR, Huhtaniemi IT, Finn JD, Lee DM, O'Neill TW, *et al.* Late-onset hypogonadism and mortality in aging men. *J Clin Endocrinol Metab* 2014; 99: 1357–66.
- Kenny AM, Bellantonio S, Gruman CA, Acosta RD, Prestwood KM. Effects of transdermal testosterone on cognitive function and health perception in older men with low bioavailable testosterone levels. *J Gerontol A Biol Sci Med Sci* 2002; 57: M321–5.
- Isidori AM, Giannetta E, Gianfrilli D, Greco EA, Bonifacio V, *et al.* Effects of testosterone on sexual function in men: results of a meta-analysis. *Clin Endocrinol (Oxf)* 2005; 63: 381–94.
- Srinivas-Shankar U, Roberts SA, Connolly MJ, O'Connell MD, Adams JE, *et al.* Effects of testosterone on muscle strength, physical function, body composition, and quality of life in intermediate-frail and frail elderly men: a randomized, double-blind, placebo-controlled study. *J Clin Endocrinol Metab* 2010; 95: 639–50.
- McGill JJ, Shoskes DA, Sabanegh ES. Androgen deficiency in older men: indications, advantages, and pitfalls of testosterone replacement therapy. *Cleve Clin J Med* 2012; 79: 797–806.
- Moss JL, Crosnoe LE, Kim ED. Effect of rejuvenation hormones on spermatogenesis. *Fertil Steril* 2013; 99: 1814–20.
- Sui Z, Zhang L, Huo Y, Zhang Y. Bioactive components of velvet antlers and their pharmacological properties. *J Pharm Biomed Anal* 2014; 87: 229–40.
- He G, Wang BX, Zhang W, Wang XN. Effects of velvet antler polypeptide on the secretion of luteinizing hormone and testosterone in male mice. *Chin Tradit Pat Med* 1994; 17: 33–4.
- He G. Research development of pharmacological actions clinical applications of velvet antler. *J Yanbian Med Coll* 1992; 15: 231–4.
- Yang ZY, Qin HB, Cheng HL, Zhu Q. Effects of elk antler ethanolic fluidextract on behavior and immune function of aging model mice. *China J Tradit Chin Med Pharm* 2010; 25: 221–5.
- Qin HB, Yang ZY, Zhu Q, Zhu YF. Studies on anti-ageing effect of elk antlers ethanolic fluidextract. *Chin Tradit Pat Med* 2004; 26: 322–4.
- Guo YJ, Zhou QL, Liu P, Wang Y, Fang JR, *et al.* The research of pilose antler polypeptides promoting osteoblast precursor cells and chondrocytes proliferation. *Chin J Biochem Pharm* 1998; 19: 74–6.
- Zhou QL, Guo YJ, Wang LJ, Wang Y, Liu YQ, *et al.* Velvet antler polypeptides promoted proliferation of chondrocytes and osteoblast precursors and fracture healing. *Zhongguo Yao Li Xue Bao=Acta Pharmacol Sin* 1999; 20: 279–82.
- Lee HS, Jung EY, Bae SH, Kwon KH, Kim JM, *et al.* Stimulation of osteoblastic differentiation and mineralization in MC3T3-E1 cells by yeast hydrolysate. *Phytother Res* 2011; 25: 716–23.
- Zang ZJ, Ji SY, Dong W, Zhang YN, Zhang EH, *et al.* A herbal medicine, saikokaryukotsuboreito, improves serum testosterone levels and affects sexual behavior in old male mice. *Aging Male* 2015; 18: 106–11.
- Robertson KM, Schuster GU, Steffensen KR, Hovatta O, Meaney S, *et al.* The liver X receptor-(beta) is essential for maintaining cholesterol homeostasis in the testis. *Endocrinology* 2005; 146: 2519–30.
- Huhtaniemi I. Late-onset hypogonadism: current concepts and controversies of pathogenesis, diagnosis and treatment. *Asian J Androl* 2014; 16: 192–202.
- Morales A, Lunenfeld B, International Society for the Study of the Aging Male. Investigation, treatment and monitoring of late-onset hypogonadism in males. Official recommendations of ISSAM. International society for the study of the aging male. *Aging Male* 2002; 5: 74–86.
- Nieschlag E, Swerdloff R, Behre HM, Gooren LJ, Kaufman JM, *et al.* Investigation, treatment and monitoring of late-onset hypogonadism in males. ISA, ISSAM, and EAU recommendations. *Eur Urol* 2005; 48: 1–4.
- Wang C, Nieschlag E, Swerdloff R, Behre HM, Hellstrom WJ, *et al.* Investigation, treatment and monitoring of late-onset hypogonadism in males. *Int J Androl* 2009; 32: 1–10.
- Corona G, Isidori AM, Buvat J, Aversa A, Rastrelli G, *et al.* Testosterone supplementation and sexual function: a meta-analysis study. *J Sex Med* 2014; 11: 1577–92.

- 27 Michihara S, Shin N, Watanabe S, Morimoto Y, Okubo T, *et al*. A Kampo formula, *saikokaryukotsuboreito*, improves serum testosterone levels of castrated mice and its possible mechanism. *Aging Male* 2013; 16: 17–21.
- 28 Kim KS, Choi YH, Kim KH, Lee YC, Kim CH, *et al*. Protective and anti-arthritis effects of deer antler aqua-acupuncture (DAA), inhibiting dihydroorotate dehydrogenase, on phosphate ions-mediated chondrocyte apoptosis and rat collagen-induced arthritis. *Int Immunopharmacol* 2004; 4: 963–73.
- 29 Chen X, Wang Y, Wu Y, Wang L, Li W. Protective effects of peptides from velvet antler of *Cervus nippon* on acute ischemic myocardial injury in rats. *Zhongguo Zhong Yao Za Zhi* 2009; 34: 1971–4.
- 30 Kawtikwar PS, Bhagwat DA, Sakarkar MD. Deer antlers-traditional use and future perspectives. *Indian J Tradit Knowl* 2010; 9: 245–51.
- 31 Dai TY, Wang CH, Chen KN, Huang IN, Hong WS, *et al*. The antiinfective effects of velvet antler of Formosan sambar deer (*Cervus unicolor swinhoei*) on *Staphylococcus aureus*-infected mice. *Evid Based Complement Alternat Med* 2011; 2011: 534069.
- 32 Gilbey A, Perezgonzalez JD. Health benefits of deer and elk velvet antler supplements: a systematic review of randomised controlled studies. *N Z Med J* 2012; 125: 80–6.
- 33 Zhang LZ, Xin JL, Zhang XP, Fu Q, Zhang Y, *et al*. The anti-osteoporotic effect of velvet antler polypeptides from *Cervus elaphus* Linnaeus in ovariectomized rats. *J Ethnopharmacol* 2013; 150: 181–6.
- 34 Luo XD, Pan FG, Zhang TH, Zhang MD, Song G, *et al*. Effects of pilose antler polypeptide on ability of mice anti-anoxia and anti-fatigue. *Food Sci* 2008; 29: 386–8.
- 35 Zhang R, Zhao YH, Wang ZZ. Anti-fatigue effects of antler velvet water extract in mice. *Sci Technol Food Ind* 2011; 32: 365–7.
- 36 Shao MJ, Wang SR, Zhao MJ, Lv XL, Xu H, *et al*. The effects of velvet antler of deer on cardiac functions of rats with heart failure following myocardial infarction. *Evid Based Complement Alternat Med* 2012; 2012: 825056.
- 37 Clark JT, Kalra SP, Kalra PS. Effects of a selective alpha 1-adrenoceptor agonist, methoxamine, on sexual behavior and penile reflexes. *Physiol Behav* 1987; 40: 747–53.
- 38 Benelli A, Bertolini A, Zoli M, Leo G, Filafiero M, *et al*. Pharmacological manipulation of brain galaninergic system and sexual behavior in male mice. *Psychopharmacology (Berl)* 2002; 160: 325–30.
- 39 Christenson LK, Strauss JF 3<sup>rd</sup>. Steroidogenic acute regulatory protein: an update on its regulation and mechanism of action. *Arch Med Res* 2001; 32: 576–86.
- 40 Stocco DM. StAR protein and the regulation of steroid hormone biosynthesis. *Annu Rev Physiol* 2001; 63: 193–213.
- 41 Simpson ER. Cholesterol side-chain cleavage, cytochrome P450, and the control of steroidogenesis. *Mol Cell Endocrinol* 1979; 13: 213–27.
- 42 Simard J, Ricketts ML, Gingras S, Soucy P, Feltus FA, *et al*. Molecular biology of the 3beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase gene family. *Endocr Rev* 2005; 26: 525–82.
- 43 Ratnasooriya WD, Fernando TS. Effect of black tea brew of *Camellia sinensis* on sexual competence of male rats. *J Ethnopharmacol* 2008; 118: 373–7.
- 44 Yakubu MT, Afolayan AJ. Effect of aqueous extract of *Bulbine natalensis* (Baker) stem on the sexual behaviour of male rats. *Int J Androl* 2009; 32: 629–36.
- 45 Yakubu MT, Akanji MA, Oladiji AT. Aphrodisiac potentials of the aqueous extract of *Fadogia agrestis* (Schweinf. Ex Hiern) stem in male albino rats. *Asian J Androl* 2005; 7: 399–404.
- 46 Ajiboye TO, Nurudeen QO, Yakubu MT. Aphrodisiac effect of aqueous root extract of *Lecaniodiscus cupanioides* in sexually impaired rats. *J Basic Clin Physiol Pharmacol* 2014; 25: 241–8.
- 47 Zanolli P, Benelli A, Zavatti M, Rivasi M, Baraldi C, *et al*. Improved sexual behavior in male rats treated with a Chinese herbal extract: hormonal and neuronal implications. *Asian J Androl* 2008; 10: 937–45.
- 48 Kamtchouing P, Mbongue FG, Dimo T, Jatsa HB. Evaluation of androgenic activity of *Zingiber officinale* and *Pentadiplandra brazzeana* in male rats. *Asian J Androl* 2002; 4: 299–301.
- 49 Weinbauer GF, Nieschlag E. Gonadotrophin-releasing hormone analogue-induced manipulation of testicular function in the monkey. *Hum Reprod* 1993; 8 Suppl 2: 45–50.
- 50 McLachlan RI, O'Donnell L, Meachem SJ, Stanton PG, de K, *et al*. Hormonal regulation of spermatogenesis in primates and man: insights for development of the male hormonal contraceptive. *J Androl* 2002; 23: 149–62.
- 51 Watcho P, Kamtchouing P, Sokeng SD, Moundipa PF, Tantchou J, *et al*. Androgenic effect of *Mondia whitei* roots in male rats. *Asian J Androl* 2004; 6: 269–72.
- 52 Vigen R, O'Donnell CI, Baron AE, Grunwald GK, Maddox TM, *et al*. Association of testosterone therapy with mortality, myocardial infarction, and stroke in men with low testosterone levels. *JAMA* 2013; 310: 1829–36.
- 53 Garnick MB. Testosterone replacement therapy faces FDA scrutiny. *JAMA* 2015; 313: 563–4.
- 54 Harris OL. FDA declines approval of testosterone drug for third time. *Lancet Diabetes Endocrinol* 2013; 1 Suppl 1: s14.