RESEARCH ARTICLE

Aphrodisiac Effect of Cultured *Cordyceps militaris* in Aged Male Rats

Suphaphorn Saksopha[1], Watcharaporn Preedapirom[2], Natthapong Singpoonga[3], Peerasak Chaiprasart[4], Pornnarin Taepavarapruk[1]

[1] *Department of Physiology, Faculty of Medical Sciences, Naresuan University, Phitsanulok, Thailand*
[2] *Division of Physiology, School of Medical Science, University of Phayao, Phayao, Thailand*
[3] *Faculty of Science and Technology, Nakhon Sawan Rajabhat University, Nakhon Sawan, Thailand*
[4] *Department of Agricultural Science, Faculty of Agriculture, Natural Resources and Environment, Naresuan University, Phitsanulok, Thailand*

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Abstract

Cordyceps militaris has been well known as an elixir since ancient times. Due to its highly priced and its scarcity of this natural fungus, the cultured *C. militaris* (CCM) has been developed and increasingly consumed for health-promoting effects. In this study, *C. militaris* was cultured in the cereal grain culture medium. Since its effect has not yet been extensively studied, this study aimed to investigate the effects of CCM focusing on its aphrodisiac effect in aging rat model with erectile dysfunction (ED). The results showed that aged rats treated with CCM (0.1, 0.5, and 1.0 g/kg BW) and sildenafil citrate (5 mg/kg BW) by gavage demonstrated significant improvements in their mating behavior and intracavernosal pressure responses to cavernous nerve stimulation. Serum testosterone level of these rats also increased markedly and a significant increase was seen in rats treated with CCM 1.0 g/kg BW. The level of penile nitric oxide synthase activity of aged rats treated with CCM 1.0 g/kg BW showed significantly higher than that of the aged vehicle group. Malondialdehyde levels in testicular tissues of aged rats treated with CCM were significantly reduced. The results obtained from this study indicated that CCM are able to restore erection functions in aged rats. Therefore, CCM could be used as aphrodisiacs for enhancing sexual performance and for the treatment of ED, especially in aged men.

Keywords: Aphrodisiac activity, *Cordyceps militaris*, aging, erectile dysfunction

Address correspondence and reprint request to: Pornnarin Taepavarapruk, Department of Physiology, Faculty of Medical Sciences, Naresuan University, Phitsanulok 65000, Thailand.
E-mail address: pornnarint@nu.ac.th; taepavap@yahoo.com

ฤทธิ์เสริมสมรรถภาพทางเพศของเห็ดถั่งเช่าสีทองเพาะเลี้ยงในหนูแก่เพศผู้

สุภาภรณ์ ศักดิ์โสภา[1], วัชราภรณ์ ปรีดาภิรมย์[2], ณัฐพงษ์ สิงห์ภูงา[3], พีระศักดิ์ ฉายประสาท[4], พรนรินทร์ เทพาวราพฤกษ์[1]

[1] ภาควิชาสรีรวิทยา คณะวิทยาศาสตร์การแพทย์ มหาวิทยาลัยนเรศวร จังหวัดพิษณุโลก ประเทศไทย
[2] สาขาสรีรวิทยา คณะวิทยาศาสตร์การแพทย์ มหาวิทยาลัยพะเยา จังหวัดพะเยา ประเทศไทย
[3] คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยราชภัฏนครสวรรค์ จังหวัดนครสวรรค์ ประเทศไทย
[4] ภาควิชาเกษตรศาสตร์ คณะเกษตรศาสตร์ ทรัพยากรธรรมชาติและสิ่งแวดล้อม มหาวิทยาลัยนเรศวร จังหวัดพิษณุโลก ประเทศไทย

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บทคัดย่อ

เห็ดถั่งเช่าสีทอง (*Cordyceps militaris*) เป็นที่รู้จักกันมาตั้งแต่อดีตกาลว่าเป็นยาอายุวัฒนะ แต่เนื่องจากราคาที่ค่อนข้างสูงและหายากตามธรรมชาติ จึงทำให้มีการพัฒนาการเพาะเลี้ยงเห็ดถั่งเช่าสีทองขึ้นในห้องปฏิบัติการและมีการบริโภคเพื่อช่วยเสริมสุขภาพกันมากขึ้น ในการศึกษาครั้งนี้ เห็ดถั่งเช่าสีทองถูกเพาะเลี้ยงในอาหารเลี้ยงเชื้อผสมเมล็ดธัญพืช แต่เนื่องจากการศึกษาสรรพคุณยังมีไม่มากนัก การศึกษานี้จึงมีจุดประสงค์เพื่อทดสอบฤทธิ์ของเห็ดถั่งเช่าสีทอง โดยเน้นที่ฤทธิ์เสริมสมรรถภาพทางเพศในหนูแก่เพศผู้ที่มีภาวะเสื่อมสมรรถภาพทางเพศ ผลการทดสอบพบว่า หนูแก่ที่ได้รับเห็ดถั่งเช่าสีทอง (0.1, 0.5 และ 1.0 ก./กก. น้ำหนักตัว) และยาซิเดลนาฟิล (5 มก./กก. น้ำหนักตัว) โดยการป้อนลงกระเพาะ แสดงพฤติกรรมการผสมพันธุ์และการตอบสนองของแรงดันภายในแกนองคชาติหลังการกระตุ้นไฟฟ้าที่เส้นประสาทคาร์เวอนัสดีขึ้น ระดับฮอร์โมนเทสโทสเตอโรนในเลือดของหนูแก่เหล่านี้สูงขึ้นอย่างมากเมื่อเทียบกับหนูแก่กลุ่มควบคุมและพบการเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติในหนูแก่ที่ได้รับเห็ดถั่งเช่าสีทองขนาด 1.0 ก./กก. น้ำหนักตัว ระดับการทำงานของเอนไซม์ไนตริกออกไซด์ซินเทส ในเนื้อเยื่อองคชาตของหนูแก่ที่ได้รับเห็ดถั่งเช่าสีทองขนาด 1.0 ก./กก. น้ำหนักตัวมีค่าสูงกว่าหนูแก่กลุ่มควบคุมอย่างมีนัยสำคัญ ระดับของมาลอนไดอัลดีไฮด์ในเนื้อเยื่ออัณฑะของหนูแก่กลุ่มที่ได้รับเห็ดถั่งเช่าสีทองลดลงอย่างมีนัยสำคัญ ผลที่ได้จากการศึกษานี้แสดงให้เห็นว่า เห็ดถั่งเช่าสีทองที่เพาะเลี้ยงสามารถฟื้นสมรรถภาพทางเพศในหนูแก่ได้ ดังนั้น เห็ดถั่งเช่าสีทองน่าจะสามารถใช้เป็นสารกระตุ้นความต้องการทางเพศและใช้รักษาภาวะหย่อนสมรรถภาพทางเพศโดยเฉพาะในผู้ชายที่มีอายุมากได้

คำสำคัญ: ฤทธิ์เสริมสมรรถภาพทางเพศ, เห็ดถั่งเช่าสีทอง, ความชราภาพ, ภาวะเสื่อมสมรรถภาพทางเพศ

Introduction

Erectile dysfunction (ED), also known as impotence, is a condition in which men are unable to get or keep an erection firm enough for satisfactory sexual intercourse.[1] ED is the most common sex problem that its prevalence and incidence are associated with aging as well as several comorbidities, such as cardiovascular disease, diabetes, metabolic syndrome, and neurological disorders.[2] Oral agents that have been considered as first line therapies are phosphodiesterase type 5 (PDE5) inhibitors which increase the level of cyclic guanosine monophosphate (cGMP), resulting in smooth muscle relaxation. However, common side effects of three approved PDE5 inhibitors (sildenafil, vardenafil and tadalafil) such as headache, dizziness, blurred vision, insomnia, muscle pain, back pain and upset stomach have been reported.[3] Some patients may prefer alternatives such as aphrodisiacs for the treatment of ED. An aphrodisiac is defined as any food or drug that arouses the sexual instinct, induces venereal desire and increases pleasure and performance.[4] Several medicinal plants including fungi have been reported to have aphrodisiac properties.[5-7] *Cordyceps militaris* (*C. militaris*), belonging to the family Clavicipitaceae, is a complex fungus which parasitic on pupae and larvae of Lepidoptera. Traditional Chinese medicine believes that *C. militaris* can be used to treat impotence, seminal emission, and infertility, and invigorate kidney and lungs.[8] *C. militaris* is also a potential source of bio-metabolites for herbal drugs and evidences are available about its applications for revitalization of various systems of the body from ancient times. Chemical and pharmacological researches have shown that *C. militaris* contains a variety of active ingredients, including cordycepin, nucleosides, adenosine, carotenoids, ergosterol, polysaccharides, and other compounds.[9]

The fruiting bodies of wild *C. militaris* in the form of the fungus and dead insect have been used as a traditional Chinese medicine for centuries. Because of host specificity and rarity in nature, this wild fungus is very expensive. So far, this medicinal fungus can be cultured artificially with similar identities of active components for mass scale production to support the markets as health supplements or nutraceuticals. Several studies have investigated pharmacological activities of cultured mycelia and fruiting bodies of *C. militaris* especially their anti-inflammatory and anticancer activities.[10-12] Its positive effects in sexual function have also been elucidated in young rats[13] and middle-aged rats focusing on testicular function as well as sperm production and sperm motility.[14] In this study, the aphrodisiac property of CCM was examined by measuring mating behavior as well as the intracavernosal pressure (ICP) following electrical stimulation of the cavernous nerve of aged rats. Its mechanism of action was also investigated.

Materials and Methods

Fungal strain and inoculum preparation

Cultured *C. militaris* (CCM) was prepared by the Department of Agricultural Science, Faculty of Agriculture, Natural Resources and Environment, Naresuan University as previously described.[15] In brief, the mycelia were cultivated on modified of potato dextrose agar (MPDA) medium and cultivated under static conditions at 22°C for 14 days. The resultant culture was then transferred to potato dextrose broth

(PDB) medium. PDB medium was autoclaved at 121℃ for 15 min and inoculated with the mycelia of *C. militaris* before incubating on a rotary shaker incubator at 120 rpm at 22℃ for 14 days. Finally, mycelia growing in PDB medium were transferred to sterilized rice culture medium that contained white rice (Sao-Hai rice) and silkworm pupae. The incubated medium was cultured at 22℃ in the dark for 7-14 days. To induce fruiting bodies, each bottle with the inoculum was kept at 18℃ under 12 h light and 12 h dark and 60-70% humidity until the mycelia was transformed into the fruiting body primordia. Subsequently, flasks were held at 22℃ and 80-90% relative humidity for 64 days. The fruiting bodies were harvested and kept frozen at -20℃ in order to stabilise the active compounds until use. *C. militaris* was weighed and daily prepared to a smooth consistency by blending with reverse osmosis (RO) water.

Experimental animals

Procedures involving animals were approved by the Naresuan University Animal Care and Use Committee (NUACUC; approval no. 58 01 006), with an effort to minimize animal suffering. All experiments were carried out in accordance with the Ethical Principles for the Use of Animals for Scientific Purposes at Naresuan University Centre of Animal Research (NUCAR) that has been accredited by AAALAC International. Ten young male Sprague Dawley (SD) (age 5-6 months), 15 young female SD rats (age 3-4 months), and 50 aged male SD rats (age 15-18 months) were used in this study. All rats were purchased from M-CLEA Bioresource Co., Ltd. (Samut Prakan, Thailand) and housed under controlled temperature at 22±1°C and relative humidity of 55±10% with 12:12 h of reverse light and dark cycle (light cycle: 09:00 PM to 09:00 AM; dark cycle: 09:00 AM to 09:00 PM) for the purpose of mating study. They were fed *ad libitum* with normal laboratory pellet diet (CP No. 082; C.P. Company, Bangkok, Thailand) and allowed free access to RO water. Aged male rats were divided into 5 groups of 10 rats per group. Three treated groups received 0.1, 0.5 and 1.0 g/kg BW of CCM, respectively. The positive control group received sildenafil citrate (5 mg/kg BW), while the negative control group received RO water. One group of young control rats (n=10) receiving RO water was used to compare and confirm age-related ED. All rats were orally administered either RO water, CCM or sildenafil citrate once daily for 21 days before conducting mating behavior test. Based on our previous dose-response study, 5 mg/kg BW sildenafil citrate was the optimal erectogenic dose in this animal model.

Procedures for bilateral ovariectomy in female rats

At least two weeks before mating behavior test, all female rats were subjected to bilateral ovariectomy surgery to prevent pregnancy. Rats were anesthetized with 2.5% isoflurane combined with oxygen and received an intramuscular injection of 5 mg/kg BW tramadol for pain reduction. After deep anesthesia, their abdominal areas were shaved and cleaned with alcohol pad. The lower abdominal skin and muscle were opened and the uterus tubes were pulled out and fastened. Both sides of the ovaries were then removed and lower abdominal muscle and skin were sutured by sterile silk. Betadine® was applied onto the wound before covered with Fixomull stretch®. Ovariectomized rats were given at least a two-week recovery period. Estrus

cycle stage was induced by subcutaneous injections of estradiol benzoate (100 mg/kg) and progesterone (1 mg/kg) 48 h and 4 h before mating behavior tests, respectively.

Procedures for mating behavior test

At the end of treatment regimens, mating behaviors were evaluated using a previously described method with a slight modification.[16] In brief, a male rat was placed at the centre of the glass box (50×35×35 cm) at the beginning of the test and allowed to acclimatize in the box for 5 min. After that, an ovariectomized female rat was introduced to the male rat. Their behaviors were constantly monitored and recorded by a digital video recorder for 30 min. The following male mating behavior parameters were calculated after monitoring for 30 min: (i) mount latency (ML): the time interval between the introduction of the female and the first mount by the male, (ii) mount frequency (MF): the number of mounts without intromission in a session, (iii) intromission frequency (IF): the number of intromissions in a session, (iv) intromission latency (IL): the time interval from the time of introduction of the female to the first intromission by the male, (v) ejaculation frequency (EF): the number of ejaculations in a session, and (vi) ejaculatory latency (EL): the time interval between the first intromission and ejaculation. All behavioral data were scored by two observers blinded to individual subject data and treatment group.

In vivo evaluation of erectile function

Erectile function was evaluated by measurement of intracavernosal pressure/ mean arterial pressure (ICP/MAP). Following mating behavior test, the male rats were anesthetized with 2-4% isoflurane combined with oxygen. The ventilation rate, pulse rate, temperature and heart rate were measured using PhysioSuite® (Kent Scientific Corporation, Torrington, CT, USA). Carotid artery was cannulated with a polyethylene tube filled with heparinized saline (250 U/mL) for measuring blood pressure. The penis was inserted with a 23G needle connected with polyethylene tube for measuring ICP. The cavernous nerve was stimulated by electrostimulation via a copper bipolar electrode linked to the PowerLab® (ADInstruments, New South Wales, Australia). The nerve was evoked with 20 Hz for 60 s, starting with 0.25, 0.50, 0.75, 1, 2, 3, 4, 5 or 10 V. Each stimulation was separated by a 5-min rest period between subsequent stimulations. Maximum rise in ICP was computed. Results were expressed as ICP/MAP where MAP is the mean arterial pressure computed at the same time. The results were recorded and computed using Labchart version 7.3.7 (ADInstruments, New South Wales, Australia).

Testosterone Measurement

After completion of ICP measurement, rats were euthanized by an overdose of sodium pentobarbital (100 mg/kg; intraperitoneal injection). The blood samples (3-5 mL) were collected from abdominal aorta with an 18G needle into a non-anticoagulated tube and stored at 4°C shortly before sending to Biolab Medical Technic Clinic (Phitsanulok, Thailand) for testosterone measurement.

Determination of nitric oxide synthase (NOS) activity and malondialdehyde (MDA) levels

NOS activity in penile samples was determined using the Calbiochem NOS assay kit (Darmstadt, Germany). The penile tissue was weighed and homogenized in phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 10,000 g for 20 min. The supernatant was ultracentrifuged at 100,000 g for 15 min. The sample was assayed and processed according to the manufacturer's instructions. The values were estimated per tissue weight and per amount of protein in the tissue. Protein was determined using Pierce® BCA Protein Assay Reagent Kit (Thermo Fisher Scientific, Rockford, IL, USA).

For MDA analysis, the supernatant of testicular tissues was centrifuged at 3,000 g 4ºC for 10 min. Supernatant (100 µL) was then added with 1,500 µL of 20% acetic acid (pH 3.5), 200 µL of 8.1% sodium dodecyl sulphate (SDS), and 1,500 µL of 0.8% of thiobarbituric acid (TBA). The mixture was incubated at 95°C for 60 min and then centrifuged at 10,000 g for 5 min. The MDA concentration was measured spectrophotometrically at a wavelength of 532 nm using UV-VIS spectrophotometer. The degree of lipid peroxidation was determined by an MDA standard curve and expressed as MDA equivalent in nmol/g protein. Finally, MDA level was normalized by the protein contents of the testicular tissues using Pierce® BCA Protein Assay Reagent Kit (Thermo Fisher Scientific, Rockford, IL, USA).

Statistical analysis

Recorded values were analyzed using GraphPad Prism (version 7.0, GraphPad Software, San Diego, CA, USA) and expressed as mean±standard deviation (SD) or standard error of the mean (SEM). Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. $P<0.05$ was considered to have a statistically significant difference among groups.

Results

Effects of CCM on mating behavior

ML shows the time interval from the introduction of the female up to the first mount. The mean ML was highest in the aged rats administered with vehicle, indicating a reduction in the sexual motivation in the aged control rats as reflected by a significant increase in ML compared to the young rats (Table 1). Additionally, the present study revealed that CCM could enhance male sexual behavior in aged rats for all doses (0.1, 0.5 and 1.0 g/kg BW) administered. Aged rats received CCM at all doses for 21 days showed a significant decrease in the ML compared to the aged control rats ($p<0.05$). Although administration of CCM decreased ML when compared to vehicle groups, this decline was not in a dose-dependent manner and the values of MF, IL, IF, EL, and EF were not significantly different among groups. The aged rats treated with sildenafil citrate for 21 days showed significantly lower ML but relatively higher MF, IL and EL than the aged vehicle group. The one-way ANOVA showed that treatment on aged rats with CCM or sildenafil citrate did not have any significant effect on MF, IL, IF, EL, and EF ($p>0.05$) when compared to those of the aged vehicle group.

Table 1. Effects of CCM (0.1, 0.5 and 1.0 g/kg BW) or sildenafil citrate (5 mg/kg BW) on mating behavior in aged male rats.

Groups	Mating behavior parameters					
	ML (s)	MF (count)	IL (s)	IF (count)	EL (s)	EF (count)
Young vehicle (n=10)	3.95±0.60*	9.15±1.66	25.10±17.57	34.20±2.94	1143.80±142.93	1.30±0.27
Aged vehicle (n=8)	124.25±82.02	9.00±1.89	76.81±17.71	22.13±4.42	837.38±167.73	1.88±0.30
Aged						
+0.1 CCM (n=10)	36.05±16.93*	8.80±2.39	111.05±37.29	26.60±4.21	1410.80±167.69	0.70±0.33
+0.5 CCM (n=9)	9.39±2.81*	8.67±2.10	43.78±19.68	35.28±4.70	1196.89±176.88	0.78±0.22
+1.0 CCM (n=10)	52.00±23.40*	12.75±2.00	83.10±31.73	33.05±4.99	1269.25±198.82	1.30±0.42
+Sildenafil (n=10)	36.70±7.23*	13.35±2.33	104.75±19.45	28.05± .94	1291.65±158.59	1.00±0.31

s=second(s); ML=mount latency; MF=mount frequency; IL= intromission latency; IF=intromission frequency; EL=ejaculatory latency; EF=ejaculation frequency. Values are expressed as mean±SEM. *Statistical significance ($p < 0.05$) compared to aged vehicle group.

Effects of CCM on erectile responses

To assess the effect of CCM on aged rat penile erectile hemodynamics, in vivo erectile responses to electrical stimulation of cavernous nerve at increasing voltage setting were investigated after completion of mating behavior test. Different voltages (0.25, 0.50, 0.75, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 V) were used to stimulate the cavernous nerve for 1 min with a 5-min pause between each voltage. The ICP and MAP were measured at the same time.

Following electrical stimulation of cavernous nerve, it was found that the ICP and ICP/MAP of the young vehicle group increased with an increase in voltage. The ratio of the maximal ICP/MAP of the rats in aged vehicle group after electro-stimulation was significantly lower than that of the young vehicle group (Figure 1). Aged rats treated with CCM (0.1, 0.5 and 1.0 g/kg BW) or sildenafil citrate (5 mg/kg BW) showed significant increases in the pressure index at all levels of electrical stimulation compared with those of aged vehicle group ($p<0.05$).

Typical ICP tracings (5 V for 60 s) are presented in Figure 2A. At the voltage of 5 V, our results showed that the aged vehicle group had significantly decreased ICP and ICP/MAP ratio compared with the young vehicle rats. In addition, aged rats treated with CCM (0.1, 0.5 and 1.0 g/kg BW) or sildenafil citrate (5 mg/kg BW) showed significant increases in ICP/MAP ratios compared with the young vehicle rats (Figure 2B).

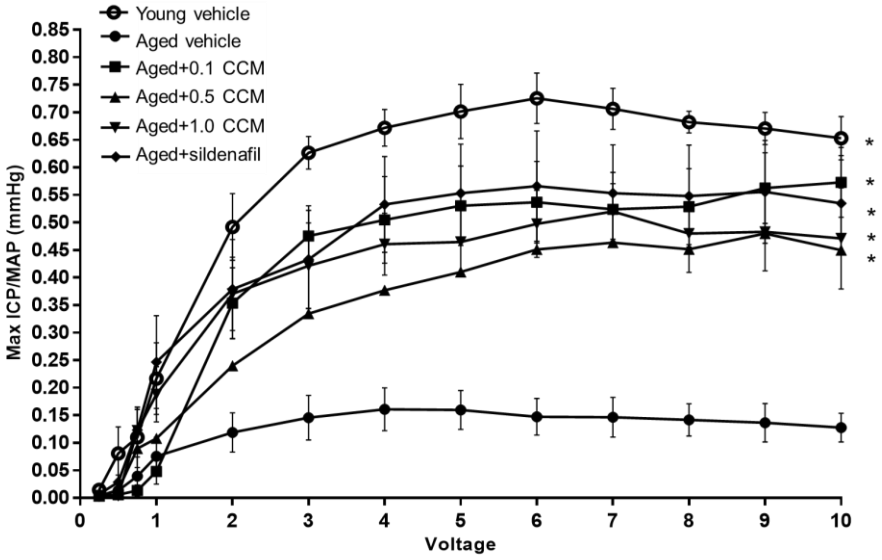

Figure 1. Comparison of ICP/MAP following cavernous nerve stimulation with 0.25-10 V (20 Hz, 60 s) among young vehicle, aged vehicle, aged+CCM (0.1, 0.5 and 1.0 g/kg BW), and aged+sildenafil. Values are expressed as mean±SD. *Statistical significance (p<0.05) compared to aged vehicle rats.

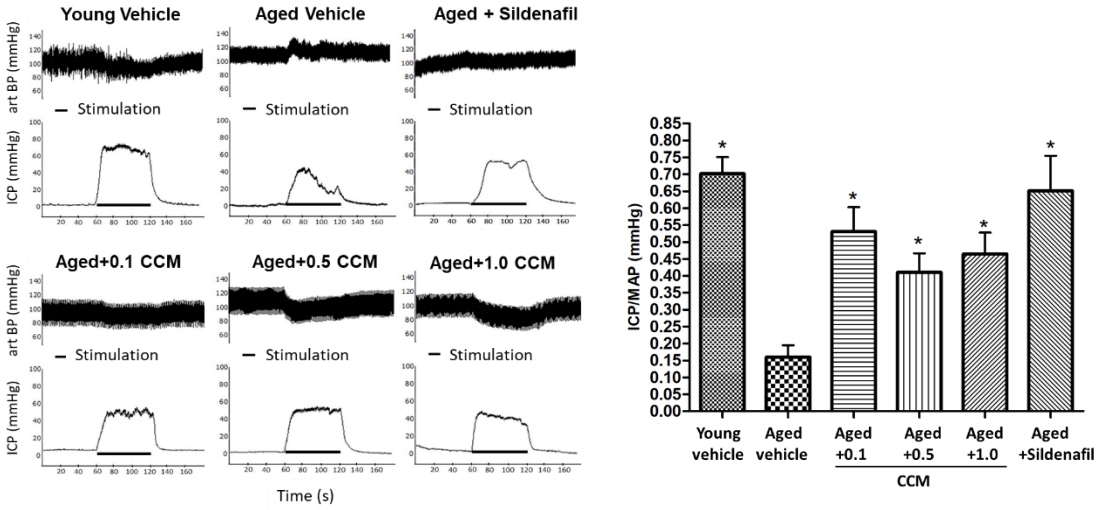

Figure 2. (A) Representative ICP and MAP tracings induced by cavernous nerve stimulation at 5 V (20 Hz, 60 s) of young vehicle, aged vehicle, aged+ CCM (0.1, 0.5 and 1.0 g/kg BW), and aged+sildenafil groups. Black bar represents duration of electrical stimulation. (B) The ICP/MAP during electrical stimulation of the cavernous nerve at 5 V (20 Hz, 60 s) among all groups (n=7). *Statistical significance (p<0.05) compared to aged vehicle rats.

Effects of CCM on serum testosterone concentration

Serum testosterone levels showed significant changes with increasing age. The total testosterone level tended to decrease with aging. The testosterone level in aged vehicle rats was significantly lower than that in young vehicle rats (1.18±0.79 *vs.* 2.45±0.21 ng/mL) (Figure 3). Aged rats treated with CCM at all doses or sildenafil citrate showed higher testosterone levels than aged vehicle rats, but only aged rats treated with CCM at a dose of 1.0 g/kg BW (2.53±1.78 ng/mL) showed a statistically significant difference when compared to the aged vehicle group ($p<0.05$). Although administration of CCM enhanced testosterone levels when compared to the aged vehicle groups, these rises were not in a dose-dependent manner. In aged rats treated with sildenafil citrate 5 mg/kg BW, the serum testosterone level was also increased (2.12±1.37 ng/mL) but not reaching statistical significance ($p<0.05$).

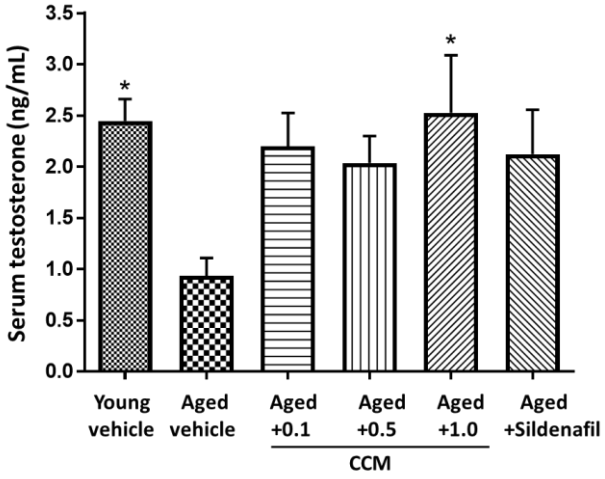

Figure 3. Effect of CCM on serum testosterone level in aged rats treated with vehicle, CCM (0.1, 0.5 and 1.0 g/kg/day) or sildenafil citrate (5 mg/kg/day) for 21 days. Data are presented as mean±SD (n=8-10). *Statistical significance ($p<0.05$) compared to aged vehicle group.

Effects of CCM on NOS activity in the penile tissue

To evaluate the effect of CCM on NO production, NOS activity in penile tissue was measured. As shown in Figure 4, the enzyme activity of penile NOS was reduced in the aged vehicle group (5.95±0.53 μmol/L) compared to the young vehicle group (6.95±2.06 μmol/L). Administration of CCM (0.1, 0.5 and 1.0 g/kg BW) increased the activity of NOS in a dose-dependent manner (6.37±1.19, 7.90±2.93, and 11.39± 2.39 μmol/L, respectively) and the NOS activity in the penile tissue of aged rats treated with CCM 1.0 g/kg was significantly higher ($p<0.05$) compared with those observed in the aged vehicle group. There were no statistically significant differences between the other treatment groups. In aged rats treated with 5 mg/kg BW sildenafil citrate, the NOS activity was 5.79±0.95 μmol/L which was not significantly different from the aged vehicle group.

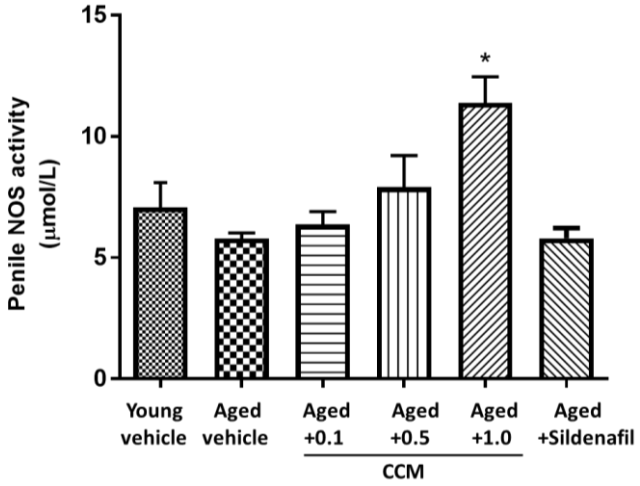

Figure 4. NOS activity in penile tissues in young and aged rats treated with vehicle, CCM (0.1, 0.5 and 1.0 g/kg/day) or sildenafil citrate (5 mg/kg/day) for 21 days. Data are presented as mean±SEM (n=5). *Statistical significance (p<0.05) compared to aged vehicle group.

Effects of CCM on MDA level of testicular tissues

In this study, the end product of lipid peroxidation, MDA level was determined in rat testicular tissues as an indicator of the oxidative stress. It was found that testicular MDA levels in aged vehicle rats were higher than those of the young vehicle rats (3.10±0.94 *vs.* 2.35±1.26 nmol/g protein) (Figure 5). Treatment of CCM at the dose of 0.5 or 1.0 g/kg/day for 21 days induced significant decreases in testicular MDA levels (1.46±0.62 and 1.81±0.60 nmol/g protein, respectively), whereas a dose of 0.1 g/kg/day caused no significant effect when compared to the aged vehicle group (2.32±1.09 *vs.* 3.10±0.94 nmol/g protein).

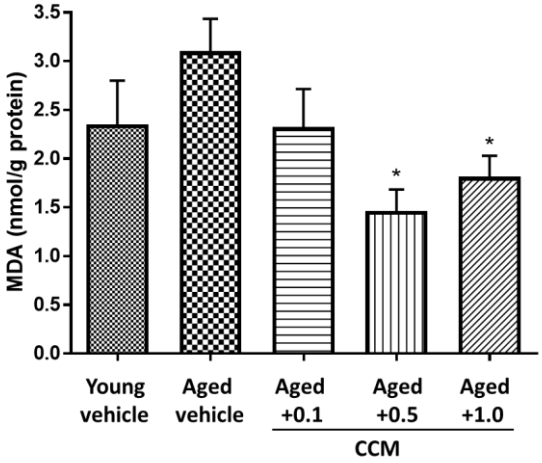

Figure 5. The MDA level in the testicular tissues of young and aged rats treated with vehicle or CCM at the doses of 0.1, 0.5 and 1.0 g/kg BW. Data are presented as mean±SEM (n=8). *Statistical significance (p<0.05) compared to aged vehicle group.

Discussion

ED is a natural consequence of aging that the incidence increased with age. Available information shows that ED is common with a worldwide prevalence of over 150 million men in 1995 and a prevalence of approximately 322 million men are estimated to be affected by ED by 2025.[17] The treatment option for men with ED are mainly FDA-approved oral PDE5 inhibitors which work by inhibiting PDE5 activity, resulting in dilating the corpora cavernosa of the penis and facilitating erection with sexual stimulation. However, some unwanted side effects of these medications have been reported.[3]

In recent years, there has been an increasing interest in medicinal plants and fungi that are recognized as aphrodisiacs. Cordyceps species, including *C. militaris*, are potential sources of bio-metabolites for herbal drugs and research evidences are available about its powerful health benefits including aphrodisiac property.[18] A number of literature and pharmacological studies on effects of *C. militaris* suggest that its biological active component, cordycepin, can be used to treat a wide array of disease conditions due to its pharmacological effects such as antitumor, antioxidant, antidiabetic, anti-inflammatory and antimicrobial activities.[19-23] In the previous study, biochemical analyses of cordycepin and adenosine contents of *C. militaris* cultured in laboratory were 4.80 and 1.26 mg/g respectively.[15] The amount of cordycepin is relatively similar to that cultured in silk worm pupae medium (4.17±1.66 mg/g) and higher than that obtained in brown rice medium (2.98±1.41 mg/g) as reported by Kang et al.[24] This CCM was tested for the ability to improve erectile response in aged rats. It is well established that sexual behavior declines with age in male rats. In this study, an increase in latency to mount as observed in aged vehicle rats indicating lower sexual motivation in aged rats. This result is consistent with a previous study reporting increased latency to mount and intromission in old male rats.[25] Treatment of CCM at a dose of 1.0 g/kg BW for 21 days significantly reduced ML and increased MF, indicating an enhancement of sexual desire in aged male rats. Treatment with sildenafil citrate in aged rats showed similar sexual behavioral results. Our result agrees with the previous study that aged rats showed poor sexual motivation and treatment of sildenafil citrate in aged rats could induce improvement in sexual performance.[26]

To study the age-associated ED, erectile function was assessed in the present study by measuring the rise in ICP following cavernous nerve electrostimulation. Aged vehicle rats exhibited significantly decreased ICP/MAP ratio at all tested voltages compared with young vehicle rats, which confirmed decreased erectile function with age. These observations were supported by a recent study of Li et al.[27] Aged rats treated with either CCM or sildenafil citrate improved erectile function as evidenced by the significant increases in ICP/MAP values. This indicates that CCM could potentiate the erectile response. Additionally, aged rats that received sildenafil citrate showed the highest magnitude of ICP among the treated groups. This is in agreement with the study of Gurbuz et al.[28] that reported the increases in both ICP and area under the curve of a rat model of ED treated with sildenafil citrate. Kovac et al.[29] also reported that a single administration of sildenafil citrate at a dose of 5 mg/kg improved penile pressure in response to cavernous nerve electrostimulation

in aged rats (13-15 months old) that reached similar levels as observed in young control rats (4-5 months old).

Male sexual behavior is dependent on testosterone. Depletion of testosterone has been shown to have a deleterious effect on erectile function. Cordycepin from *C. militaris* has been reported to effectively restore the decline of testicular function with age.[14] Recently, the study of Kopalli et al.[30] demonstrated that cordycepin treatment could ameliorate aging-induced testicular dysfunction at the gene expression level, particularly those related to spermatogenesis, antioxidant defense, acetylation, and autophagy-related activity.

Aged rats used in this study treated with CCM or sildenafil citrate showed markedly elevated serum concentration of testosterone and significant effect was observed in the group receiving 1.0 g/kg of CCM. Our present result is in agreement with the previous study that *C. militaris* fruiting body fed to the SD rats for 4 weeks significantly increased serum testosterone concentration. Additionally, Hong et al.[31] documented the stimulatory effect of *C. militaris* on testosterone production in male rats. The *C. militaris* supplementation has also been reported to result in an increase in serum cordycepin concentration, which simultaneously enhanced the testosterone and estradiol-17 as well as increased the percentage of sperm.[13] Tuli et al.[32] also explained the mechanism of *C. militaris*-induced steroidogenesis through the effect of cordycepin (3′-deoxyadenosine), an adenosine analog that increased the plasma testosterone concentration and is associated with adenosine receptors to activate the cAMP-PKA-StAR signaling pathway and steroidogenesis in mouse Leydig cells.

NO activates soluble guanylate cyclase in cavernosal smooth muscle to generate cGMP which, in turn, promotes vasorelaxation. Normal erection is dependent on sufficient vasorelaxation induced by stimulation of the nervous system to overcome vasoconstriction of corporal smooth muscle. Aging alters erectile functioning as well as endothelial cell functioning, resulting in a reduction of NO level and subsequent impairment in penile smooth muscle relaxation. Decreased level of NOS has been reported in aged rats.[33] In this study, penile tissue was evaluated for NOS activity. The obtained data demonstrated that NOS activity in penile tissue of aged rats was lower than that in young vehicle rats. Administration of 1.0 g/kg CCM significantly increased NOS activity in these aged rats. Our data also demonstrated that in the testes, the level of MDA, the most commonly used marker for lipid peroxidation, markedly increased with respect to age of rats. This confirmed the role of oxidative stress in the pathological changes seen within the reproductive organ during aging.[29] Interestingly, CCM could significantly decrease the elevated MDA level in aged rats.

Taken together, this study indicates that CCM has an aphrodisiac activity. The improvement of mating behavior and erectile function by CCM could be, in part, due to the effect of its major bioactive, cordycepin, through activation of steroidogenesis, causing increased serum testosterone concentration. Testosterone then could mediate the erectile response in the rat penis by stimulating NOS.[34,35] Besides cordycepin, antioxidant properties of CCM may also account for the reduction in oxidative stress, resulting in less cellular damage, especially in reproductive organs, and restoring the normal mechanisms of cellular signaling. Therefore, it is possible that improvement of ED observed in this study may result from increased serum testosterone level induced by adenosine and cordycepin as well as antioxidant activities of CCM.

Conclusion

Model for human ED related to aging has been characterized in the rats. In this study, the aged rats exhibited poor sexual motivation and showed a reduction in erectile response to cavernous nerve electrostimulation. Aphrodisiac effect of CCM against ED in aged rats was observed based on behavioral, physiological and biochemical evidences. Collectively, CCM had capacity to increase sexual desire, erectile response, serum testosterone concentration and penile NOS activity as well as decrease testicular MDA level. Thus, this medicinal fungus might have aphrodisiac potential for promoting sexual activity, especially in aged men with ED.

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