In vivo and in vitro stimulatory effects of *Cordyceps sinensis* on testosterone production in mouse Leydig cells

Chih-Chao Hsu\(^a\), Yuan-Li Huang\(^a\), Shaw-Jeng Tsai\(^b\), Chia-Chin Sheu\(^c\), Bu-Miin Huang\(^{a, *}\)

\(^a\)Department of Cell Biology and Anatomy, College of Medicine, National Cheng Kung University, #1 University Road, Tainan 701, Taiwan  
\(^b\)Department of Physiology, College of Medicine, National Cheng Kung University, Tainan, Taiwan  
\(^c\)Simpson Biotech Co, Taipei, Taiwan, ROC

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**Abstract**

The in vivo and in vitro effects of *Cordyceps sinensis* (CS) and its extracted fractions on the secretion of testosterone in mice were studied. CS, F2 (water soluble protein), and F3 (poorly water soluble polysaccharide and protein) significantly stimulated in vitro testosterone production in purified mouse Leydig cells. However, F1 (water soluble polysaccharide) had no effect \((p>0.05)\). F2 and F3 stimulated in vitro testosterone production in dose- and time-dependent relationships with maximal responses at 3 mg/ml for 3 h \((p<0.05)\). An in vivo study illustrated that testosterone levels in plasma were significantly increased by CS, F2, and F3, respectively \((p<0.05)\). Because CS, F2, and F3 stimulated both in vitro and in vivo testosterone secretions in mice, it is possible that CS might contribute to an alternative medicine for the treatment of some reproductive problems caused by insufficient testosterone levels in human males.

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**Keywords:** *Cordyceps sinensis*; Mouse Leydig cells; Steroidogenesis; Testosterone

**Introduction**

For decades, modern physicians have treated men with insufficient testosterone secretion with modern medicines and techniques, such as the injection of gonadotropin hormones and testosterone to restore the
reproductive function (Zitzmann and Nieschlag, 2000; Huff et al., 2001; Bouloux et al., 2002). On the other hand, traditional healers have been using a wide variety of complementary (alternative) medicines to improve sexual performance and fertility for millennia. Alternative approaches, such as the intake of plants, fungi, and insects, or their extracts, have been practiced to enhance sexuality and ameliorate illness all around the world with notable successes (Rege et al., 1997; Veal, 1998; Crimmel et al., 2001). However, the scientific evidence documenting the mechanisms and efficacy of these alternative medicines is both scarce and all too often unconvincing.

It is well documented that testosterone plays an essential role in controlling male sexual activities and reproductive functions. Testosterone secretion from Leydig cells is finely regulated by luteinizing hormone (LH), which is released from the anterior lobe of the pituitary gland (Saez, 1994). The secretion of LH is further controlled by gonadotropin-releasing hormone (GnRH) from the hypothalamus. LH binds to its receptor on Leydig cells to activate the cyclic AMP-protein kinase A (PKA) signal transduction pathway (Moger, 1991) and thus induce de novo synthesis of proteins for the production of testosterone (Stocco and Clark, 1996). However, this hypothalamus-pituitary-gonad axis in the human male is profoundly influenced by physical factors—aging and disease, for example—as well as by social and psychological factors. The negative effects of these factors often lead to infertility due to low or no testosterone secretion (Sinclair, 2000; Roscoe et al., 2001).

*Cordyceps sinensis* (CS), a fungal parasite on the larvae of *Lepidoptera*, has been used as a tonic herb in Chinese traditional medicine for centuries (Zhu et al., 1998) because it is believed that CS possesses many important pharmacological activities related to reproduction. It has been reported that CS can enhance reproductive activity and restore impaired reproductive function in both sexes (Zhu et al., 1998). In previous studies we demonstrated that CS stimulated in vitro steroidogenesis in both mouse normal and tumor Leydig cells (Huang et al., 2000, 2001a,b). What remains elusive, however, is which component of CS does the job and whether CS or its components have any in vivo effect on Leydig cell steroidogenesis. Thus, a search for the components in CS with in vivo and in vitro stimulatory effects on mouse Leydig cells is the goal of the present study. Purified mouse Leydig cells were treated with CS and its extracted fractions—F1 (water soluble polysaccharide), F2 (water soluble protein) and F3 (poorly water soluble polysaccharide and protein)—to determine their in vitro effects on steroidogenesis. More importantly, the fractions of CS with positive effects were further used to feed the mice to determine their in vivo effect on the levels of testosterone in plasma.

**Materials and methods**

*Chemicals*

Culture mycelium of *Cordyceps sinensis* was supplied by the Simpson Biotech Co. Ltd. (Taipei, Taiwan). M199 medium, fetal bovine serum, Dulbecco's PBS, and gentamicin sulfate were purchased from Gibco (Grand Island, NY). Tissue-culture grade sodium bicarbonate, bovine serum albumin, HEPES, penicillin, Percoll gradient solution, streptomycin, nicotinamide, b-nicotinamide adenosine dinucleotide, dehydroepiandrosterone, nitroblue tetrazolium, testosterone, ether, and charcoal were purchased from Sigma Chemical Co. (St. Louis, MO). ³H-testosterone used for radioimmunoassay was purchased from DuPont-New England Nuclear (Boston, MA). Antiserum to
testosterone was a kind gift from Dr. Paulus S. Wang (National Yang-Ming University, Taipei, Taiwan).

**Preparation of CS fractions**

One hundred grams of crude CS was extracted with 800 ml distilled water and shaken at 37 °C for 72 hr. The solution was then centrifuged at 12,000 × g at 4 °C for 30 min to collect the pellet named fraction 3 (F3). The supernatant was applied in the G150 gel filtration column (3 × 100 cm) with 50 mM CH₃COONH₄ buffer at pH 6.0. Two peaks were collected; the first peak was designated F1 and the second peak, F2. The yield percentage of F1, F2, and F3 were 1.69%, 13.46%, and 84.85%, respectively. The main content of those CS fractions were F1 with water-soluble low molecular weight polysaccharides, F2 with water-soluble low molecular weight proteins, and F3 with relatively poor water-soluble polysaccharides and proteins.

**Animals**

Male B6 (C57BL/6NCrj) mice, 4–5 weeks old, were purchased from National Cheng Kong University Animal Center (Tainan, Taiwan). B6 mice should be less than 8 weeks old for the experiments to exclude the exposure of Leydig cells to luteinizing hormones. All animals were housed in groups of 4 to 6 in 29 × 18 × 13-cm polyethylene cages. The animal room was maintained at 22–24 °C under a constant 12L:12D cycle. Purina mouse chow (Ralston-Purina, St. Louis, MO) and water were always available. For in vivo study, 15 animals were randomly divided into 3 groups of 5 with infusions of water and 0.02 or 0.2 mg/g (body weight) of CS, F2, or F3, respectively, for 7 days. The age at the beginning of experiment was 5 weeks old of immature mice. Animals were sacrificed by cervical dislocation and trunk blood was collected. The blood samples were kept on ice and then centrifuged for 1 min at 12,000 × g to separate plasma. Plasma was collected and stored at −20 °C for later determination of testosterone.

**Primary leydig cell isolation**

The testes were removed from the sacrificed mice and decapsulated in medium 199 containing 4 mM NaHCO₃, 25 mM HEPES, 0.06 g penicillin, 0.05 g streptomycin, and 0.2% BSA, pH 7.35. After decapsulation, the testes were then incubated in a shaking water-bath (120 cycles/min) at 37 °C in medium 199 containing 1% BSA and 100 units/ml collagenase (Type II; Worthington Biochemical Corp., Lakewood, NJ) for 10 min. After incubation, cold medium 199 was added to stop the action of the collagenase. Seminiferous tubules were separated from interstitial cells by gravity sedimentation. Cells were then collected by centrifugation (300 × g for 6 min) and resuspended in 2 ml of medium 199 containing 0.1% BSA. This suspension, which did not contain seminiferous tubules and was composed of interstitial cells, contained 20–30% Leydig cells. The interstitial cell preparation described above was layered onto a Percoll gradient and then centrifuged at 800 × g at 4 °C for 20 min. The gradient, which was preformed by centrifugation at 25000 x g for 30 min, contained 10 ml of isotonic Percoll solution and 15 ml medium 199 plus 0.1% BSA and 25 mM HEPES (Huang et al., 2001b). A 1-ml fraction of gradient was collected from the top. Mouse Leydig cells were mainly distributed from fraction 23 to 25. The total number of cells and the percentage of 3α-hydroxysteroid
dehydrogenase-positive cells were determined in this Leydig cell preparation (Huang et al., 2001b). The purity of the Leydig cells was 80–85%.

**Cell culture**

Cells were maintained at 37 °C in a humidified environment containing 95% air and 5% CO₂ for all of the following experiments. Approximately 5 × 10⁴ cells/100 μl of medium 199 were plated into each well of 96-well plates. After 2 h, cells were washed twice with medium without any serum and then treated with various doses of CS or CS fractions for different amounts of time. At the end of the incubation, the media were withdrawn and testosterone levels were determined by radioimmunoassay (Huang et al., 2001b).

**Radioimmunoassay**

Media from cultures with different treatments were collected and diluted with medium to fall within the standard curves for the respective assays. Twenty-five μl of diluted sample was withdrawn to a glass tube and 100 μl each of testosterone antiserum and ³H-testosterone were added. An equilibrium reaction occurred at room temperature for 2 h and was stopped by putting the tubes in ice. Charcoal was added and incubated for 15 min at 4 °C and then centrifuged for 10 min to spin down the charcoal bound with free ³H-testosterone. The supernatant was poured into 3 ml of scintillation fluid and samples were counted in a beta-counter for 2 min.

Testosterone levels in plasma were determined by established radioimmunoassay after 10-μl plasma samples were diluted to 250 μl and extracted with ether (Huang et al., 2001c). Recovery after ether extraction averaged 85%. The intra- and inter-assay coefficients of variation for the testosterone assay were 4.1% and 4.7% with a sensitivity of 5 pg per assay tube. Concentrations of testosterone were expressed as ng/ml plasma.

![Fig. 1. The effects of CS and CS fractions (F1, F2, and F3) on testosterone production in purified normal mouse Leydig cells. The cells were treated with CS and CS fractions (3 or 10 mg/ml) for 3 h incubation.](image)
Statistics

Each data point in the figures represents the mean ± SEM of testosterone production of three separate experiments with triplicates in each in vitro treatment or plasma testosterone of 5 mice in in vivo study. Statistically significant differences between treatments and controls were determined by one-way ANOVA and the Fisher-PLSD multiple comparison procedure. Statistical significance was set at $p < 0.05$.

Results

The in vitro effects of CS and CS fractions on testosterone production in normal mouse Leydig cells

To investigate the effects of CS and CS fractions on testosterone production in mouse, the purified Leydig cells were treated with CS, F1, F2, and F3 at dosages of 3 mg/ml for 3 h. Testosterone production

![Graph of testosterone production](image1)

**Fig. 2.** Dose effects of F2 on testosterone production in primary mouse Leydig cells. The cells were stimulated for 3 h with increasing concentrations of F3 (0.1–10 mg/ml).

![Graph of testosterone production](image2)

**Fig. 3.** Time effect of F2 on testosterone production in primary mouse Leydig cells. The cells were treated with 3 mg/ml CS, and the media were collected after 0, 1, 2, 3, and 6 h.

![Graph of testosterone production](image3)
was significantly increased by CS, F2, and F3 (1398 ± 181, 1165 ± 179, and 1440 ± 190 pg/ml, respectively) compared to the control (273 ± 24 pg/ml) (p < 0.05) (Fig. 1). In contrast, treatment with F1 at 3 mg/ml failed to induce testosterone production (332 ± 30 pg/ml) (p > 0.05).

**In vitro dose and time course effect of F2 on testosterone production in normal mouse Leydig cells**

Based on the above results, F2 and F3 were further investigated to determine the dose and time course effects on steroidogenesis in normal mouse Leydig cells. F2 stimulated testosterone production in a dose-dependent manner (p < 0.05) that leveled off at dosages from 3 to 10 mg/ml (Fig. 2).

Fig. 3 demonstrates the temporal effect of F2 on purified mouse Leydig cell steroidogenesis. F2 at 3 mg/ml significantly increased testosterone production at 1 h (335 ± 25 pg/ml versus control 121 ± 3.2

![Fig. 4. Dose effects of F2 on testosterone production in primary mouse Leydig cells. The cells were stimulated for 3 h with increasing concentrations of F3 (0.1–10 mg/ml).](image1)

![Fig. 5. Time effect of F2 on testosterone production in primary mouse Leydig cells. The cells were treated with 3 mg/ml CS, and the media were collected after 0, 1, 2, 3, and 6 h.](image2)
pg/ml; p < 0.05) and reached the maximal response between 2 to 6 h (515 ± 68 to 614 ± 20 pg/ml versus control 152 ± 6.6 to 227 ± 9.1 pg/ml; p < 0.05).

In vitro dose and time course effect of F3 on testosterone production in normal mouse Leydig cells

Purified mouse Leydig cells treated with various concentrations of F3 (0.1-10 mg/ml) for 3 h showed a dose-dependent relationship for testosterone production (p < 0.05) with the greatest amounts found at 1 and 3 mg/ml (509 ± 72 and 523 ± 86 pg/ml, respectively) (Fig. 4). At 6 and 10 mg/ml dosages, however, testosterone production was not stimulated (158 ± 39 and 51 ± 13 pg/ml, respectively, versus basal level 129 ± 10 pg/ml; p > 0.05).

Fig. 6. Mice were randomly divided into three groups of 5. The administration of water plus 0.02 or 0.2 mg/g-body weight of (a) CS, (b) F2, or (c) F3 lasted for 7 days. Trunk blood was collected and plasma testosterone was determined.
Fig. 5 demonstrates the temporal effect of F3 on purified mouse Leydig cell steroidogenesis. F3 at 3 mg/ml significantly increased testosterone production at 1 h (657 ± 70 pg/ml versus control 223 ± 18 pg/ml) (p < 0.05) and reached the maximal response between 2 to 6 h (914 ± 127 to 1076 ± 121 pg/ml versus control 263 ± 23 to 278 ± 19 pg/ml) (p < 0.05).

In vivo effect of CS, F2 and F3 on plasma testosterone level in mice

In the present experiment, immature mice with none or very low testosterone level in blood was used to simulate dysfunctional animal in reproduction, which was able to determine if CS, F2 and F3 could stimulate testicular functions. Fig. 6 illustrated the in vivo effects of CS, F2, and F3 on plasma testosterone levels in mice. Based upon their in vitro positive effects on Leydig cell steroidogenesis, CS, F2, or F3 was fed to the mice to examine changes in plasma testosterone levels. Mice were fed with water and 0.02 or 0.2 mg/g-body weight of CS, F2, or F3, respectively, for 7 days. At 0.02 and 0.2 mg/g, CS significantly elevated plasma testosterone levels (3.83 ± 0.756 and 3.69 ± 0.885 ng/ml, respectively) compared to untreated control mice (1.38 ± 0.047 ng/ml; p < 0.05) (Fig. 6A). However, only mice fed with higher concentrations (0.2 mg/g-body-weight) of F2 or F3 (Fig. 6B and 6C, respectively) had greater plasma testosterone levels than controls (5.34 ± 2.03 vs. 0.95 ± 0.11 and 2.66 ± 1.33 vs. 0.59 ± 0.03 for F2 and F3, respectively).

Discussion

In the present study, we found that CS, F2, and F3 stimulated in vivo and in vitro testosterone secretion in mice. Indeed, there were differences in the stimulatory effects on mouse Leydig cells by different CS fractions. F1 had no effect on Leydig cell steroidogenesis, but F2 and F3 both stimulated steroidogenesis in normal mouse Leydig cells. F2 induced testosterone production in a dose- and time-dependent relationship and showed a maximal response to 3 mg/ml at 3 h. F3 also induced testosterone production in a dose- and time-dependent relationship, but as the concentration of F3 increased to 6 and 10 mg/ml, testosterone levels returned to basal level. This is similar to the effect of CS (Huang et al., 2001b). It is possible that CS and F3 contain stimulatory as well as inhibitory substances and that, at high concentrations, the latter neutralize the former, whereas F2 lacks inhibitory components.

Polysaccharides extracted from herbs have pharmacological properties (Ukai et al., 1983), and the polysaccharide components of CS also have these properties (Yoshida et al., 1989). Polysaccharides, found in interstitial tissues as structural molecules (cell wall, chitin, receptor, etc.) with the functions of recognizing or connecting molecules for cell interaction and communication, play important roles in living organisms (Ruoslahti, 1989). It is well known that the carbohydrate moiety of the glycoprotein LH/hCG plays an important role in recognizing LH receptor to activate a signal pathway for steroidogenesis (Pigny et al., 1992). Similar to the function of LH/hCG on Leydig cells (Huang et al., 1995), F2 and F3 had their maximal stimulatory effect at 3 h in the current study, which strongly suggests that F2 and F3 have acute regulatory properties similar to those of LH/hCG on Leydig cell steroid production. Thus, it is possible that the polysaccharides and/or glycoproteins in CS, F2, and F3 may be similar to LH in structure, have the ability to recognize LH receptors on Leydig cells, and stimulate steroid production. However, this remains to be elucidated.
Consistent with the in vitro results, the present study illustrated that CS, F2, and F3 could induce in vivo testosterone secretion in plasma. CS at both dosages (0.02 and 0.2 mg/g) did have similar stimulatory effects, whereas only the higher dose (0.2 mg/g) of F2 and F3 had stimulatory effects on plasma testosterone secretion. Indeed, F2 at 0.2 mg/g seemed to have a stronger stimulatory effect than CS and F3, which indicates that the factors, which activate Leydig cell steroidogenesis, may be more abundant in F2. Theoretically, F2 at 0.02 mg/g should also have a significant stimulatory effect on testosterone production, but it had no effect. We cannot explain this; elucidation will require further in vivo investigations on the finer substances in CS.

LH/hCG stimulates in vivo testosterone secretion (Padron et al., 1980). In the present study, we demonstrated that ingestion of CS, F2, and F3 also stimulated in vivo testosterone plasma levels. These results indicate that CS, F2, and F3 still have the effect on Leydig cells even after passing through the digestive system. Thus, present studies strongly imply that CS, F2, and F3 contain substances that, like LH/hCG, can stimulate Leydig cell steroidogenesis even during the metabolic processing they undergo in the gastrointestinal tract. Therefore, the finer substances and/or the factors in CS after digestion are worth investigating.

In conclusion, we have found that CS, F2 and F3 stimulated mouse Leydig cell testosterone production in dose-dependent and temporal relationships. Moreover, CS, F2, and F3 could also significantly induce in vivo testosterone secretions. The combined results of in vitro and in vivo studies may contribute an alternative treatment for male reproductive dysfunction in humans.

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