

Improvement of Sperm Production in Subfertile Boars by *Cordyceps militaris* Supplement

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Abstract: *Cordyceps* species have been traditionally used for the enhancement of sexual function, however, there is few direct evidence to prove this. We investigated the spermatogenic effect of *Cordyceps militaris* (CM) by supplementation with CM mycelium to subfertile boars. Seventeen Duroc and 12 Landrace boars (29 to 40 months old) were selected to feed with regular diet (control groups, n = 8 and 6, respectively) or diet supplemented with CM mycelium (treatment groups, n = 9 and 6, respectively) for 2 months. Semen was collected once a week. The quality of fertile sperm (normally greater than 62% of motility and 70% of normal morphology) and the quantity (semen volume, and total sperm number) were compared in these boars. The result showed that sperm production was enhanced significantly at the end of first month ($p < 0.05$), peaked at the second month ($p < 0.01$) of supplementation with CM and was maintained for 2 weeks after stopping the treatment ($p < 0.01$). Plasma cordycepin concentration was detected in boars supplemented with CM but not in the controls. More

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importantly, the percentages of motile sperm cells and sperm morphology were also improved significantly in most of treated boars during the second month of supplementation ($p < 0.01$) and 2 weeks after the treatment ($p < 0.05$) as compared to their initial values. These results indicate that supplementation with CM mycelium improves sperm quality and quantity in subfertile boars and may partly support the role of Cordyceps in sexual enhancement.

Keywords: Boar; Cordycepin; *Cordyceps militaris*; Sperm Production.

Introduction

Cordyceps species, including *Cordyceps sinensis* (CS), *C. militaris* (CM), and others, are valuable traditional medicinal materials from Ascomycetes fungus parasitic to *Lepidoptera* larvae. CS has been traditionally used as nutritious food for the enhancement of sexual performance and the restitution of impaired sexual function in Chinese society (Ng and Wang, 2005). The chemical constituents include cordycepin (3'-deoxyadenosine) and its derivatives, ergosterol, polysaccharides, a glycoprotein and peptides containing alpha-aminoisobutyric acid. They have anti-tumor, anti-metastasis, immunomodulatory, antioxidant, and anti-inflammatory effects (Aman *et al.*, 2000; Zhang *et al.*, 2005; Yang *et al.*, 2006). CS mycelium is believed to enhance libido and fertility in both sexes, however, its effect and mechanism has been reported only recently. CS can induce the steroidogenic enzyme 17 β -estradiol (E2) expression in human granulosa-lutein cells (GLC) and testosterone in primary mouse Leydig cells and MA-10 mouse Leydig tumor cells (Hsu *et al.*, 2003a; Huang *et al.*, 2004a). CS significantly induces plasma testosterone levels in mice (Hsu *et al.*, 2003b; Huang *et al.*, 2004b). CM contains higher concentration of cordycepin than CS (Yu *et al.*, 2006). Cordycepin from CM has been reported to have acute anti-inflammatory, anti-nociceptive, anti-angiogenesis and immunoregulatory activities (Won and Park, 2005; Kim *et al.*, 2006).

Nutrition is important for the reproductive performance and some studies reported that supplementation with selenium and vitamin E improved sperm quality in boars (Marin-Guzman *et al.*, 1997; 2000) and vitamin E increased the concentration of spermatozoa in semen (Brezczynska-Slebodzinska *et al.*, 1995). However, a recent study showed that supplement of vitamin C or vitamin E for 5 weeks failed to affect the sperm production in young (6–10 months) boars (Audet *et al.*, 2004). Similarly, the effect of vitamin C on the reproduction of boars is also inconsistent (Lin *et al.*, 1986). Reproductive performance of sperm relies on the quality of sperms that have a limit value of the motility greater than 62% and morphological abnormalities less than 30% (Flowers, 1998). However, the effect of nutrients on reproductive performance in adult (29–40 months old) boars has not been reported and particularly in the improvement of sperm quality. Since CS has been traditionally used as nutrition for the enhancement on sexual function in Chinese society (Ng and Wang, 2005), therefore, the aim of this study was to evaluate the effect of CM mycelium supplementation in adult boars with poor sperm quality.

Materials and Methods

Materials

Cordyceps militaris (CM) mycelium was obtained from COMSUN Company (Taoyuan, Taiwan). Vitamin and mineral premix was obtained from Premix Inve Export (Baasrode, Belgium) and semen extender (Well Sperm) from Animal Technology Institute, Chunan, Taiwan.

Animals and Treatments

The experiment had followed the standards for the Taichung Veterans General Hospital Animal Study Protocol. The experimental boars were bred by Huei Huang swine farm (Maoli, Taiwan). Twenty-nine boars (17 Duroc and 12 Landrace), grouped as the control and the supplement, with mean age \pm SE: 38 ± 10 vs. 40 ± 15 months for Duroc and 32 ± 4 vs. 29 ± 2 months for Landrace, were housed individually in pens on semi-slatted floors. The subfertile animals with poor normal sperm morphology (less than 70%) were assigned to the experiment group with CM mycelium supplement for 2 months. Average BW of Duroc boars was (mean \pm SE) 215 ± 14 vs. 229 ± 28 kg and Landrace boars, 278 ± 21 vs. 267 ± 12 kg at the beginning of the experiment, respectively for the control and supplement groups. Basal diet (Table 1) contained a premix providing vitamin and mineral concentrations corresponding to the industry average. The dietary composition of *Cordyceps militaris* mycelium contains cordycepin and other nutrients (Table 2). The daily food allowance for the whole experimental period was 2 kg per boar and the supplement of CM mycelium powder was given at a rate of 10 g per boar.

Table 1. Composition of Diet

Item ^a	%
Corn	68
Soybean meal	22.5
Oat hull	3
Fish meal	5
Vitamin and mineral premix	2
Bile salt	0.05
NaCl	0.25
Ca	0.7
P	1.4

^aCrude protein 15%, ME 3,180 kcal/kg, lysine 2.69%, methionine 0.61%, threonine 2.03%. Premix: Vitamin A, 2.7×10^5 I.U.; vitamin D₃, 3.4×10^4 ; vitamin E, 580 mg; vitamin K₃, 33 mg; vitamin Bs: B₁, 25 mg; B₂, 130 mg; B₃, 470 mg; B₆, 30 mg; B₁₂, 1000 mcg; niacine, 580 mg; choline, 8500 mg; Co, 8.5 mg; Cu, 2100 mg; Fe, 1800 mg, Mn 1330 mg, Se 2.5 mg; Zn 1800 mg.

Table 2. Composition of *Cordyceps militaris* Mycelium

Composition	Unit/100 g
Cordycepin	1,892 mg
Cordycepic acid	2,162 mg
Polysaccharide	4,200 mg
Superoxid dismutase	31.2 U
Ca	251 mg
Mg	162 mg
Zn	23 mg
Se	0.23 mg
Essential amino acids (without Trp)	0.1–0.9 g
Non-essential amino acids	0.1–1.1 g
Fatty acids	
Palmitoleic acid	1.8 mg
Gadoleic acid	3.7 mg
Vitamins	
A	0.08 mg
B ₁	0.12 mg
B ₂	0.49 mg
B ₁₂	0.21 mg
C	16.2 mg
β-Carotene	0.43 mg

Plasma Concentration of Cordycepin

Blood samples were taken from the jugular vein by venipuncture during the experiment to measure the plasma concentration of cordycepin in the supplement and the control boars. The plasma cordycepin was detected using the method of Hsu *et al.* (2002). After filtering through a 0.2 µm filter, samples (10 µl) were subjected to total cordycepin analysis via L-6000 HPLC (Hitachi, Japan) with a pre-packed LiChrospher 100 RP-18 column (4 × 250 mm, 5 µm particle size) of Merck (Darmstadt, Germany). The mobile phase was a mixture of methanol/0.02 M KH₂PO₄ (15:85). Elution was performed at a flow rate of 1 ml/min and detection was determined using a variable-wavelength UV detector (L-4250) at 260 nm. A known quantity of cordycepin was used as an internal control.

Semen Production

All boars had been trained to mount the dummy for semen collection and semen were collected once a week. The collected semen was strained through a sterile gauze in a prewarmed insulated container kept at 37°C, to remove the gelatinous phase. Semen was immediately diluted by adding a commercial extender (Well Sperm). The sperm concentration was estimated using a photometer (Minitub SpermaCue, Tifenbach, Germany) calibrated with a hemacytometer. Each semen sample was diluted to a final concentration of 3 × 10⁹ spermatozoa per dose. Sperm production was measured as sperm concentration, semen volume and total sperm number.

Sperm Quality

Semen quality was determined using computer-aided semen analysis (Hamilton Thorne IVOS; Hamilton Thorne Bioscience, Beverly, MA, USA). Normal traits include sperm motility, sperm concentration, percentages of normal sperm, sperm with persistent proximal plasma droplets, sperm with persistent distal plasma droplets, and abnormal sperm. Sperm morphology was made under a microscopic field ($\times 1000$) for 200 cells in the slides.

Statistical Analyses

Data were analyzed by repeated-ANOVA measurement and post hoc LSD test, and student's t-test. For the sperm production and the sperm quality, analyses were performed at the beginning, during the supplementation, and 1 month after the stop of the supplementation. Differences were considered significant at $p < 0.05$.

Results

Sperm Production

This study was aimed to evaluate the effect of Cordyceps on sexual function. Therefore, old subfertile Duroc and Landrace boars with poor sperm quality were included in the study. There was no difference of the total sperm production at the beginning of the experiment: for Duroc, $5.0 \pm 1.7 \times 10^{10}$ vs. $4.6 \pm 1.5 \times 10^{10}$ cells and for Landrace, $6.3 \pm 1.7 \times 10^{10}$ vs. $6.5 \pm 0.7 \times 10^{10}$ cells (means \pm SEM) for the control and the supplement, respectively. After the CM treatment, total sperm number for Duroc was increased by 35% and 70%; and for Landrace, 18% and 35%, at 1 and 2 month of supplementation respectively, from the initial levels (Fig. 1). Total sperm number for Landrace was still increased (+17% from the initial level) and remained high (+53%) for Duroc, 2 weeks after stopping the CM supplement.

Sperm Quality

During the supplementation with CM, the percent change of motile sperm cells was +15% and +27% ($p < 0.01$), respectively for subfertile Duroc and Landrace boars at 8 weeks (Fig. 2). The mean sperm motility from the treated Duroc was closed to the healthy range of the control boars (from 73% up to 81% vs. control's 80% and 81%, at 0 week to 8 weeks, respectively). Similarly, the mean sperm motility from the treated Landrace was increased from 67% to 78% as compared with the control's 85% and 85%, at 0 week and 8 weeks, respectively. The mean motility of sperm cells for Duroc and Landrace boars were still significantly increased ($p < 0.05$) from their initial levels, 2 weeks after stopping the supplement (78% and 74%, respectively).

The value for the normal morphology in a good sperm should be above 70% (Flowers, 1998). The treatment significantly improved the percentage of sperm cells with normal

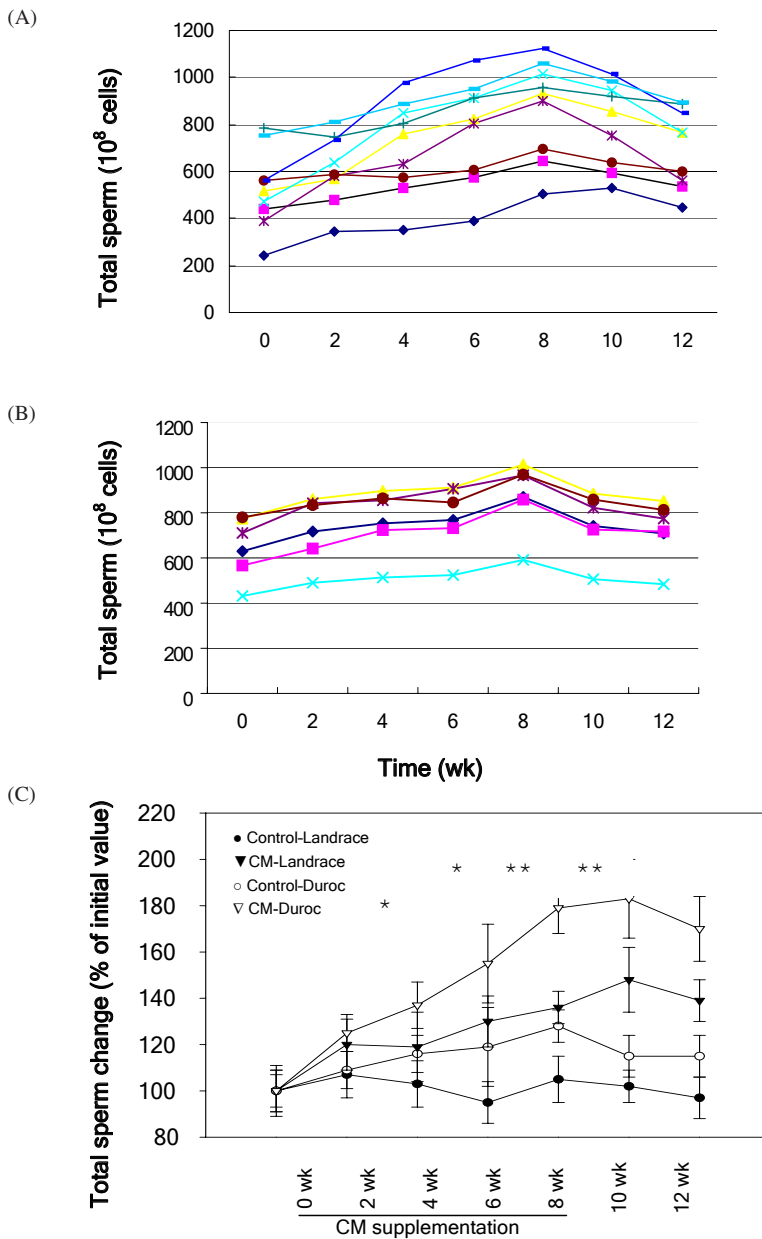


Figure 1. Effect of supplementation with *Cordyceps militaris* (CM) mycelium on sperm production. CM supplement enhanced the sperm production from each Duroc (A) and Landrace boars (B). Percent changes of total sperm numbers (C) before and after the CM supplementation. The total sperm production before the experiment for Duroc (○, ▽) was $5.0 \pm 1.7 \times 10^{10}$ vs. $4.6 \pm 1.7 \times 10^{10}$ cells and for Landrace (●, ▼), $6.3 \pm 1.7 \times 10^{10}$ vs. $6.5 \pm 0.7 \times 10^{10}$ cells (mean \pm SEM for the control and the supplement, respectively). Total numbers and percentage change from the initial value (0 week) of sperm production for both boars with CM supplement were significantly different at 8 weeks ($p < 0.01$).

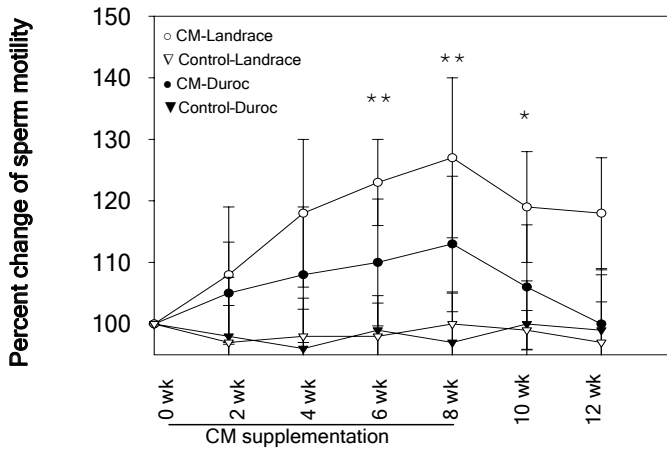


Figure 2. Effect of CM supplement on sperm motility in adult Duroc (○, ▽) and Landrace (●, ▼) boars. Percent changes in the sperm motility were shown before, during and after the CM supplementation (mean ± SEM). Sperm motility of both CM supplement groups was significantly increased from its initial level at 8 weeks (**p < 0.01).

Table 3. Effect of CM Supplementation on Morphology of Boar Spermatozoa

Group	Time (weeks)	Percent (% , mean ± SE)			
		Normal	PCD ^a	DCD ^b	Abnormal
Duroc					
Control	0	65 ± 6.8	8 ± 3.4	11 ± 3.6	16 ± 4.6
	4	65 ± 8.6	9 ± 3.3	11 ± 2.7	15 ± 3.8
	8	66 ± 9.1	9 ± 2.5	9 ± 3.7	16 ± 4.8
	12	65 ± 9.6	8 ± 2.6	11 ± 3.5	16 ± 4.1
CM	0	48 ± 7.2	24 ± 6.7	17 ± 4.2	11 ± 1.0
	4	60 ± 7.6*	18 ± 5.5*	14 ± 2.6*	8 ± 0.6*
	8	67 ± 7.9**	17 ± 5.7**	9 ± 2.5**	7 ± 0.8**
	12	46 ± 6.9	24 ± 6.0	15 ± 2.6	15 ± 1.3
Landrace					
Control	0	75 ± 3.4	4 ± 1.2	6 ± 1.5	15 ± 1.2
	4	73 ± 2.5	4 ± 1.1	6 ± 1.6	17 ± 2.3
	8	76 ± 3.8	4 ± 0.9	6 ± 1.4	14 ± 2.4
	12	72 ± 2.9	4 ± 0.9	8 ± 0.9	16 ± 2.5
CM	0	45 ± 9.4	8 ± 4.9	9 ± 0.6	38 ± 7.9
	4	52 ± 10*	7 ± 5.4	9 ± 1.8	32 ± 7.2*
	8	58 ± 8.7**	5 ± 4.1**	7 ± 0.7**	30 ± 6.8**
	12	45 ± 8.0	7 ± 5.1	11 ± 1.7	37 ± 5.4

^aPCD: proximal cytoplasmic droplets.

^bDCD: distal cytoplasmic droplets.

*p < 0.05, **p < 0.01, as compared with the initial value (0 weeks).

morphology from $52.8 \pm 6.7\%$ to $68.8 \pm 10\%$ (mean \pm SEM, $p < 0.05$) and $64.2 \pm 7.1\%$ to $66.2 \pm 8.2\%$, respectively for Duroc and Landrace boars at 2 month of CM supplement (Table 3). The change of normal morphology of sperm cells was significant in both Duroc and Landrace boars ($p < 0.01$). However, two of the Duroc boars with less than 2% of normal sperm morphology remained poor quality (up to 6% at 2 months), although their sperm number increased to 1.32×10^{-10} and 2.06×10^{-10} cells. The CM supplement boars had the mean plasma cordycepin concentration of $0.49 \pm 0.21 \mu\text{M}$ ($n = 7$; $p < 0.01$), after 2 months of supplementation as compared to $0.01 \pm 0.01 \mu\text{M}$ of the control boars ($n = 9$).

Discussion

Sperm production is influenced by many factors such as nutrition, season, collection frequency, breed and age. The average number of sperm cells produced per boar per week may differ more than 30% within one artificial insemination (AI) station, depending on the breed. Sperm morphology in boars tends to be changed by aging and environmental factors (Suriyasomboon *et al.*, 2005; Kunavongkrit *et al.*, 2005). Boar selection and boar management markedly influence the efficiency of sperm production (Colenbrander *et al.*, 1993). Currently, boars with less than 70% of normal morphology and motilities are typically culled and not used for breeding in the USA. Therefore, any practice to enhance sperm production and qualities would be valuable to an AI farm management. The present result showed that total sperm production from Landrace and Duroc boars was significantly increased 19–38% and 36–79%, respectively after 1 and 2 months supplementation with CM mycelium (Fig. 1). Importantly, the percentages of motilities and normal morphology of sperm cells were increased significantly at 2 months of the supplementation with CM. Most of the treated boars reached to the normal range from the subfertile status. In contrast, those with very poor normal morphology (less than 2%) only improved slightly but compensated with increased total sperm number after the treatment.

The plasma concentration of cordycepin was increased in the boars supplemented with CM mycelium for 2 months. It is possible that CM supplement might affect spermatogenesis through the effect of cordycepin, which is the major component in both CM, and CS. CS can induce the steroidogenic enzyme E2 expression (Huang *et al.*, 2004a) and plasma testosterone level *in vivo* (Hsu *et al.*, 2003; Huang *et al.*, 2004b). Testosterone, follicle-stimulating hormone (FSH), and E2 are all involved in the spermatogenic process. These hormonal effects on sperm cells are not direct, but are mediated through Sertoli cells. The Sertoli cell plays a pivotal role in the development of a functional testis. FSH is the major mitotic factor for Sertoli cells (Colenbrander *et al.*, 1993; Saez *et al.*, 1986). A negative relationship exists in mature boars between FSH secretion and testicular size, testicular and epididymal weights, and daily sperm production (Ford *et al.*, 1997; Franca *et al.*, 2005). Although cordycepin can induce the steroidogenic enzyme E2 and testosterone (Hsu *et al.*, 2003; Huang *et al.*, 2004), its effect on FSH has not been reported and awaits future study. Boars with high testes weight had a more rapid increase in E2 concentrations than did boars with low testes weight (Schinckel *et al.*, 1984a, 1984b; Zanella *et al.*, 1999). The sperm transit through the epididymis takes approximately 10 days in pigs. Therefore,

the high supporting capacity of Sertoli cells and the short duration of the spermatogenic cycle are the main factors responsible for the comparatively high spermatogenic efficiency in pigs (Franca *et al.*, 2005; Okwun *et al.*, 1996). Our present result showed that the effect of CM supplement was more prominent at the second month of supplementation and slightly after stopping the CM supplement for 2 weeks. This result is in agreement with the fact that the spermatogenic cycle and the entire spermatogenic process which lasts 8.6–9.0 days and approximately 40 days, respectively in boars (Franca *et al.*, 2005). This result suggests that cordycepin may affect hormones, and which in turn, may affect the spermatogenic cycle and the entire spermatogenic process; but the precise mechanism awaits future study.

The CM mycelia contained 3,120 U/g superoxide dismutase (SOD) that had a strong antioxidative activity (Li *et al.*, 2001). However, SOD might not retain the antioxidative effect after digestive enzymes. Whether it can affect the sperm quality awaits further study. Selenium (Se) has a role in establishing the number of boar spermatozoal reserves and Sertoli cells (Marin-Guzman *et al.*, 2000). Se has been well recognized for its role in preventing oxidative damage, but its role in male reproduction is less well-defined. Our CM mycelium contained 0.23 mg/100g of Se that was far below its level in the feed. Therefore, the influence of Se from CM mycelium on sperm production in adult boars might be minimal.

In conclusion, the present study shows that the CM supplement increased the total sperm number, the percentage of motile sperm cells and normal morphology in subfertile adult boars. The beneficial effect of CM supplement continued after stopping the supplement for 2 weeks. Since plasma concentration of cordycepin was increased by the supplementation with CM mycelium, it is suggested that cordycepin might be responsible for the increased semen production and sperm quality in boars. The effect of CM supplement on sperm production awaits further study to understand its precise mechanism.

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