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CORDYCEPS SINENSIS BIOMASS PRODUCED BY SUBMERGED FERMENTATION IN HIGH-FAT DIET FEED RATS NORMALIZES THE BLOOD LIPID AND THE LOW TESTOSTERONE INDUCED BY DIET

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ABSTRACT

This study investigated the effect of *Cordyceps sinensis* biomass supplementation obtained from submerged fermentation on blood lipid and low testosterone induced by high-fat diet (HFD). The experiments were carried out using a long-term intake of HFD and HFD plus Simvastatin or *C. sinensis* (4 months). Our results show that plasma cholesterol, triglycerides and LDL were decreased by *Cordyceps sinensis* biomass supplementation (CSBS). A long-term intake of HFD caused a significant liver damage which has been reverted by CSBS. CSBS normalized decreasing testosterone levels observed in high-fat diet feed rats. All these findings lead us to suggest that *C. sinensis* was able to decrease blood lipid concentration, increase hepatoprotective activity and normalize testosterone levels.

Keywords: *Cordyceps sinensis*, high-fat diet, hypolipidemic effect, testosterone

INTRODUCTION

The biological properties of *C. sinensis* mycelium, including anti-inflammatory and anti-tumor activities, have previously been investigated *(*Liu et al., 2011; Yan et al*.*, 2011).

Interestingly, in addition to their mycelium effects obtained from solid-state fermentation, *C. sinensis* biomass obtained from submerged fermentation (Chimilovski et al., 2011) could be an effective agent in lipid metabolism. This hypothesis was supported by some evidences about glucan isolated from the *C. sinensis* (Wu et al., 2005, 2007). Remarkably, hypocholesterolemic effects of glucans have been shown in various studies (Tiwari and Cummins, 2011;

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Lei et al., 2012). Similarly, submerged fermentation is a conventional approach to produce high quality biomass and this technique possesses particular advantages such as superiority in process control and easy recovery of biomass (Sun and Xu, 2009).

Current interest in the effect of glucans on lipid metabolism main is centered on the possibility that the glucans could entrap bile acids in the intestine and thus increase bile acid exclusion in the feces (Bowles et al., 1996). Second, cholesterol uptake could be inhibited by glucan in the intestinal wall (Drozdowski et al., 2010). Third, glucans undergo a fermentation process to produce short-chain fatty acids that can inhibit cholesterol synthesis (Drozdowski et al., 2010; Turunen et al., 2011).

Atherosclerosis, the complex interaction of macrophages with serum cholesterol in arterial wall, is the leading cause of cardiovascular disease worldwide and it has become a serious social problem. Among various factors leading to atherosclerosis, high low-density lipoprotein cholesterol, triglycerides and total cholesterol have been considered to be the major risk factors in its pathogenesis (Østerud and Bjørklid, 2003; Ding et al., 2012; Zha et al., 2012).

Since increased cholesterol and other lipid parameters may affect the development of atherosclerosis and cholesterol build-up in the coronary arteries (Zha et al., 2012), we assumed that hypolipidemic activity must be studied in *C. sinensis* biomass obtained from submerged fermentation.

Current knowledge concerning the role played by chronic liver disease on testosterone levels has been demonstrated. Studies have shown that low testosterone levels are common in men with severe liver disease (Grossmann et al., 2012). The pathogenesis of low testosterone levels in men with chronic liver disease involves dysregulation of the hypothalamo-pituitary-gonadal axis at multiple levels (Grossmann et al., 2012). Thereby, the severity of chronic liver disease could become obviously a serious problem for boys in pubertal stage.

Here we investigated whether a long-term intake of fat diet could decrease testosterone levels through liver damage (caused by liver-fat deposition) and the beneficial effects of CSBS, produced by submerged fermentation, on hyperlipidemia pattern and low testosterone observed in the high-fat diet feed rats.

EXPERIMENTAL

Diet preparation

The basal animal diet used (Labina, Purina®, São Paulo, Brazil) was modified by supplementation with the following ingredients (g/100 g): lard, 14 and hydrogenated vegetable fat, 6. To prepare it, we have mixed pulverized standard diet and melted lipids (lard and hydrogenated vegetable fat). A daily average of the food intake was determined by adding the food consumed each day by all of the rats of each group and dividing it by the number of rats per group.

Study design

All procedures involving animals were approved by the Positivo University Committee for Animal Welfare. Forty male *Wistar* rats, 30 days weighing 110 g (± 10 g) were divided into four groups (ten per group). The animals were kept in the animal house at a temperature of 24 ±2 °C with a 12/12 hour light/dark cycle for 4 months and fed with the respective diets and water *ad libitum*. Control group was fed with basal diet without modification, HDF and HFD + Simvastatin (Medley, Campinas–SP, Brazil) or *C. sinensis* groups were fed with modified basal diet and modified basal diet + Simvastatin or *C. sinensis* respectively. When required, Simvastatin and *C. sinensis* biomass were added together with modified basal diet. The dosage of drug and biomass were 10.36 mg/Kg and 10 % (w/w) (drug or biomass/feed) for a total of 14 weeks respectively.

Biochemical determinations

At the end of the experiments, the animals were anesthetized through ethereal

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inhalation, and blood samples were collected through cardiac puncture for measuring the plasma cholesterol, triglycerides, LDL, AST activity, urea and testosterone.

The plasma lipid, urea and creatinine measurements were performed in an ADVIA 1650 automated system (Bayer AG, Leverkusen, Germany). Testosterone measurements were performed by direct immunoassay on the Roche E170.

Liver lipid hydroperoxides

Dosage of lipid hydroperoxides was carried out on methanolic extract of liver tissue as described by Nourooz-Zadeh et al. 1994. A 300 mg portion of the liver right lobe was homogenized in 1 mL methanol, using an electric homogenizer (GGS 27, Bosch). After centrifugation (5000 g, 5 min, 4 °C), a 50 µL aliquot of the supernatant (cell-free extract) was stored for further measurement of the total proteins, and 90 µL aliquots were disposed into six centrifuge vials (1,5 mL). To three of these vials, 10 µL of methanolic 10 mM triphenylphosphine was added, thereby generating three blanks. To the other three vials, 10 µL of methanol was added. All the six vials were vortexed and then incubated for 30 min at room temperature. After that, 900 µL FOX 2 (100mM xylenol orange, 4 mM BHT, 25 mM sulfuric acid and 250 mM ammonium ferrous sulfate, 90 % methanol, 10 % ultrapure water) was added to all vials. After mixing, the samples were incubated for another 30 min at room temperature. The absorbance was measured at 560 nm using a spectrophotometer (Ultrospoc 2000, Pharmacia Biotech). The results were corrected for the extract protein concentration.

Liver total proteins

The method described by Bradford was carried out for this measurement (Bradford, 1976). Briefly, 250 µL Bradford reagent was added to 10 µL of cell-free extract in a microplate. After 5 min at room temperature, absorbance at 595 nm was measured using a microplate spectrophotometer (Benchmark, Bio-Rad). Protein concentration was determined interpolating absorbance values in a standard curve generated by known concentrations of bovine serum albumin.

Histopathology and staining

We performed biopsies of the livers of animals from different experimental groups: (A) Control, (B) HFD and (C) HFD plus *C. sinensis*. The biopsy specimens were fixed in formalin, embedded in paraffin, and cut in cryostat with serial sections of 3 to 6 µm after freezing. Thereafter, they were stained with Sudan black.

Statistical analysis

The data are presented as mean ± SEM values. Statistical analysis was performed by one-way ANOVA, followed by the Tukey test. The value of $p < .05$ was taken to indicate statistical significance.

RESULTS

Figure 1 shows the lipid parameters (plasma cholesterol, triglycerides and LDL) of rats fed HFD and HFD supplemented with Simvastatin and biomass (*C. sinensis).* In the diet supplementation experiment levels of lipid parameters were calculated using enzymatic-colorimetric method. High-fat diet feed rats showed an increase in plasma cholesterol and LDL levels; however, the triglyceride levels exhibited no changes compared with control animals. Simvastatim and *C. sinensis* administered to high-fat diet feed rats as a diet supplement was well tolerated and caused positive response (significant decrease) such as plasma cholesterol, triglycerides and LDL levels. Interestingly, Simvastatin (synthetic hypolipidemic) and *C. sinensis* showed similar trends.

From the HFD group, the diet increased liver-fat deposition (Figure 2), which remained significantly higher than in control group at the end of the experiment. In contrast, rats treated with *C. sinensis* showed a lower liver-fat deposition.

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Figure 2 shows liver cryosections stained with Sudan black for specific labeling of lipids. As shown in Figure 2, lipids accumulate predominantly in HFD group, while in HFD plus *C. sinensis* is spared. Aspartate aminotransferases were lower in the treated groups than those of the HFD group (Figure 3A). Plasma urea in the HFD group or HFD plus treatment was decreased compared with control group (Figure 3B). Measurement of liver plasma hydroperoxide concentrations were realized by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine (Nourooz-Zadeh et al., 1994). Liver plasma hydroperoxides of treated groups were lower than those of the HFD group (Figure 3C).

The results of this study show how repeated HFD (able to increase lipid parameters), without increasing body weight (data not shown), results in lower testosterone levels and possibly promote the delayed onset of signs of pubertal maturation in male rats. Interestingly, plasma testosterone was identical for control and treated groups (Figure 4).

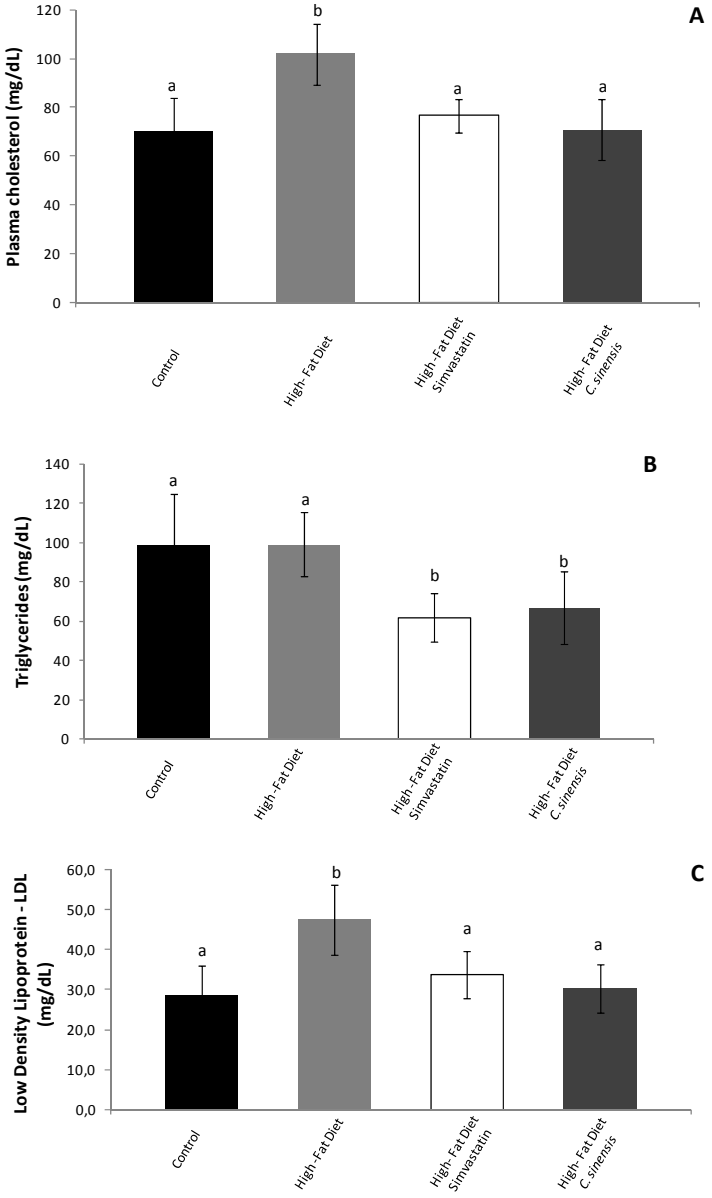

Figure 1: Plasma cholesterol, triglycerides and LDL in rats fed HFD and HFD supplemented with Simvastatin and biomass (*C. sinensis).* Data are mean ± SEM values of ten rats per treatment group. (A,B,C) [b] $p < .05$ compared to control group

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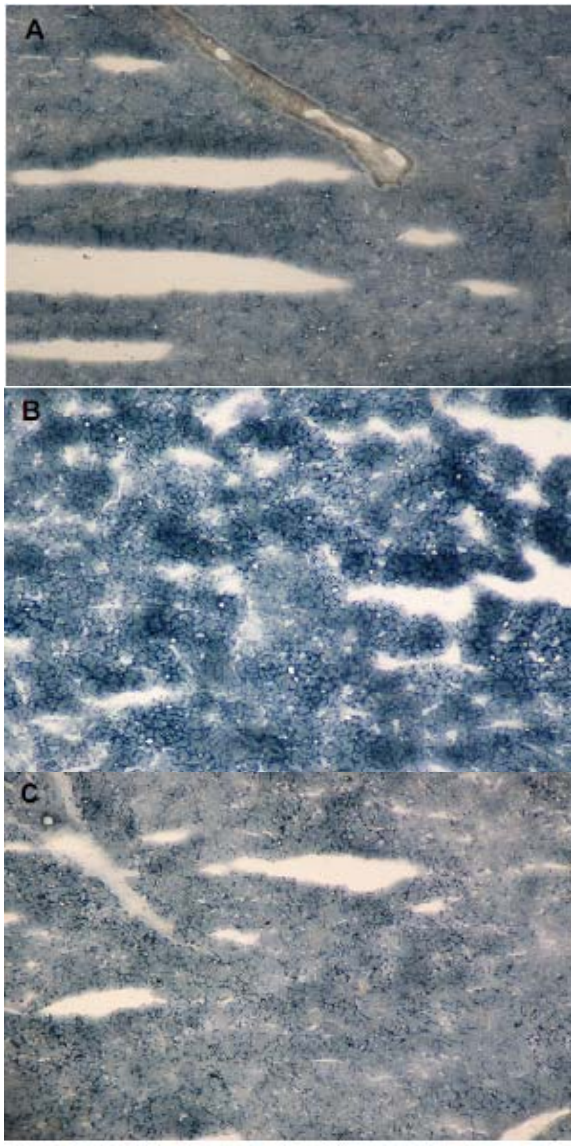

Figure 2: Livers of animals from different experimental groups: (A) Control, (B) HFD and (C) HFD plus *C. sinensis.* Histopathological analysis (Sudan black, 10x magnification).

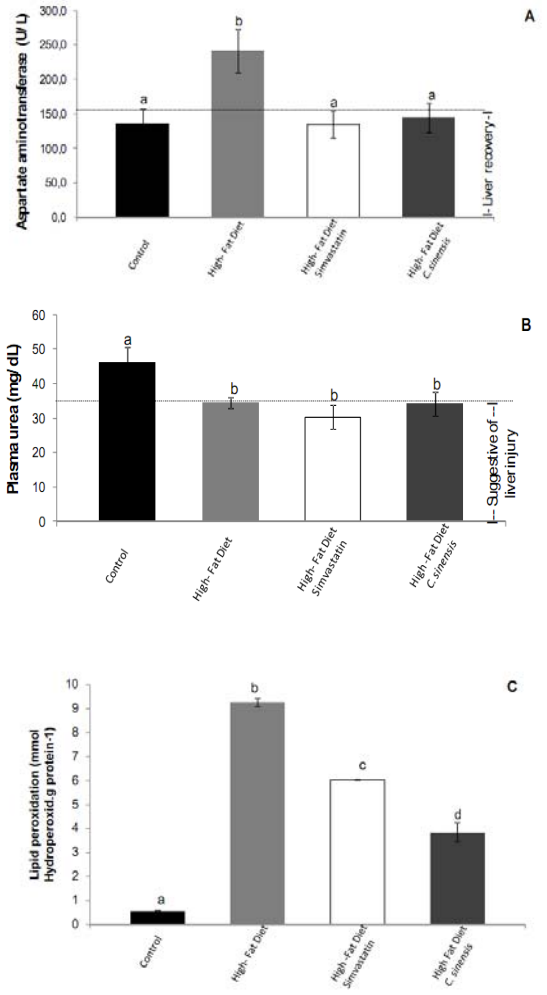

Figure 3: Effect of HFD on aspartate aminotransferase, plasma urea and lipid peroxidation. Data are mean ± SEM values of ten rats per treatment group. [b,c,d] $p < .05$ compared to control group. Horizontal lines demarcate groups with similar mean.

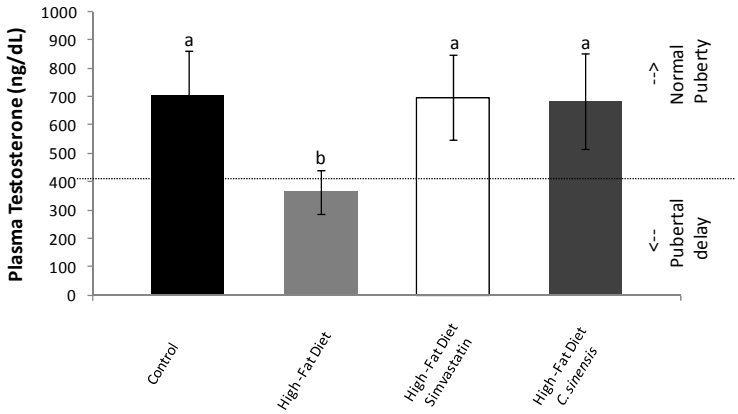

Figure 4: Effect of HFD on plasma testosterone. Data are mean ± SEM values of ten rats per treatment group. [b] $p < .05$ compared to control group

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DISCUSSION

Here we investigated whether a long-term intake of fat diet supplemented with *C. sinensis* biomass, produced by submerged fermentation, given to rats would modify the hyperlipidemia pattern and low testosterone observed in the high-fat diet feed rats.

The findings in this study confirm that *C. sinensis* biomass in our experimental conditions is sufficient to decrease lipid parameters such as plasma cholesterol, triglycerides and LDL (Figure 1). Notably, Simvastatin (synthetic hypolipidemic) and *C. sinensis* showed similar trends, which may reflect their potential use against hyperlipidemia and atherosclerosis. It is possible that compounds (lovastatin, glucans, steroids, niacin, CETP inhibitors and cordycepin) derived from the cell body *C. sinensis* may be the active hypolipidemic materials (Wu et al., 2005, 2007; Rozman and Monostory, 2010; Leu et al., 2011; Tiwari and Cummins, 2011; Lei et al., 2012). In addition, all statins have been observed to cause myopathy, and the risk of adverse effects on muscle increases with the use of high doses (Rallidis et al., 2012). Therefore, *C. sinensis* can be an alternative treatment option as far as it could decrease the doses of statins used in therapeutic regimes. One reason can be devised for invariable levels of triglycerides in high-fat fed rats: our experimental diet was prepared with laboratory animal feed enriched with commercial hydrogenated lard which contained high concentrations of conjugated linoleic acid (CLA). Notably, hypotriglyceridemia effect of CLA has been shown in various studies (Andreoli et al., 2009; Shu-Chiun et al., 2012). Thus, CLA could alleviate hypertriglyceridemia caused by HFD. At any rate, the treated groups had a positive effect on triglycerides levels (Figure 1B).

Our diet conditions showed liver damage by accumulation of lipid droplets, which may reflect hepatic steatosis (Figure 2) (Amacher, 2011). All other analyses also showed liver damage such as aspartate aminotransferase activity, plasma urea and lipid peroxidation (HFD group) (Figure 3). From the treated groups, plasma urea did not show improved levels. Despite that Simvastatin and *C. sinensis* could be considered biologically inactive against liver damage when we observed plasma urea levels (Figure 3B), it is possible that treatment period was not able to restore urea cycle enzymes. However, aspartate aminotransferase activity, liver histopathology analyses and lipid peroxidation demonstrated that treated groups, especially in the HFD plus *C. sinensis*, exhibited hepatoprotective activity (Figure 2 and Figures 3A, C). The assessment of oxidative damage by the lipid peroxidation in tissue exposed to oxidative stress has been proposed to assess tissue damage (Wang et al., 2012). Correlation between HFD and improved oxidative stress was reported (Chaudhari et al., 2012).

An interesting effect was observed in the plasma testosterone of animals from HFD group (Figure 4). HFD promoted lower testosterone levels which probably could delay onset of signs of pubertal maturation in male rats. Insight into the relationship between HFD and lower testosterone levels may be provided by at least two reasons: (a) dysregulation of the hypothalamo-pituitary-gonadal axis at multiple levels since liver damage (caused by HFD) was observed in our study - low testosterone levels in men with chronic liver disease have been demonstrated (Grossmann et al., 2012), (b) liver disease could effectively decrease the insulin-like growth factor 1 (IGF-1) production. The IGF-1 is a single chain polypeptide consisting of 70 amino acids and regulated by liver. The liver is the central organ of the endocrine growth hormone/insulin-like growth factor (GH/IGF) axis. The IGF system is involved in all aspects of male reproductive physiology and it increases during the onset of puberty (Caregaro et al., 1997; Flores et al., 1998; Lackey et al., 2000; Donaghy et al., 2002; Yoon et al., 2011). Still on reproductive function, we believe that the negative effects of high-fat diet feed rats on testosterone levels showed

in our study may be due to no weight gain demonstrated by animals under our experimental conditions (data not shown) since the weight gain could be correlated with the higher leptin levels which suggest higher testosterone levels or precocious puberty (Terasawa et al., 2012; Wagner et al., 2012). The beneficial effect of *C. sinensis* on testosterone level disorders in HFD rats may be provided by liver recovery and the regulation of steroidogenesis by *C. sinensis* in rats Leydig cells (Hsu et al., 2003; Huang et al., 2004; Wong et al., 2007).

CONCLUSIONS

Our results suggest that *C. sinensis* biomass supplementation in high-fat diet feed rats for 4 months normalizes the blood lipid and the low testosterone levels induced by HFD. Probably, *C. sinensis* biomass supplementation cannot replace the use of currently available drug regiments for lipid reduction, but can complement them. They may also enable the use of lower doses of therapeutic drugs, thereby decreasing the risk of dose-related side effects. Our observations also contribute to validity the current knowledge concerning the role played by chronic liver disease on lower testosterone levels. Further studies should be made in order to evaluate IGF levels, as well as other pubertal parameters to assess the delayed onset of signs of pubertal maturation.

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DECLARATION OF INTEREST

The authors report no declaration of interest.

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