Effects of *Cordyceps sinensis* on macrophage function in high-fat diet fed rats and its anti-proliferative effects on IMR-32 human neuroblastoma cells

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Abstract: Macrophages have been considered an elusive yet emerging therapeutic target in tumor development since they are an important component in tumor microenvironment. The purpose of the present study was to evaluate the effect of *C. sinensis* on macrophage function (a component of tumor microenvironment which can alter the virulence of cancer) in high-fat diet fed rats. IMR-32 human neuroblastoma cell cytotoxicity was also investigated. The following parameters were observed to evaluate macrophage function: superoxide anion, hydrogen peroxide, nitric oxide, lysosomal volume and phagocytic capacity. High fat diet (HFD) plus *C. sinensis* supplementation promoted a decreased superoxide anion and hydrogen peroxide levels as well as lysosomal volume and phagocytic capacity. Nitric oxide was increased in the same group. In summary, *C. sinensis* offered an important anti-tumoral perspective from the standpoint of the tumor microenvironment and *in vitro* IMR-32 cytotoxicity.

Keywords: Anti-tumoral, *Cordyceps sinensis*, high-fat diet, macrophage funcion.

INTRODUCTION

Currently, medical mushrooms are becoming increasingly popular as foods and supplements with special health properties. Of all the medicinal mushrooms, *C. sinensis*, an ascomycete, is officially recognized as a Chinese medicinal treasure (Choi *et al*., 2010). Recently, previous studies suggest that the *C. sinensis* strongly inhibits T cells activity and reduce interferon gama (IFN-γ) production (Jordan *et al.,* 2008). Notoriously, IFNs play a central role in the modulation of immune responses and macrophage activation. Furthermore, previous studies demonstrated that aqueous extract of *C. sinensis* significantly inhibited the activity of macrophage phagocytosis assessed by colloidal carbon clearance assay as well as the *C. sinensis* increased nitric oxide concentration. (Jordan *et al.,* 2008; Zhang *et al.,* 2011). Thus, we are mainly interested in the effects of *C. sinensis* on other aspects of macrophage activation such as oxidative burst as well as the activity of macrophage phagocytosis by other method such as zymosan phagocytosis assay.

Macrophages are well known to play an important role in innate immune system as initiators and effectors (Wang *et al*., 2012). However, macrophages also markedly increase the virulence and progression of cancer because activated macrophages can enhance tumor cell invasion, migration and angiogenesis which are mainly involved in the tumerogenesis process (Linde *et al*., 2012; Medrek *et al*., 2012; Schmieder *et al*., 2012; Wagner *et al*., 2012). Thereby, current interest in the effect of potential agents such as *C. sinensis* on modulation of the macrophage function is centered on the possibility that the *C. sinensis* could have a beneficial effect on virulence and progression of cancer through a reduction of macrophage activation.

Evaluation of macrophage function has been linked to certain parameters such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and nitric oxide (NO) production as well as lysosomal volume and phagocytic capacity (Nunes *et al.*, 2008; Rubel *et al*., 2010). Only in the past decade the importance of these oxidative burst parameters (H_2O_2, O_2^- and NO) and phagocytic capacity became more and more apparent in the occurrence and progression of cancer (Jaganjac 2010; Medrek *et al*., 2012; Rai *et al.,* 2012).

Similarly, HFD is the most prominent cause of hyperlipidemia for both men and women in most developed countries and it is considered to put an individual at greater risk of hypertension, heart disturbances, diabetes and obesity. In addition, HFD is

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also associated with an increased contribution to virulence and incidence of cancer because there is clear correlation between body fat and elevated tumor growth hormones or HFD and higher tumor volume (Cottam *et al.*, 2010; Koike *et al.,* 2012). Most important, studies have been demonstrated that HFD can promotes an altered macrophage polarization which could reflect on an altered virulence of cancer (Ji *et al*., 2012; Wagner *et al*., 2012). Thus, add an aggravating factor such as HFD which is correlated with altered macrophage polarization can be interesting to evaluate the oxidative burst and morphological parameters of macrophages in hyperlipidemic animals and its speculations for anti-cancer potential agents (Ji *et al*., 2012; Mutoh *et al*., 2006; Reichwaldt *et al.*, 2010).

Neuroblastoma is a neoplasm of the sympathetic nervous system. This disease is the second most common extracranial malignant tumor of childhood and the most common solid tumor of infancy. Surgical resection, chemotherapy, or radiotherapy procedures are indicated upon patient's risk stratification. Survival rates for patients who have International Neuroblastoma Staging System (INSS) stage 1 are excellent with surgery alone. The introduction of new agents, when INSS >1, is essential to reduce the use of chemotherapy and radiotherapy (Bhatnagar and Sarin, 2012).

In this study the main focus will address the outcome of *C. sinensis* on anti-tumor perspectives through the evaluation of macrophage function (a component of tumor microenvironment which could alter the virulence of cancer) in hyperlipidemic rats (fig. 1). Finally, cytotoxicity test against IMR-32 human neuroblastoma cells after treatment with water extract of Cordyceps sinensis were evaluated.

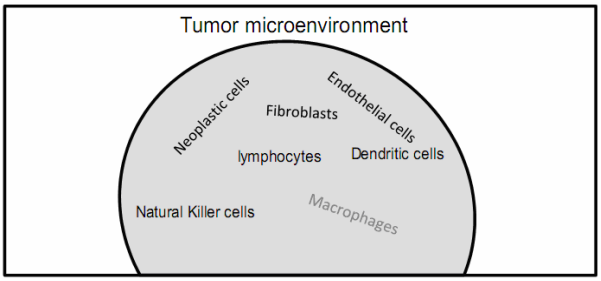

Fig. 1: Tumor microenvironment is composed of proliferating neoplastic cells, extra cellular matrix produced by fibroblasts, a vascular network of endothelial cells and cellular components of immune system such as macrophages.

MATERIALS AND METHODS

Fungal strain

Cordyceps sinensis PPGEBB was obtained from the *Banco de Cepas do Departamento de Engenharia de Bioprocessos e Biotecnologia*, UFPR (Curitiba, Brazil). The strain was maintained on nutrient agar slants.

Submerged culture conditions

Cultures were carried out in 1L Erlenmeyer flasks, with 400mL of a basal medium containing per liter: dextrose 30g, peptone 8g, KH_2PO_4 1g, $MgSO_4.7H_2O$ 0.5g at 25°C shaken at 120rpm, pH was adjusted to 5.5. For experimental diets, mycelium was then removed by filtration on filter paper (Choi *et al*., 2010).

Experimental diets

HFD was prepared using a laboratory animal feed (Labina, Purina®, São Paulo, Brazil) with the following ingredients: lard, 14% and hydrogenated vegetable fat, 6%. To prepare it, we have mixed pulverized standard diet and melted lipids (lard and hydrogenated vegetable fat). Control group was fed with basal diet without modification. When required, *C. sinensis* biomass was added together HFD. The dosage of biomass was 20% (w/w) (biomass/feed) for a total of 16 weeks (Dos Santos *et al*., 2012).

Animals

All procedures involving animals were appreciated by the Positivo University Committee for Animal Welfare. Thirty male Wistar rats, 30 days of age, weighing 110g (10±g) were distributed into three 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 water *ad libitum* and the respective diets.

Peritoneal macrophages activity

Peritoneal macrophages were obtained by peritoneal cavity washing (5mL of phosphate buttered saline - PBS) followed by centrifugation (200 g for 8 min. at 4°C). Then the macrophages were counted using trypan blue solution (1%) and were resuspended (10^6 cells/mL) in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum containing antibiotic solution (10 U/mL streptomycin and 20 U/mL penicillin). Peritoneal macrophages activity was evaluated using superoxide anion, hydrogen peroxide and nitric oxide production, as well as phagocytic capacity and lysosomal volume (Dos Santos *et al.*, 2013; Rubel *et al.*, 2010). Chemicals and cell culture medium used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Superoxide anion production ($O_2^{\bullet-}$)

Superoxide anion production was evaluated by the reduction of nitroblue tetrazolium (NBT). Peritoneal macrophages (10^5 cells in 0.45 mL of PBS) were incubated for 1h at 37°C in the presence of 0.03 mL of phorbol myristyl acetate (5 µM) and 0.1% (wt/v) nitroblue tetrazolium. Then the mixture was centrifuged (453 g for 5 minutes), the supernatant was discarded, and the peritoneal macrophages were fixed by adding 100 µL of methanol (50%) for 10 minutes. The plate was dried and 120 µL of KOH (2M) and 140 µL of dimethyl

sulfoxide were added to the wells. After 30 minutes the reduction of NBT resulted in the formation of blue formazan. The absorbance was read at 550 nm (Dos Santos *et al*., 2013; Liao *et al.*, 2011).

Hydrogen peroxide production (H_2O_2)

H_2O_2 production was evaluated using an assay based on the horseradish per oxidase-dependent conversion of phenol red into a colored compound by H_2O_2. Peritoneal macrophages (100µL) containing 10^5 cells were incubated in the presence of horseradish per oxidase (8.5U/mL), phenol red solution (0.56mM) and glucose (5mM) in the dark for 1 hour at 20°C. H_2O_2 production was detected spectrophotometrically at 620 nm (Dos Santos *et al*., 2013; Hayat *et al.,* 2012).

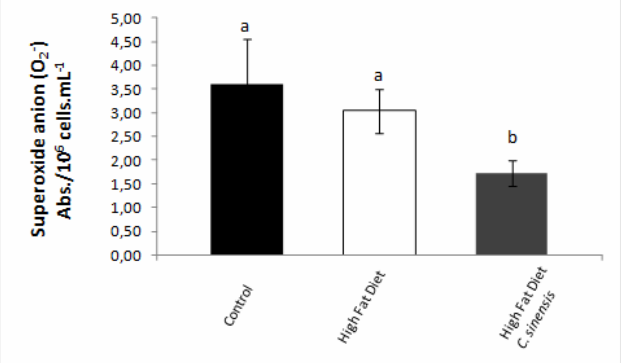

Fig. 2: Effects of *C. sinensis* on measured superoxide anion by nitro blue tetrazolium reduction assay. Statistical significance was evaluated by student´s t-test. [b] P<0.05 vs. control.

Lysosomal volume

The lysosomal volume of the peritoneal macrophages was assessed by the uptake of the cationic dye neutral red which concentrates in macrophage lysosomal system. 20 µL of 3% neutral red in PBS were added to 0.1mL of peritoneal macrophages suspension per plate well during 30min. The cells were then washed doubly and centrifuged at 453g for 5min. The neutral red stain was solubilized by adding 0.1mL of 10% acetic acid plus 40% ethanol solution. The absorbance was read at 550 nm and lysosomal volume was showed as absorbance (Dos Santos *et al.*, 2013; Rubel *et al.*, 2010).

Phagocytic capacity

0.1mL of peritoneal macrophages suspension containing 10^5 cells were added to the wells of a 96-well flat bottomed tissue culture plate. Then 10µL of neutral-red stained zymosan (10^8particles/mL) were added to each well. The plate was incubated for 30min. After incubation time, the cells were fixed with Baker´s formol-calcium solution (4% formaldehyde, 2% sodium chloride and 1% calcium solution) for 30 min. The wells were then washed doubly and centrifuged at 453g for 5min. 0.1mL of acidified alcohol (10% acetic acid, 40% ethanol and distilled water q.s) was utilized to solubilize neutral-red stain. After 30 min, the absorbance of each well was read on a plate reader at 550 nm (Dos Santos *et al*., 2013; Guerra and Otton, 2011).

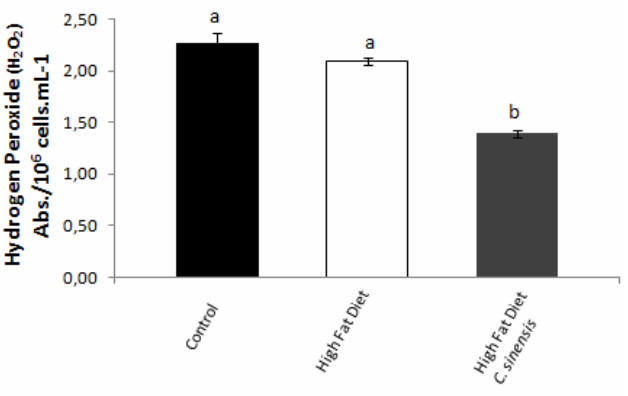

Fig. 3: Effects of *C. sinensis* on measured hydrogen peroxide by horseradish per oxidase. Statistical significance was evaluated by student´s t-test. [b] P<0.05 vs. control.

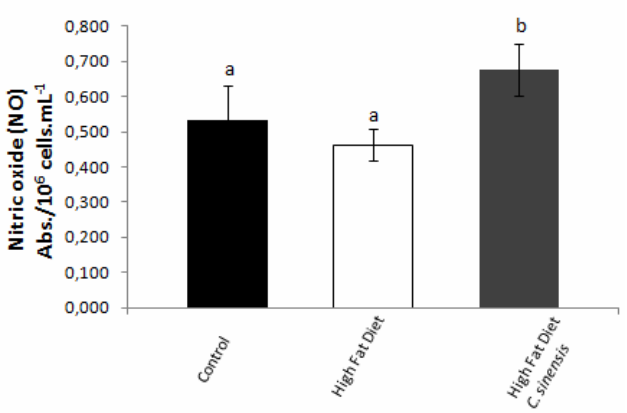

Fig. 4: Effects of *C. sinensis* on measured nitric oxide by Griess reaction. Statistical significance was evaluated by student´s t-test. [b] P<0.05 vs. control.

Nitric oxide production (NO)

NO production was evaluated as nitrite (NO_2^-) by the Griess reaction. Macrophages (100 µL) were incubated for 2 h at 37°C in a 96-well flat-bottomed tissue culture plate. Then the plate was washed twice with PBS to remove the nonadherent cells. The remaining cells were incubated for 24h in the presence and absence of lipopolysaccharide (10µg/mL). 100µL of Griess reaction were added for 10 minutes at room temperature and the absorbance was measured at 550 nm (Dos Santos *et al*., 2013; Tsikas, 2007).

Cytotoxicity assay

The IMR-32 human neuroblastoma and fibroblasts cells were obtained from the cell bank of the Pelé Pequeno Príncipe Institute. The cells were cultived in 25 cm^2 culture flasks in Dubelcco´s Modified Eagle Medium (DMEM; Gibco). The medium was supplemented with 5% fetal bovine serum (Gibco). The cells were grown in a

CO_2 (5%) incubator that was humidified at 37°C. The MTT (Thiazolyl Blue Tetrazolium Bromide) assay was performed according to the method described by Mosmann (Mosmann, 1983). IMR-32 and fibroblasts cells were seeded in a 96-well culture plate (10^5 and 3.10^3 cells/well respectively) and incubated for a period of 24 h to stabilize. Next, the cells were then treated with various dilutions from hot water extract (HWE) of Cordyceps sinensis biomass (40g dry biomass powder was mixed with 600 mL distilled water and heated at 90°C in a water bath for 4h, followed by centrifugation (453g for 5 min)). Next , the treatment medium was discarded, and the plate was incubated at 37°C for 4h with MTT diluted in phosphate buffered saline. The MTT was then discarded, and dimethyl sulfoxide (DMSO) was added to dissolve the formazan. The optical density was measured at 550 nm using a spectrophotometer plate reader.

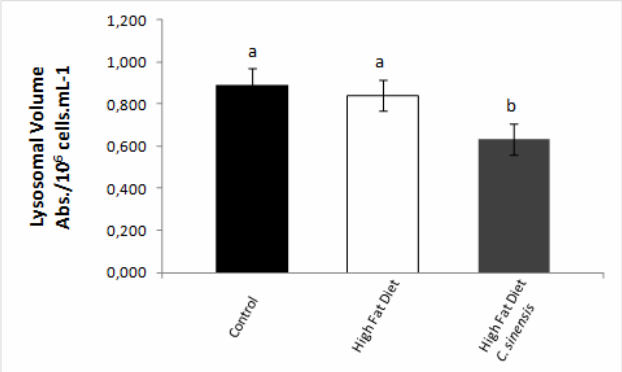

Fig. 5: Effects of *C. sinensis* on measured lysosomal volume by uptake of the cationic dye neutral red. Statistical significance was evaluated by student´s t-test. [b] P<0.05 vs. control.

Treatment of data

The statistical significance of the differences between parameters obtained in the experiments was assessed by student´s test. Treatment of data was carried out at the 5% significance level. Data are expressed as means ± SEM.

RESULTS

The effect of *C. sinensis* on macrophage function was made by observing certain parameters such as hydrogen peroxide, super oxide anion and nitric oxide production as well as lysosomal volume and phagocytic capacity. Physiological characteristics representing HFD induced hyperlipidemia have previously been published (Dos Santos *et al*., 2012). Reaction mechanisms are referenced in Section Material and Methods.

Oxidative burst- super oxide anion, hydrogen peroxide and nitric oxide

For evaluation of oxidative burst, super oxide anion, hydrogen peroxide and nitric oxide were observed. The results are shown in fig. 2, 3 and 4. We have found that, at the end of the experiments (4 months), *C. sinensis* decreased super oxide anion and hydrogen peroxide (fig. 2 and 3). However, according to the fig. 4, *C. sinensis* increased nitric oxide levels. Interestingly, HFD group did not show effects on super oxide anion, hydrogen peroxide and nitric oxide levels.

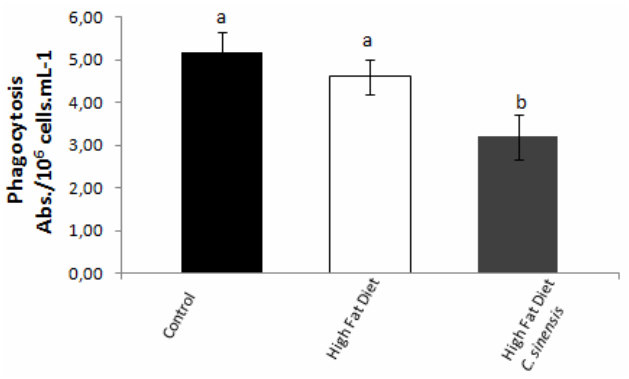

Fig. 6: Effects of *C. sinensis* on measured phagocytosis by zymosan assay. Statistical significance was evaluated by student´s t-test. [b] P<0.05 vs. control.

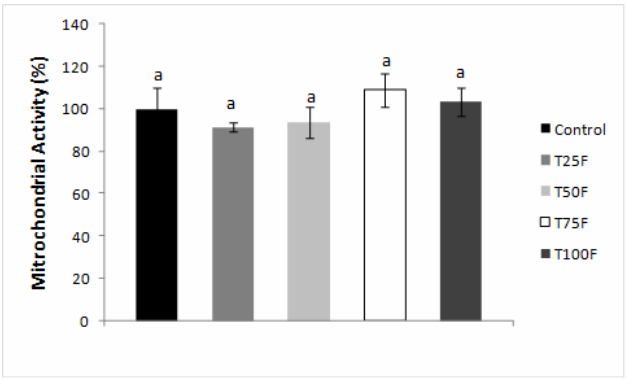

Fig. 7: Effects of water extract of C. sinensis on measured fibloblasts activity by MTT. Statistical significance was evaluated by student´s t-test. [b] P<0.05 vs. control. T25F = 25% (v/v) of HWE of *Cordyceps sinensis* biomass *et coetera.*

Morphological parameters- lysosomal volume and phagocytic capacity

Fig. 5 and 6 shows that oxidative burst changes were accompanied by a decrease in morphological parameters as can be seen in lysosomal volume and phagocytic capacity. Thereby, *C. sinensis* showed inhibitory effects on oxidative burst and morphological parameters. Once again, HFD did not alter lysosomal volume and phagocytic capacity.

Antitumor activity

Fig. 7 and 8 shows the cell response for the treatment effects from water extract of *Cordyceps sinensis* against IMR-32 and fibloblasts cells. Water extract of *Cordyceps sinensis* exhibited moderate antitumor activity with about 20% inhibition of the IMR- 32 cell growth. Compared to

the fibloblasts cytotoxicity assay, the water extract of *Cordyceps sinensis* is evidently non-toxic.

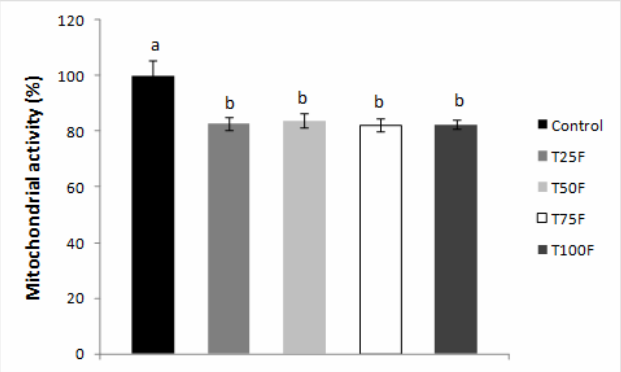

Fig. 8: Effects of water extract of C. sinensis on measured IMR-32 neuroblastoma cell activity by MTT. Statistical significance was evaluated by student´s t-test. [b] P<0.05 vs. control. T25F = 25% (v/v) of HWE of *Cordyceps sinensis* biomass *et coetera.*

DISCUSSION

The main objective of the present study was to evaluate the influence of *C. sinensis* on the macrophage functions by peritoneal macrophages. In spite of the well known effect of *C. sinensis* on macrophage phagocytosis assessed by colloidal carbon clearance assay and nitric oxide concentration, here we extend these data in macrophage oxidative burst and macrophage phagocytosis capacity by zymosan phagocytosis assay this assay provides substantial assistance as an additional parameter in the evaluation of phagocytosis. Although no tumor has yet been induced in our current experimental design, macrophages can be a new target since they can appears in tumor microenvironment. New treatments that have additional targets are interesting therapeutic approaches (Avnet *et al*., 2009). In addition, there is no information about IMR-32 cells when subjected to water extract from Cordyceps sinensis. Up till now, *C. militaris*, *C. takaomontana* and *C. sphecocephala* were evaluated against Neuro 2A and SK-N-SH cells (Lee *et al*., 2011; Lee *et al*., 2009; Young *et al.*, 2008).

The hypothesis of our study was that *C. sinensis* would alter macrophage function for which there a strong correlation with anti-tumoral potential. For a long time macrophages has been known as a component of innate immune system (Wang *et al*., 2012). However, previous study have been demonstrated that macrophages also markedly increase the virulence and progression of cancer because activated macrophages can enhance tumor cell invasion, migration and angiogenesis which are mainly involved in the tumerogenesis process (Linde *et al*., 2012; Medrek *et al*., 2012; Schmieder *et al*., 2012; Wagner *et al*., 2012). Thus, the reduction of macrophage activation could serve as alternative therapy to complement currently available drug regiments for tumerogenesis since their prominent role in tumor initiation, development and metastasis (Ghosh and Basu, 2012; Linde *et al*., 2012; Medrek *et al*., 2012; Schmieder *et al*., 2012; Wagner *et al*., 2012).

Our data collectively demonstrate that *C. sinensis* decreased macrophage function as observed by lower levels of super oxide anion, hydrogen peroxide, lysosomal volume and phagocytic capacity (zymosan phagocytosis assay). Lower levels of phagocytic capacity by colloidal carbon clearance assay are also implicated in previous studies about effect of *C. sinensis* on macrophage function (Zhang *et al*., 2011). Overall, *C. sinensis* was able to reduce oxidative burst and morphological parameters. These striking observations may change positively the tumor microenvironment. Nevertheless, controlled-delivery systems for targeting tumor using this alternative therapy are necessary to release the agents exactly where they should act to avoid systemic distribution and adverse effects (Dos Santos *et al*., 2012; Shen *et al*., 2012).

The close correlation between low levels of superoxide anion and hydrogen peroxide provided the evidence supporting that the inhibition mechanism occurs at level of enzymes that catalyze the superoxide anion production which will reflect in lower hydrogen peroxide prodution since the hydrogen peroxide is a end product from superoxide anion metabolism (Li *et al*., 2009; Root and Metcalf, 1977).

Nitric oxide is key mediator involved in many pathological and physiological processes. Our findings show that there was an increase in nitric oxide levels in group treated with HFD and *C. sinensis*. Previous studies also found an increase in NO production by *C. sinensis* (Jordan *et al*., 2008). Despite higher levels of nitric oxide, super oxide anions were decreased in same group the toxicity of nitric oxide is linked to its ability to combine with super oxide anions to form per oxynitrite which is an oxidizing free radical that can cause DNA fragmentation (Dahiya *et al*., 2012). High levels of peroxynitrite, a metabolic derivative from nitric oxide, can modify functional proteins leading to tumor development (Dahiya *et al*., 2012; Li *et al*., 2012). Moreover, increased levels of nitric oxide has been revealed interesting features such as participation in anti-tumor mechanisms of potential agents (Ling *et al*., 2010; Takeda *et al*., 2012). Thus, nitric oxide promotes an antitumorogenic environment.

We have further observed that *Cordyceps sinensis* leads to regression of IMR-32 neuroblastoma cell growth. Water extract of *Cordyceps sinensis* exhibited moderate antitumor activity with about 20% inhibition of the carcinogenic cell growth. Other fungal genres such as *C. militaris*, *C. takaomontana* and *C. sphecocephala* have

already been demonstrated to have effectiveness in lowering neuroblastoma cell activity (Neuro 2A and SK-N-SH cell). Therefore, these studies previously reported that Cordyceps spp. may exert their antitumor activity against neuroblastoma cell through the mechanism of inducing apoptosis (Lee *et al*., 2011; Lee *et al*., 2009; Young *et al*., 2008).

Our understanding of HFD effect on macrophage function is very limited. Previous study underscores a distinct macrophage polarization upon short term high fat diet feeding (Ji *et al*., 2012). Classical (M1) and alternative macrophage polarization (M2) induce pro-inflammatory and anti-inflammatory cytokines respectively. M1 macrophages play a high activity against microorganisms while studies suggest an important role of M2 macrophages in tumor progression (Dall'Asta *et al*., 2012). M1 form may also be present in cancer and has been associated with its survival time (Ma *et al*., 2010). The observation about an altered macrophage polarization upon short term high fat diet feeding leaves room to the intriguing hypothesis that HFD could also exert some effect on oxidative burst in tumor-associated macrophages. Reactive oxygen species which can be produced by oxidative burst/oxidative stress in tumor associated macrophages has become widely viewed as an underlying condition in cancer and tumors (Baskar *et al*., 2012; Jaganjac, 2010; Otsuji *et al*., 1996). HFD is believed to promote pro-inflammatory responses such as macrophage activation in adipose tissue, which contributes significantly to obesity-associated complications (Ji *et al*., 2012). Contrary to our expectations, the results showed that HFD did not affect the macrophage parameters. In our experimental design, we did not observe alteration in body weight (data not shown) although the lipid profile has changed (Dos Santos *et al*., 2012). This may explain why we did not visualize in HFD group alteration in macrophage parameters since macrophage activation has been linked to body fat (Roos, 2012; Zhuang *et al*., 2012). This clearly suggests that HFD as an aggravating factor to evaluate anti-tumoral potential through macrophage function in experimental design should be useful as long as the body fat increase.

CONCLUSIONS

The main finding from the results presented here is that *C. sinensis* displayed strong effect on macrophage function, particularly for super oxide anion, hydrogen peroxide, lysosomal volume and phagocytic capacity which were decreased and nitric oxide which was increased. Thus, our data demonstrate a decrease in macrophage function although nitric oxide has increased. Interestingly, macrophages have been associated with tumor development and apparently, new therapies which are specially directed against tumor-associated macrophages could offer benefits for treatment of tumor. A high level of nitric oxide was considered important since it show interesting features such as participation in anti-tumor mechanisms. *C. sinensis* not only decreased macrophage function, but also IMR-32 neuroblastoma cell activity. Unfortunately, we did not observe any affect of HFD as we had hypothesized at the beginning of our study, probably due to non-alteration in body weight. We do not intend to replace the use of currently available drug regiments for treatment of tumor, but complement them through integration of substances which have effect on macrophage function a component of tumor microenvironment. We are interested in further investigating the effect of *C. sinensis* on tumor development *in vivo* and examine proinflammatory and anti-inflammatory cytokines and markers for macrophage subtypes.

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