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IMMUNOMODULATING EFFECTS OF HOT-WATER EXTRACT FROM *Pleurotus ostreatus* MYCELIUM ON CYCLOPHOSPHAMIDE TREATED MICE

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ABSTRACT

With the view of developing new immunotherapeutic agents, there has been a recent upsurge of interest in edible mushrooms. Hot-water extract (F-I: 43.6% w/w carbohydrate and w/w protein) prepared from the mycelium of *Pleurotus ostreatus* was administered intraperitoneally (i.p.) at 100 mg/kg for 7 days to Balb/c mice and cyclophosphamide (CY) at 10 mg/kg was injected on the fifth day. The influence of F-I administration on the immunosuppression caused by CY was evaluated on the eighth day. CY treated mice exhibited less pronounced immunosuppression and more rapid haematopoietic recovery when administered with F-I. The i.p. injection of F-I increased bone marrow cellularity (4.1 x 10⁶ vs. 1.5 x 10⁶ per femur in saline control group, P< 0.01), the white blood cell counts (7.6 x 10⁹ vs. 4.8 x 10⁹ cells/l, P< 0.05) and led to a two-fold increase in the number of endogenous macroscopic colonies of hemopoietic tissue on the parietal surface of spleen (P< 0.05). F-I enhanced the murine reticuloendothelial system as judged by the shorter rate of carbon clearance (4.23 vs. 6.18 min, P< 0.05). F-I increased the number of peritoneal exudate cells (P< 0.01) and stimulated *in vivo* murine macrophage phagocytic ratio (15.65% vs. 4.70%, P< 0.01) and phagocytic index (1.06 vs. 0.12, P< 0.05). No toxicity signs such as hepatosplenomegaly were observed in F-I treated animals. These effects suggest that F-I could enhance host-defense mechanisms *in vivo*.

Key words: *Pleurotus ostreatus*, edible mushrooms, mycelium, cyclophosphamide, macrophage, immunomodulator.
EFECTOS INMUNOMODULADORES DE UN EXTRACTO EN AGUA CALIENTE DEL MICELIO DE *PLEUROTUS OSTREATUS* EN RATONES TRATADOS CON CICLOFOSFAMIDA

RESUMEN

Con el objetivo de desarrollar nuevos agentes inmuquimioterapéuticos, se ha producido un reciente resurgimiento en el interés por los hongos comestibles. Un extracto en agua caliente (F-I: 43.6%, w/w, de carbohidratos y un 28.0%, w/w de proteínas), preparado a partir del micelio de *Pleurotus ostreatus*, fue administrado intraperitonealmente (i.p.) en dosis de 100 mg/kg durante 7 días a ratones Balb/c y la ciclofosfamida (CY), a razón de 10 mg/kg, fue inoculada el quinto día. La influencia de la administración de F-I sobre la inmunosupresión causada por CY fue evaluada el octavo día. Los ratones tratados con CY mostraron una inmunosupresión menos pronunciada y una recuperación hematopoyética más rápida cuando se les administró F-I. La inyección i.p. de F-I incrementó la celularidad de la médula ósea (4.1 x 10⁶ vs. 1.5 x 10⁶ por fémur en el grupo control tratado con solución salina, P< 0.01), los conteos leucocitarios (7.6 x 10⁹ vs. 4.8 x 10⁹ cells/l, P< 0.05) y duplicó el número de colonias macroscópicas endógenas de tejido hematopoyético en la superficie parietal del bazo (P< 0.05). F-I potenció el sistema reticuloendotelial murino, a juzgar por el menor tiempo de aclaramiento del carbón (4.23 vs. 6.18 min, P< 0.05). F-I incrementó el número de células presentes en el exudado peritoneal (P< 0.01) y estimuló in vivo el porcentaje de macrófagos fagocitantes (15.65% vs. 4.70%, P< 0.01) y el índice fagocítico (1.06 vs. 0.12, P< 0.05). No fueron observados signos de toxicidad, como hepatoesplenomegalia, en los ratones tratados con F-I. Estos efectos sugieren que F-I es capaz de potenciar los mecanismos de defensa del huésped in vivo.

INTRODUCTION

The most important aim of cancer therapy is to increase the survival time of cancer patients, enabling them to live in comfort. Anticancer chemotherapy, however, is generally accompanied by severe toxic side effects and reduces the host’s resistance to cancer and infectious diseases, especially destroying lymphoid cells and bone marrow cells. Therefore, potentiating of host resistance should be one of the most important objectives in the development of cancer and AIDS therapy⁵. Recently, immunochemotherapy has been attempted in various animal models and human cancer patients to support chemotherapy⁸.

In view of developing new immunotherapeutics with low toxic potential, numerous polysaccharides isolated from edible mushrooms such as lentinan⁶, schizophyllan¹⁰, grifolan¹⁷, krestin or PSK¹, ganoderan¹⁵ and others obtained from *Armilariella tabescens¹²* and *Phellinus linteus⁹* have been investigated for their antitumor and immunomodulating activities. There has been a recent upsurge of interest in mushrooms as a source of biologically active compounds of medicinal value and efforts to find new immunomodulator polysaccharides are on-going ⁴,¹⁶.
**IMMUNOMODULATION BY *Pleurotus ostreatus***

*Pleurotus ostreatus* is a popular cultivated edible mushroom with medicinal properties. The effects of this mushroom on pathological changes in dimethylhydrazine-induced rat colon cancer and their antioxidant and antitumor activities have been reported. However, some biological activities of *P. ostreatus* may yet be clarified.

In Cuba, the implementation of technologies for the cultivation of *Pleurotus* on a large or small scale in agricultural communities will generate a food for human consumption and open new research activities towards mushroom pharmaceuticals. The Center of Studies for Industrial Biotechnology (CEBI) has begun studies to enhance our understanding of the potential applications of *Pleurotus*-derived preparations for immunotherapy.

The present study examined the immuno-modulating effects of a hot-water extract prepared from the mycelium of *P. ostreatus* on the immunosuppression caused by cyclophosphamide in mice.

**MATERIALS AND METHODS**

*Preparation of the hot-water concentrate.* Mycelium of *Pleurotus ostreatus* f. sp. florida (P-184) was grown on wheat kernels in jars of 200 g. This strain is adapted to tropical conditions, and is deposited at the culture collection of the Center of Studies for Industrial Biotechnology (CEBI). Mycelium (272 g) was extracted with 1.1 l of boiling water for 10 h and the extract was concentrated at 100 C. The concentrate designated as F-I, contained 43.6% (w/w) carbohydrate and 28% (w/w) protein. Carbohydrate was determined by the phenol-sulphuric method and protein by the Folin phenol reagent. F-I was obtained at a yield of 15 g (5.5%).

*Animals.* Pathogen-free male Balb/c mice were purchased from LABEX® (Santiago de Cuba). The 20-25 g mice were fed a standard diet and acidified water *ad libitum*. Fifteen mice were divided into two groups. Hot-water F-I extract was administered intraperitoneally (i.p.) at 100 mg/kg for 7 days to ten Balb/c mice and cyclophosphamide (CY) USP 23 for injection, obtained from JSLYP (China), at 10 mg/kg was given i.p. on the fifth day. The control group, comprised of five mice, was injected i.p. with physiological saline instead of F-I. On the eighth day, blood was collected from the orbital vein and animals were then bled to death.

*Haematological methods.* The blood specimens were analysed for haemoglobin and white blood cell count. Femoral bone marrow cells were withdrawn with Hanks’ solution and counted with a Neubauer chamber (Germany).

*Carbon clearance test.* On the eighth day, 0.2 ml of colloidal carbon solution consisting of 3 ml of Perikan drawing ink 17 black (Perikan AG, Germany), 4 ml of saline and 4 ml of 3% gelatine solution was administered intravenously (i.v.) via the tail vein to the selected mice. A 50 µl aliquot of the blood was taken every 5 min from the orbital vein with a heparinized haematocrit tube after the injection of the carbon solution, then immediately mixed with 4 ml of 0.1% (w/v) Na$_2$CO$_3$. The concentration of the colloidal carbon was estimated by absorbance at 675 nm. The clearance rate of carbon is expressed as the half-life of carbon in the blood ($t_{1/2}$, min), calculated by means of the following equation:

$$t_{1/2} = \frac{\ln 2(t_2 - t_1)}{\ln OD_1 - \ln OD_2}$$

where $OD_1$ and $OD_2$ are the optical densities at times $t_1$ and $t_2$, respectively.

*The number of peritoneal exudate cells.* Peritoneal exudate cells were collected from the peritoneal cavity of mice by washing with...
Hanks’ solution. The number was counted with a Neubauer chamber.

**In vivo macrophage phagocytic activity.** On the eighth day, the selected mice were injected i.p. with 0.5 ml of 1% sheep red blood cells (SRBCs) suspension. SRBCs were purchased from LABEX®. Thirty minutes later, intra-peritoneal cells were washed out with saline. Cells were fixed onto slides and stained with Giemsa’s solution. The percentage of macrophages that ingested SRBC (phagocytic ratio) and the number of SRBC in the macrophage (phagocytic index) were calculated by counting 100 macrophages under a microscope.

**Autopsy and measurement of organ weights.** The animals were autopsied; livers and spleens isolated, and weighed using an electronic balance (ER-182 A, Japan). The per cent ratios of organ weight to body weight were determined.

**Spleen cellularity.** The spleen cell suspension was prepared by gently teasing the tissue with ice-cold Hanks’ solution and passing it through antiseptic gauze (Johnson & Johnson Medical, TX, USA). The number was counted with a Neubauer chamber. The number of endogenous macroscopic colonies of haemopoietic tissue on the parietal surface of the spleen (ESC) of selected mice were determined after 24 h fixation in Bouine’s solution22.

**Liver protein concentration.** After weighing, 0.1 g liver samples were homogenized in 2 ml of ice-cold Hanks’ solution. Total protein was measured according to the Lowry’s method14.

**Statistical analysis.** Statistical comparisons between groups were carried out employing Student’s *t*-test21.

**RESULTS AND DISCUSSION**

During the experimental period, no environmental factors other than the stated variables were thought to have affected the results of the study. No deaths occurred in either the control or the F-I administered groups.

Cyclophosphamide is probably the most common antineoplastic used in cancer chemotherapy and is an essential component of several effective chemotherapeutic formulas. However, cyclophosphamide shows potent immunosuppressing properties8. As expected, cyclophosphamide severely impaired the haemopoietic tissue of our mice, but the extract from *P. ostreatus* (F-I) was found to have an active protective effect in mice, when administered before cytostatic.

The haematological parameters assayed on the eighth day are shown in Table 1. Haemoglobin levels of the F-I group were significantly higher than those of the control group (P< 0.05), and the recovery of bone marrow cellularity after CY injection, as well as the leucocyte count in peripheral blood proceeded at a faster rate than in animals treated with saline. A significant contribution to the protective effect of F-I on haemopoiesis, may be in the differentiation of bone marrow haemopoietic cells and a more rapid recovery of this series of haemopoietic cells in animals protected by F-I.

Administration of PGG (betafectin), a genetically modified glucan from *Saccharomyces cerevisiae*, to mice accelerated recovery from drug-induced neutropenia with cyclophosphamide13. Carboxymethyl-glucan (CMG) in two different degrees of substitution of carboxymethyl groups administered to cyclophosphamide treated mice led to a less pronounced immunosuppression and more rapid haematopoietic recovery24. Based on those studies, we can infer that one of the mechanisms of increased cyclophosphamide tolerance of animals is the activation effect on haemopoiesis by F-I.

The effect of the murine reticuloendothelial system on the phagocytic activity was examined by the carbon clearance test.
Table 1. Effects of *Pleurotus ostreatus* hot-water extract (F-I) administration on haemopoiesis and tissue parameters of cyclophosphamide challenged Balb/c mice.

<table>
<thead>
<tr>
<th></th>
<th>F-I</th>
<th>Saline control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>10.6 ± 2.7*</td>
<td>8.6 ± 0.7</td>
</tr>
<tr>
<td>Bone marrow cellularity (#x10⁶/femur)</td>
<td>4.1 ± 1.6**</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>White blood cell count (#x10⁹/L)</td>
<td>7.6 ± 1.5*</td>
<td>4.8 ± 2.2</td>
</tr>
<tr>
<td>Clearance rate of carbon (t1/2, min)</td>
<td>4.23 ± 1.06*</td>
<td>6.18 ±0.86</td>
</tr>
<tr>
<td>Peritoneal exudate cells (#x10⁶/mouse)</td>
<td>4.9 ± 1.4**</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>Phagocytic ratio (%)</td>
<td>15.65 ± 4.21**</td>
<td>4.70 ±1.27</td>
</tr>
<tr>
<td>Phagocytic index</td>
<td>1.06 ± 0.50*</td>
<td>0.12 ±0.01</td>
</tr>
<tr>
<td>Spleen weight (g/100 g body wt.)</td>
<td>0.60 ± 0.09</td>
<td>0.79 ±0.17</td>
</tr>
<tr>
<td>Spleen cellularity (#x10⁷/spleen)</td>
<td>5.15 ± 0.70</td>
<td>4.93 ±0.37</td>
</tr>
<tr>
<td>Number of ESC</td>
<td>19.5 ± 7.2*</td>
<td>9.5 ± 3.5</td>
</tr>
<tr>
<td>Liver weight (g/100 g body wt.)</td>
<td>5.38 ± 0.68</td>
<td>5.01 ±0.36</td>
</tr>
<tr>
<td>Total protein concentration (mg/g tissue)</td>
<td>65.6 ±17.9**</td>
<td>42.6 ± 6.1</td>
</tr>
</tbody>
</table>

ESC: endogenous macroscopic colonies of haemopoietic tissue on the parietal surface of spleens. The values represent the mean ± S.E. of each group.
* Significant difference from the control, P< 0.05 or **P<0.01.

(Table 1). The rate of carbon clearance by the administration of F-I was shorter than that of the saline control group (4.23 min vs. 6.18 min, P< 0.05). This indicated that F-I possesses potentiating activity for the reticuloendothelial system. Schizophyllan shows enhancing activity for the rate of carbon clearance, which has been suggested to be related to its antitumor activity23.

Since macrophages have been suggested to play important roles in immunological surveillance, we studied the influence of the administration of F-I on the number of peritoneal exudate cells and the phagocytic activity of macrophages (Table 1). F-I extract at 100 mg/kg remarkably increased the number of peritoneal exudate cells compared with saline control (4.9 x 10⁶ vs. 2.9 x 10⁶ cells per mouse, P< 0.01). The ratio of macrophage occupied more than 50% of the peritoneal exudate cells (data not shown). The phagocytic activities were greatly enhanced as judged by the phagocytic ratio (15.65% vs. 4.70%, P< 0.01) and the phagocytic index (1.06 vs. 0.12, P<0.05). Soluble β-1,3-D-glucans activate the functions of peritoneal macro-phages such as lysosomal enzymes, phagocytic activity and H₂O₂ production. Scavenger functions are responsible for killing microorganisms and cancer cells *in vivo*19.

The spleen cellularity of mice treated with F-I extract was slightly enhanced, but no increase was observed in the spleen weight compared with saline control. An increased number of endogenous colonies of haemopoietic tissue cells was found on the parietal surface of spleens of mice receiving F-I compared to controls (Table 1). No
augmentation was detected in the liver weight of F-I treated group, but the liver total protein concentration was significantly higher (P<0.01) than that of saline control group (Table 1).

We measured the liver and spleen weights to elucidate whether i.p. administration of F-I was associated with toxicity signs like hepatosplenomegaly, reported in micro-particulate β-1,3-D-glucan given by the systemic route. No increase was observed in the F-I treated animals; indicating it to be a safe product. The hot-water extract from P. ostreatus mycelium had been assumed to be non-toxic, since the source organism is edible.

The protective component of F-I seems to be heat-stable, since prolonged extraction with boiling water was required to obtain the active compound(s) from the mycelium. The many positive properties suggest that there should be further study of our F-I extract. Both pharmacological and purification studies are needed.

LITERATURE CITED


