Indian Journal of Biochemistry & Biophysics
Vol. 46, April 2009, pp 161-165

Lentinula edodes (Shiitake) mushroom extract protects against hydrogen peroxide induced cytotoxicty in peripheral blood mononuclear cells

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Received 13 June 2008; revised 29 January 2009

Lentinula edodes (Berk) Pegler, commonly known as Shiitake mushroom has been used as medicinal food in Asian countries, especially in China and Japan and is believed to possess strong immunomodulatory property. In the present study, the methanolic extract of the fruit bodies of *L. edodes* was investigated for cytoprotective effect against H_2O_2-induced cytotoxicity in human peripheral blood mononuclear cells (PBMCs) by measuring the activities of xanthine oxidase (XO) and glutathione peroxidase (GPx) . H_2O_2 at a concentration of 5 μM caused 50% inhibition of PBMCs viability. The extract improved the PBMC viability and exerted a dose-dependent protection against H_2O_2-induced cytotoxicity. At 100 μg/ml of extract concentration, the cell viability increased by 60% compared with the PBMCs incubated with H_2O_2 alone. The extract also inhibited XO activity in PBMC, while showing moderate stimulatory effect on GPx. However, in the presence of H_2O_2 alone, both the enzyme activities were increased significantly. The GPx activity increased, possibly in response to the increased availability of H_2O_2 in the cell. When the cells were pretreated with the extract and washed (to remove the extract) prior to the addition of H_2O_2, the GPx and XO activities as well as the cell viability were comparable to those when incubated with the extract alone. Thus, it is suggested that one of the possible mechanisms *via* which *L. edodes* methanolic extract confers protection against H_2O_2-induced oxidative stress in PBMC is by inhibiting the superoxide-producing XO and increasing GPx activity which could rapidly inactivate H_2O_2.

Keywords: Peripheral blood mononuclear cells, *Lentinus edodes*, Oxidative stress, Cytotoxicity, Glutathione peroxidase, Xanthine oxidase

Lentinus edodes (Berk) Pegler commonly known as Shiitake has been used as medicinal food in Asian countries, especially China and Japan for centuries[1]. It possesses anti-hypertensive, cholesterol lowering, anti-viral (promotes interferon), anti-hepatitis anti-cancer and libido increasing properties. Lentinan, a metabolite from *L. edodes* has been reported to be effective in the treatment of liver, stomach, lung and ovarian cancers[2]. It shows a strong immunno modulatory effect and the use of crude extract is believed to confer a better synergistic effect[3].

The aetiology of numerous diseases is known to involve free radical induced damage[4]. Evidences indicate that the manifestation of disease complications involve both oxidative stress (free radical generation overwhelms the antioxidant defense) as well as the immune system[5,6]. Hydrogen peroxide (H_2O_2) is a common metabolite generated in the body during superoxide reduction. The superoxides which are generated by xanthine oxidase (XO) in the mononuclear cells play an important role in destroying engulfed microorganisms. When in excess, H_2O_2 is cytotoxic, as it is easily converted to the harmful hydroxyl radical. The cellular system is endowed with enzymic defense such as glutathione peroxidase (GPx) and catalase which play cooperative roles to reduce H_2O_2 to water[7].

L. edodes is commercially available in many forms. Although its medicinal properties including the immune-modulating properties have been extensively reported[8-10], the effects on the oxidant-antioxidant system in the immune cells are not known. Thus, in the present study, the cytoprotective effect of methanolic extract of *L. edodes* on H_2O_2 induced cytotoxicity has been assessed by measuring the activities of XO and GPx in human PBMCs exposed to H_2O_2

Materials and Methods

Materials

Fresh *L. edodes* was purchased from a local distributor. Sodium azide (NaN_3), glutathione reductase,

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β-NADPH, xanthine oxidase (XO), glutathione peroxidase (GPx), uricase, peroxidase, hypoxanthine, xylenol orange and trypan blue dye were from Sigma Chemicals (St. Louis, MO, USA). Ficoll (Histopaque ®-1077) was from Flowlab (Australia). All other chemicals used were of highest purity available.

Preparation of mushroom extract

The *L. edodes* fruit bodies were cut into small pieces and soaked in methanol for 48 h in a Schott bottle. The fruit bodies were removed and the methanolic extract was rotary evaporated to dryness under vacuum.

Collection of blood and isolation of peripheral blood mononuclear cells (PBMC)

Venous blood (10 ml) was drawn from healthy volunteer(s) after obtaining informed consent. The study was approved by the University Malaya Medical Centre Ethics Committee, complying with the declaration of Helsinki.

Human peripheral blood was collected in a sterile EDTA tube and separated according to previously described method[11]. The blood was layered on to Histopaque-1077 (media) in a sterile conical centrifuge tube at a ratio of 1:1 and centrifuged at 1500 × *g* for 30 min. The buffy coat containing the PBMCs was transferred into another tube, resuspended in phosphate buffer saline (PBS; pH 7.4, 0.1 M, 0.9% NaCl) and centrifuged at 1200 × *g* for 10 min to wash the cells. The washing procedure was repeated twice and the PBMCs were then resuspended in PBS and the approximate cell number was estimated using trypan blue dye exclusion method.

***L. edodes* effect on H_2O_2 induced cytotoxicity**

The PBMCs were incubated for 30 min with various concentrations of H_2O_2 in a 96-well flat bottom tissue culture plate at a density of 1×10^4 cells/100 µl/ well and the cell viability was assessed using trypan blue dye exclusion assay. The concentration of H_2O_2 that caused 50% inhibition of PBMC proliferation was deduced. The PBMCs were incubated with various concentrations of the *L. edodes* extract (solubilized in DMSO) in the presence or absence of H_2O_2 to assess the cytoprotective effect of the extract against H_2O_2-induced cytotoxicity. The final concentration of DMSO in the incubation was less than 1% and at this concentration, DMSO did not affect the PBMC viability (result not shown).

Preparation of PBMCs for assessment of GPx, XO and H_2O_2 levels

To assess the effects of H_2O_2, and the mushroom extract on the oxidant-antioxidant enzymes, as well as the endogenous H_2O_2 level, the freshly isolated PBMCs (density of 1×10^5 cells/ml) were placed in culture plates and incubated with the extract (100 µg/ml) in the presence or absence of H_2O_2 for 30 min or 4 h. The control incubation contained PBMC, PBS and 1% DMSO (solvent for the extract). In another set of experiment, the PBMCs were pre-incubated with the mushroom extract for 30 min, the cells were washed twice with PBS, H_2O_2 was added and further incubated for 30 min or 4 h. At the end of the appropriate incubation periods, the cells were washed twice and resuspended in PBS and the viable cell number was estimated as mentioned earlier. The resuspended cells were sonicated (10 s pulse, on ice), centrifuged at 13000 × *g* for 15 min. The resulting supernatant (cell lysate) was used to estimate the XO, GPx and H_2O_2 levels. At least six sets of incubation were carried out and the cells from replicate incubations were pooled to yield sufficient number of cells (one million) prior to the above mentioned procedure, so that the enzyme activities and the H_2O_2 levels in the lysate were within detectable limit.

The GPx activity of PBMC lysates was determined[12] using a coupled-enzymatic assay in which oxidation of NADPH was monitored spectrophotometrically at 340 nm. XO activity was determined based on the oxidation of the chromogen ABTS [2,2'-azino-di(3-ethylbenzthiazoline)] through coupled reactions catalyzed by uricase and peroxidase[13]. H_2O_2 was measured using ferrous ion oxidative xylenol orange version-2 (FOX-2)[14]. This assay is based on the oxidation of Fe(II) to Fe(III) ions which then binds to xylenol orange and the resulting complex can be monitored spectrophotometrically at 560 nm.

Results and Discussion

Preliminary studies showed that H_2O_2 induced toxicity (inhibited the PBMC proliferation) in a dose-dependent manner and the IC_{50} (concentration that caused 50% inhibition) was approximately 5 µM (final concentration) (result not shown). In contrast, other reports have shown that a higher concentration of H_2O_2 is required to induce toxicity in PBMCs[15,16]. One plausible explanation for this discrepancy was the low cell density (1×10^5 cells/ml) used and the incubations carried out in PBS, instead of culture media, in our present study. Culture media in itself was found to be able to reduce H_2O_2, thus requiring a higher dose of H_2O_2 for induction of PBMC toxicity (result not shown).

Lentinus edodes exerted a dose-dependent protection against H_2O_2 (5 µM) induced cytotoxicity and the maximum dose tested (100 µg/ml) showed approx 60% increase of positive control (cell proliferation in the presence of 5 µM H_2O_2 (Fig. 1). A higher dose of mushroom extract was not tested due to its poor solubility in the aqueous buffer system. It was noteworthy that in the course of this study, PBMCs from different volunteers (healthy individuals) were observed to have variations in susceptibility to H_2O_2-induced oxidative stress or toxicity. Similar observations were reported on H_2O_2-induced DNA damage of human lymphocytes[17]. Therefore in the present study, only PBMCs isolated from a single volunteer was used for each set of experiments to enable standardization and reasonable interpretation of results.

Fig. 2 shows the viability of PBMC incubated in PBS (without the culture medium) after 30 min and 4 h. The control cells showed reduced viability after 4 h, whereas the cells incubated with *L. edodes* extract showed an increased viability after 30 min, which reduced significantly after 4 h and this was comparable to the control (untreated cells) incubation. When the cells were incubated with H_2O_2 (5 µM), approx 50% of the cells were viable at 30 min and 4 h incubation (compared with the respective untreated, control incubations).

L. edodes extract prevented H_2O_2-induced cell death significantly and the effect was not significant after 30 min, but was significant after 4 h (30%). Interestingly, PBMCs preincubated with the extract for 30 min, washed (to remove the extract) and then the H_2O_2 added to induce cytotoxicity, showed significant viability which was comparable to the untreated control cells (at 4 h incubation but not at 30 min) (Fig. 2). Our preliminary studies showed that the cells preincubated with the extract, washed and then incubated without any addition of H_2O_2 had viability similar to the control (result not shown). Therefore, in the subsequent experiments, only the untreated cells were maintained as the control as depicted in Figs 2-3.

H_2O_2 is produced during normal aerobic cell metabolism. In particular, it serves as a potential source for HO•, one of the most potent radicals through the Fenton reaction in the presence of transition metal ions[18]. Addition of exogenous H_2O_2 into the cell culture is cytotoxic to most bacterial and plant cells[19]. H_2O_2 level in the human cells can serve as an indicator for the degree of oxidative stress *in vivo*[14]. In the present study, GPx activity in the cells, incubated with H_2O_2 and *L. edodes* was upregulated in response to the exogenous H_2O_2, where the increase in GPx activity was 4-fold higher at 4 h compared to 30 min incubation (Fig. 3A and B). When the cells were exposed to H_2O_2 alone, GPx activity was not increased at 30 min incubation, but elevated significantly at 4 h incubation, implicating the presence of endogenous antioxidant response.

The GPx activity in the cells increased significantly, when *L. edodes* extract and H_2O_2 were present concurrently (Figs 3A and B). The elevated GPx could

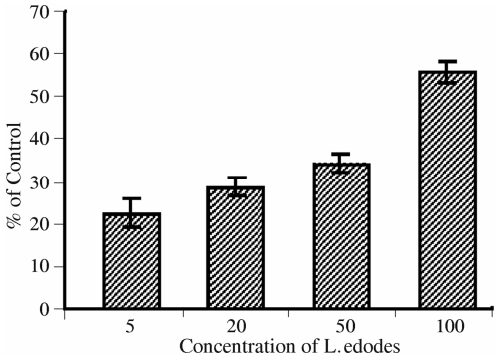

Fig. 1—Dose-response effect of *L. edodes* on H_2O_2 induced cytotoxicity in PBMCs [PBMCs at a density of 1×10^4 cells/100µl were incubated with H_2O_2 (5 µM) and various concentrations of *L. edodes* (5-100 µg/ml) for 30 min. The control incubation contained PBMCs and H_2O_2 (5 µM). The cell viability for various incubations was determined as described in the 'Materials and Methods'. Percentage increase in viability for PBMC incubations in presence of *L. edodes* compared with the control was calculated. Results expressed as mean ± SD of triplicate assays]

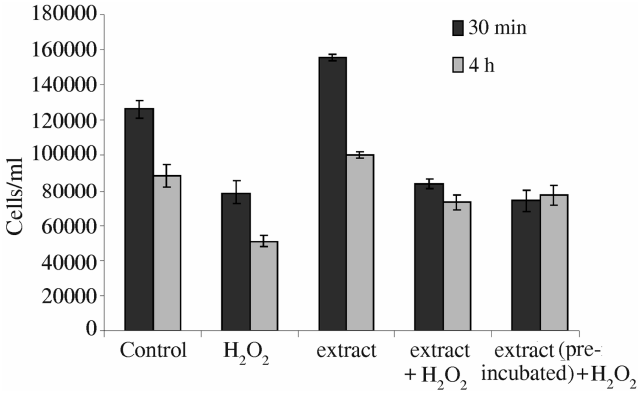

Fig. 2—PBMC viability in response to H_2O_2 and *L. edodes* [PBMCs were incubated at a density of 1×10^5 cells/ ml for 30 min or 4 h with H_2O_2 (5.0 µM), *L. edodes* extract alone, *L. edodes* extract and H_2O_2 (5.0 µM) as well as H_2O_2 (5.0 µM) added after an initial 30 min preincubation with the extract (as described in the 'Materials and Methods'). The control incubation contained untreated PBMCs. The Y-axis indicates the cell number ± S.E. of triplicate assays]

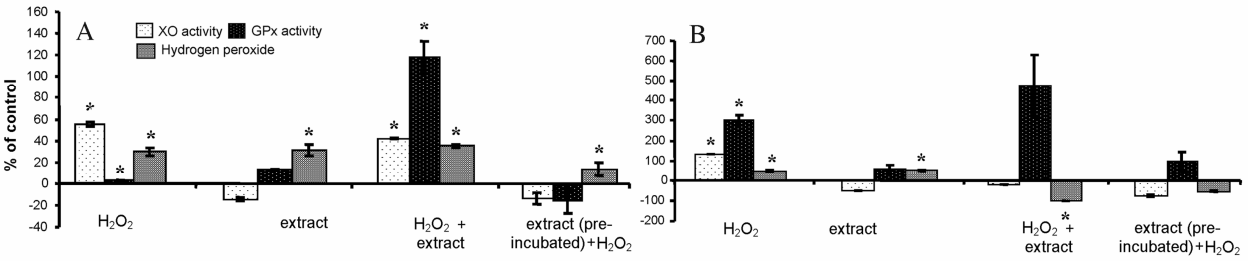

Fig. 3—Effects of *L. edodes* extract and H_2O_2 on GPx and XO activities and H_2O_2 level in PBMCs after 30 min incubation (A) and after 4 h (B) [The PBMCs (1×10^5 cells/ml) were incubated with various substances [H_2O_2 (5 μM) and *L. edodes* (100 μg/ml)] for 30 min (A) or 4 h (B) at 37^0C in 5% CO_2 incubator. The cell number was estimated using trypan blue dye exclusion method. The cell lysate was prepared as described in the 'Materials and Methods'. The various activities were calculated for one million cells and expressed as percentage of control (the corresponding activity in the cell lysate obtained from untreated PBMCs) ± S.E. The data presented were a single representation of at least 3 separate experiments carried out in triplicates. Significance of data was calculated using one sample t-test (*p< 0.05)]

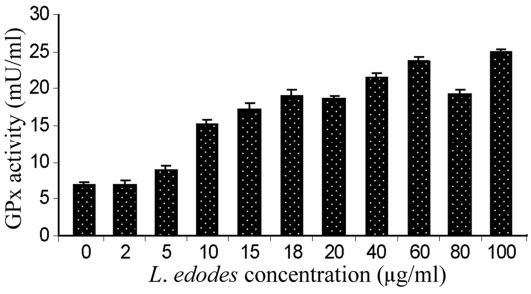

Fig. 4—Effect of *L. edodes* on GPx activity [Purified commercial GPx activity was determined in presence of *L. edodes* extract (0-100 μg/ml) to estimate the direct effect of the extract on GPx activity. Results expressed as mean ± SD of triplicate assays]

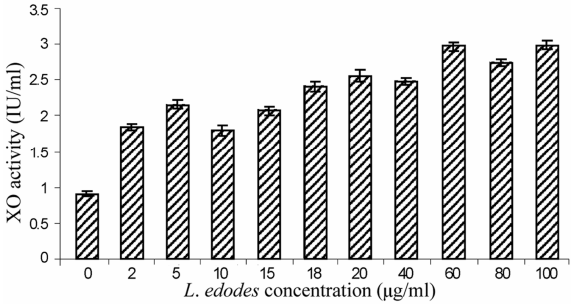

Fig. 5—Effect of *L. edodes* on XO activity [Purified commercial XO activity was determined in presence of *L. edodes* extract (0-100 μg/ml) to assess the direct effect of the extract on XO activity. Results expressed as mean ± SD of triplicate assays]

possibly utilize the H_2O_2 rapidly, which is reflected by the attenuation of endogenous H_2O_2 level (Figs 3A and B). This effect was observed even when the cells were pre-incubated with *L. edodes* extract only for 30 min (then removed by washing procedure), prior to the exposure to H_2O_2. *L. edodes* extract was able to amplify/sensitize the upregulation of the GPx upon the latent exposure to H_2O_2. This hypothesis was in agreement with our earlier study, which suggested that the normal PBMCs could handle the exogenous free radicals or oxidative stress by upregulating the antioxidant enzyme activities[20]. Conversely, GPx activity in the cells, incubated with *L. edodes* alone was insignificant at 30 min incubation, but showed approx 100% increase (compared with activity in untreated cells) at 4 h incubation. This could possibly be due to the direct stimulatory effect of extract on the GPx activity (Fig. 4).

L. edodes contains selenium as one of its active components[21] which exerts direct stimulatory effect on blood GPx activity (Kuppusamy et al., unpublished data). Our earlier study with trace elements demonstrated that antioxidant enzymes were inhibited or downregulated when the cells were proliferating and not under oxidative stress, but upregulated when subjected to oxidative stress[20]. However, the mechanisms that regulate the cellular changes of antioxidant enzyme activities in response to oxidative stress are still unclear and require further investigation.

XO is one of the major free radical producing enzymes. It can form superoxide in the body during the oxidation of hypoxanthine to xanthine and is believed to be involved in metabolic regulation and control[13]. The superoxide radicals derived from XO can be converted to H_2O_2 by SOD, which give rise to hydroxyl radicals[13]. Measurement of XO activity is difficult, due to its short half-life and low concentration in mononuclear cells of human blood[22]. However, the results in this study indicated XO activity was higher in the cells that were exposed to H_2O_2 (5 μM) both at 30 min and 4 h, but the activity was suppressed in the presence of *L. edodes* extract (Figs 3A and B). Since the extract exerted stimulatory effect on commercial XO activity (Fig. 5), the possible direct effect of the extract on XO was ruled out.

It was plausible that XO activity was downregulated when the cell viability was good (the cells were not under oxidative stress) and only upregulated in the presence of oxidative stress stimulus. Even the short duration of PBMC pre-exposure to *L. edodes* extract was sufficient to induce the protective mechanism against H_2O_2-induced oxidative stress/toxicity (Figs 2, 3A and B). We have previously reported that blood XO is elevated in oxidative stress conditions such as in diabetes[23].

In conclusion, the cells pre-incubated or incubated with the *L. edodes* methanolic extract exhibited protective effect when exposed to H_2O_2 at longer incubation period (4 h) and not at shorter period (30 min). The results clearly demonstrated that the extract used was able to protect human mononuclear cells from oxidative stress by sensitizing the H_2O_2 inactivating enzyme GPx and suppressing the superoxide-producing XO. The possible use of this extract to attenuate oxidative stress conditions *in vivo* deserves further investigation.

Acknowledgement

The work was supported by the University Malaya grant (BM 27) and IRPA grant awarded by the Ministry of Science, Technology and Environment, Malaysia (IRPA 01-02-03-1001).

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