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Protective effect of polysaccharide from the loach on the *in vitro* and *in vivo* peroxidative damage of hepatocyte

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Abstract

Misgurnus anguillicaudatus polysaccharide (MAP), a type of natural neutral polysaccharide occurring in the mucus of *Misgurnus anguillicaudatus C.*, was studied for antioxidant bioactivity. The preliminary research showed that MAP was able to remove $O_2^{\cdot-}$, $HO^{\cdot}$, H_2O_2 and other active compounds of oxygen and significantly protected DNA chains from being damaged by hydroxyl radicals. This antioxidant bioactivity was further evaluated using the model of peroxide-induced oxidative injury in rat primary hepatocytes and hepatotoxity in mice. The results demonstrated that MAP, at the concentrations of 0.10 ~ 0.40 mg/ml, significantly increased the activity of SOD and GSH-px, but decreased the consumption of reduced glutathione and the formation of malondialdehyde induced by a 30-min treatment of H_2O_2 (2.0 mmol/L). The in vivo investigation showed that oral pretreatment of MAP (100, 200 and 300 mg/kg) for 7 days before a single dose of t-BHP (0.2 mmol/kg, ip) significantly lowered the serum levels of hepatic enzyme markers (alanine and aspartate aminotransferase) and reduced oxidative liver injury. The histopathological evaluation of the liver revealed that MAP reduced the incidence of liver lesions including inflammatory, leukocyte infiltration, and necrosis induced by t-BHP in mice. Based on the results described above, a conclusion can be drown that MAP may play an important role in the prevention of oxidative damage in living systems.

Keywords: Misgurnus anguillicaudatus polysaccharide; loach; liver injury; lipid peroxidation; hepatoxicity

1. Introduction

The mucus coat of fish skin contains a variety of secretions from epidermal goblet cells and epithelial cells. These secretions have been implicated in many important biological functions [1]. The loach (*Misgurnus anguillicaudatus*) has long been employed as traditional Chinese medicine in folk remedies for the treatment of hepatitises, osteomyeitis, carbuncles, inflammations and cancers [2]. Some vertebrate lectins were purified from the skin mucus or egg of the loach (*Misgurnus anguillicaudatus*) and found to induce release of cytotoxin from fresh murine bone narrow cells or macrophages and lyse tumor cells but not normal spleen cells [3–6]. A novel antimicrobial peptide named misgurin, which consists of 21 amino acid residues, from the loach was isolated and identified [7]. The carbohydrate compositions of the skin mucus of several species of fish was examined and found that the sialic acids in loach consisted predominantly of 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN). A deaminated neuraminic acid-containing glycoprotein from the skin mucus of the loach, *Misgurnus anguillicaudatus*, was isolated and characterized [8].

Misgurnus anguillicaudatus polysaccharide (MAP), a kind of neutral free polysaccharide, was extracted from the mucus of the loach (*Misgurnus anguillicaudatus*). The preliminary research showed that MAP was able to remove $O_2^{\cdot-}$, $HO^{\cdot}$, H_2O_2 and other active compounds of oxygen and significantly protected DNA chains from being damaged by hydroxyl radicals [9]. The present study was carried to examine whether MAP could protect the rat hepatocytes against the oxidative damage induced by hydroperoxide *in vitro*. The protective effect of MAP against *in vivo* liver injury caused by t- BHP was also investigated.

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List of Abbreviations: ALT = alanine aminotransferase; AST = aspartate aminotransferase; t-BHP = *tert;* butyl hydroperoxide; GPC = gel permeate chromatography; GSH = reduced glutathione; GSH-Px = glutathione peroxidase; LDH = lactate dehydrogenase. MAP = Misgurnus anguillicaudatus polysaccharide; MDA = malondialdehyde; OPT = *o*-phthaldialdehyde; SOD = superoxide dimutase.

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2. Materials and methods

2.1. Chemicals

Thiobarbituric acid (TBA), tert-butyl hydroperoxide (t-BHP), glutathione (GSH), o-phthalaldehyde (OPT), Sephadex G-100 and 5,5′-dithiohydroxyl-2,2′-dinitrobenzoxic acid (DTNB) were purchased from Sigma Co. (St Louis, MO, USA). 1,1,3,3-tetraethoxylpropane (TEP) and pyrogallic acid (PA) were supplied by Fluka Chemical Co. Pullulan standards (P-112000, P-47300, P-22800, P-11800, P-5900, P-2700) were bought from Japan. Kits for aianine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were obtained from Institute of Jiancheng Bioengineering (Nanjing, P. R. China). Medium for cell culture and other reagents or dishes were ordered from the Chinese suppliers.

2.2. Preparation of MAP

2.5 kg of live loaches (*Misgurnus anguillicaudatus*, weight 8 ± 1.5 g, length 8.5 ± 2 cm, obtained from the market in Wuhan.) were first immersed in 2.5 L of clean tap-water and aerated for 24 hr at room temperature. Then, after being supersonicated for 1 hr on an ultrasonic shaker (model SB2200, Shanghai, China), the loaches were removed by filtration. The supersonication and filtration are not fatal. The extract was centrifugated at 0°C and 8,000 rpm for 10 min in a centrifugal machine (model RS-2 III, Tomy Seiko Co., Tokyo, Japan), and subsequently concentrated to 1/3 volume in vacuum. The residue was extracted with 50 ml/time Sevag reagent, $CHCl_3$-^{n}BuOH (V/V = 4:1) 3 times to deproteinize. After removing the Sevag reagent, 4 times the volume of absolute alcohol was added to the water phase and kept at 4°C overnight in a refrigerator to precipitate carbohydrate compounds. After filtering with a Buchner funnel in vacuum and washing with absolute alcohol, the sediment was frozen at -79.5°C overnight in a super low-temperature freezer (NUAIRE, Japan), and lyophilized to obtain 7.5 g of white powder preparation. Qualitative tests show that this sediment preparation containing free reductive polysaccharides. It had color-reaction with phenols in sulfuric acid or with 3,5-dinitrosalicylic acid, but no color-reaction with ninhydrin or biuret reagent.

A portion of the dried sediment preparation (2 g) was homogenized with 100 ml of hot distilled water, and after adding to it 50 ml of anhydrous ethanol, put into a refrigerator and kept at 4°C overnight. The MAP sediment was separated by centrifugation at 10,000 rpm, washed with anhydrous ethanol for 3 times frozen and lyophilized to yield fine MAP (300 mg). The MAP was further purified on a Sephadex G-100 gel column (1×50 cm), using HAc-NaAc buffer solution (pH 5) as eluent at the mobile rate of 0.1 ml/min. The fractions were detected by the phenol-sulfuric acid method. The eluting curve gave a single peak and suggested that MAP was purified. The content of total sugar was 95.7%.

2.3. Homogeneity and molecular weight of MAP

The homogeneity and molecular weight of MAP were determined by GPC with a Waters HPLC apparatus equipped with a TSK G-3000 SW column (300 mm × 7.5 mm), a model 410 detector and a Millennium-32 Workstation was used for the calculation of molecular weights. The Pullulan standards (P-112000, P-47300, P-22800, P-11800, P-5900, P-2700) were used for the calibration curve. The detailed experimental conditions are column temperature: 21°C (column temperature auto-control system); column pressure: 5 Mpa (model 600 pump); injection volume: 50.00 μl; sampling volume: 20 μl; Mobile phase: HAc-NaAc buffer solution (PH 5); mobile rate: 1.0 ml/min; run time: 40 min. The chromatography gave a single sharp peak and showed that the average molecular weight of MAP was 130.25 kDa.

2.4. Preparation of hepatocyte

SPF grade Wistar rats (280 ± 5 g) (Hubei Provincial Center of Medical Experiment, P. R. China) were used for the experiments. Hepatocytes were prepared by two-stage collagenase perfusion [10,11] and cultured in Williams E medium supplemented with antibiotic mixture of penicillin, streptomycin and neomycin (1%), glutamine (1%) and fetal calf serum (10%), and gassed with O_2/CO_2 (95%/5%). Cells were plated out at a density of 1×10^6 cells/60 mm dish, and treated with chemicals as indicated in the following assay 3 hr after attachment.

2.5. Microculture tetrazolium (MTT) assay

The range of non-toxic dose levels of MAP was established using the MTT assay with some modification [12]. Briefly, to the primary cultured rat hepatocytes at a density of 5×10^4 cells/dish, MAP at various concentrations of 0.01, 0.05, 0.10, 0.20 and 0.60 mg/ml were added. After a 24-hr incubation, medium was replaced by the one containing 20 μl MTT (5.0 mg/ml) with PBS washing in between. The cells were incubated for another 4 hr, then the blue crystals which are the metabolized product of MTT were extracted by isopropanol. Absorbance at 563 nm was determined and used for the measurement of the proportion of surviving cells.

2.6. GSH, GSH-Px and SOD assay

A small portion of rat liver was removed for the GSH assay by the method of Hissin and Hilf [13]. In brief, the tissue was homogenized with phosphate buffer containing 25% m-phosphoric acid (pH 8.0). After centrifugation (100,000 g, 30 min), OPT was added to the supernatant and

incubated for 15 min at room temperature. Fluorescence at 420 nm was determined with the excitation at 350 nm. The results were expressed as μg GSH per g liver tissue. GSH-Px activities were detected with DTNB [14]. The activities of SOD were analyzed by the auto-oxidation of pyrogallol [15].

2.7. Lipid peroxidation assay

After pretreatment with MAP for 0.5 hr at the final concentration of 0.01, 0.05, 0.10, 0.20, 0.30 and 0.40 mg/ml, hepatocytes were incubated with H_2O_2 (2.0 mmol/L) for 0.5 hr [16]. Next, 1 ml culture medium was removed, and the lipid peroxidation product (MDA) was assayed according to an improved thiobarbituric acid fluorometric method at 553 nm with excitation at 515 nm using 1,1,3,3-tetramethoxypropane as the standard [17,18]. The protein content was determined using a standard commercial kit (Bio-Rad Lab. Ltd) with bovine serum albumin as a standard. The results were expressed as MDA formation per mg protein.

2.8. Cytotoxicity assay of hepatocytes

Hepatocytes were pretreated with MAP, and then with H_2O_2 (2.0 mmol/L) as described above, and the activities of LDH were analyzed following the procedures provided by the commercial kits (Sigma Chemical Co.). The cytotoxicity of hepatocytes is expressed in terms of the activity of LDH released from the treated cells.

2.9. Animal treatment

SPF grade Male Kunming mice (20 $\pm$ 5 g) were used for the experiments. The rats were provided with food and water *ad lib* and divided into six groups (eight rats/group). To study the protective effect against the t-BHP-induced hepatotoxicity, MAP (50, 100, 200, 300 mg/kg) was given daily by gavage to the animals for 6 consecutive days. On day 7, t-BHP (0.2 mmol/kg) was injected (ip) to each animal, and 18 hr later the mice were killed by decapitation and the blood samples were collected for the assay of ALT and AST. The livers were excised from the animals and assayed for GSH level, MDA production and pathological histology according to the procedures described below.

2.10. Hepatotoxicity assessment

Hepatic enzymes, ALT and AST were used as the biochemical markers for the early acute hepatic damage. The serum activities of ALT and AST were determined by the colorimetric method of Reitman and Frankel [19]. The contents of GSH and MDA production in the mouse livers were analyzed by the methods as described above.

Table 1 Cytotoxisity of MAP analyzed by microculture tetrazolium assay in the rat hepatocytes

$\bar{x} \pm s$, n = 3

Treatment[a]	A_{563nm}	% of control absorbance
Control	0.825 ± 0.09	100
MAP (gL^{-1})		
0.01	0.832 ± 0.11	101
0.05	0.864 ± 0.10	105
0.10	0.957 ± 0.07	116
0.20	0.969 ± 0.09	118
0.40	0.838 ± 0.10	102
0.60	0.806 ± 0.13	98

[a] Hepatocyte culture was treated with various concentrations of MAP for 24 hr.

2.11. Pathological histology

Immediately after removal from the animals, hepatic tissues were fixed in 10% buffered formaldehyde, processed for histological examination according to the conventional methods and stained with hematoxlin and eosin (HE). The morphology of any lesions observed was classified and registered.

2.12. Statistical analysis

The data were reported as means$\pm$standard deviations from repeated determinations, and evaluated with the analysis of Student's *t-test.* Differences were considered to be statistically significant if $P < 0.05$.

3. Results

3.1. Cytotoxicity of MAP

Using the MTT assay for cytotoxicity it was shown that MAP, up to 0.60 mg/ml concentration, exhibited no toxic effect on the primary culture of rat hepatocytes (Table 1). Therefore concentrations of MAP below 0.60 mg/ml were used for the following cell culture experiments *in vitro.*

3.2. Effect of MAP on the endocytes in the injurious rat hepatocytes induced by H_2O_2

Compared with the normal control group, treatment with 2.0 mM H_2O_2 for 30 min alone significantly decreased the activity of SOD and GSH-Px, and the content of GSH in the rat hepatocytes. The Pretreatment with MAP (0.1~0.4 mg/ml) effectively and dose-dependently inhibited the decrease of these indexes caused by H_2O_2 ($P < 0.01$, Table 2). The data indicated that MAP exhibited a protective effect to the activity of SOD and GSH-Px, and the content of GSH in the primary rat hepatocytes.

Table 2 Effect of MAP on hydroperoxide-induced lipid peroxidation and cytotoxicity in the rat primary hepatocytes $\bar{x} \pm s$, n = 3

Treatment	SOD/kUg^{-1}Pro	GSH/mmolg^{-1}Pro	GSH-Px/kUg^{-1}Pro	MDA/μmolg^{-1}Pro	LDH/mU10^{-6}
Control	4.16 ± 1.08	1.74 ± 0.50	8.90 ± 1.29	2.10 ± 0.11	75.1 ± 11.4
H_2O_2 (2.0 mM)	2.32 ± 0.81[b]	1.15 ± 0.30[b]	5.58 ± 1.45[b]	4.56 ± 1.34[b]	286.3 ± 14.6[b]
MAP (gL^{-1})[a]					
0.01	2.51 ± 0.57	1.27 ± 0.45	6.72 ± 1.30	3.54 ± 1.00	272.8 ± 16.3
0.05	2.75 ± 0.87[c]	1.35 ± 0.51	7.31 ± 1.54[c]	3.38 ± 1.03	264.5 ± 19.2
0.10	3.08 ± 1.25[d]	1.47 ± 0.66[c]	7.33 ± 1.50[c]	3.14 ± 0.75[c]	257.4 ± 15.8[c]
0.20	3.27 ± 0.92[d]	1.58 ± 0.50[d]	7.70 ± 1.62[d]	2.79 ± 0.85[d]	226.7 ± 10.3[c]
0.30	3.42 ± 1.05[d]	1.63 ± 0.62[d]	7.98 ± 1.71[d]	2.65 ± 0.67[d]	209.5 ± 9.7[d]
0.40	3.16 ± 0.93[d]	1.55 ± 0.54[c]	7.69 ± 1.24[d]	2.70 ± 0.78[d]	189.2 ± 10.9[d]

[a] Primary hepatocytes were pretreated with various concentrations of MAP before the addition of hydroperoxide as described in Materials and Methods.
[b] $P < 0.01$, compared with control
[c] $P < 0.05$, compared with the sample treated with hydroperoxide alone.
[d] $P < 0.01$, compared with the sample treated with hydroperoxide alone.

3.3. Effect of MAP on the peroxidative hepatocyte damage induced by H_2O_2

Lipid peroxidation has been recognized as a potential mechanism of cell injury. The MDA production, a main index of peroxidation, was increased in the hepatocytes treated with 2.0 mM H_2O_2 for 30 min alone. Pretreatment with MAP (0.1∼0.4 mg/ml) significantly inhibited the formation of MDA in the injurious rat hepatocytes caused by H_2O_2 ($P < 0.01$, Table 2). The data suggested that the administration of MAP resulted in an effective inhibition of the H_2O_2-induced lipid peroxidation.

3.4. Effect of MAP on cytotoxicity induced by hydroperoxide

Adding MAP to the primary cultured hepatocytes effectively prevented the cells from the cytotoxity induced by hydroperoxide as expressed by the leakage of LDH (Table 2). The pretreatment of MAP significantly avoided the LDH leakage of hepatocytes induced by hydroperoxide (2.0 mM; for 30 min) at the concentration of 0.1∼0.4 mg/ml ($P < 0.05$).

3.5. Effect of MAP on t-BHP-induced in vivo hepatotoxicity

Several hepatic enzymes, such as ALT and AST, were used as the biochemical markers for the early acute hepatic damage. When a single dose of t-BHP was given to mice by ip injection, it caused an elevation of serum ALT and AST (Table 3), and an increased production of MDA in liver (Table 3). The acute hepatotoxicity reaction was significantly ($P < 0.05$) suppressed in all the animals pretreated with 100, 200 and 300 mg/kg MAP (Table 3). As the oxidative stress of tissue generally involves the GSH of each group and found that the t-BHP-treated group revealed a reduced GSH level in the liver, though not significantly. However, the groups with MAP pretreatment presented a decreased effect of t-BHP. The GSH level in the groups treated with 200 and 300 mg/kg MAP were significantly higher than that in the group treated with t-BHP alone (Table 3).

3.6. Pathological histology of the mouse livers

The treatment of t-BHP caused neutrophil infiltration, swelling of liver cells and necrosis in mice livers. However,

Table 3 Effects of MAP on serum enzymes and hepatic GSH and MDA in mice treated with tert-butyl hydroperoxide $\bar{x} \pm s$, n = 8

Group	ALT/IUL^{-1}	AST/IUL^{-1}	GSH/μgg^{-1} liver	MDA/nmolg^{-1} liver
Normal control	49 ± 4	95 ± 18	1559 ± 254	105 ± 17
t-BHP (0.2 mmol/kg)	132 ± 76[b]	1347 ± 228	187 ± 32[b]	
MAP[a] (mg/kg)				
50	124 ± 54	265 ± 92	1425 ± 203	173 ± 19
100	118 ± 31[c]	232 ± 67[c]	1612 ± 247	142 ± 14[c]
200	105 ± 29[c]	219 ± 59[c]	1704 ± 219[c]	133 ± 12[c]
300	95 ± 23[d]	185 ± 74[d]	1786 ± 255[c]	125 ± 15[d]

[a] Mice were pretreated with various concentrations of MAP by gastric tube for 7 consecutive days before the administration of t-BHP. The animals were killed 18 hr later. Then, the alanine and aspartate aminotransferase (ALT and AST) in serum, and the malondialdehyde (MDA) and reduced glutathione (GSH) in heptic tissue were determined.
[b] $P < 0.01$, compared with normal control group.
[c] $P < 0.05$, compared with the group treated with t-BHP alone.
[d] $P < 0.01$, compared with the group treated with t-BHP alone.

MAP (50 mg/kg) pretreatment reduced 65% of the incidence of the liver lesions. The histological examination showed that the groups pretreated with 100, 200 and 300 mg/kg MAP exhibited no hepatotoxicity.

4. Discussion

Recently, much attention has focus on the protective biochemical function of naturally occurring polysaccharides in biological systems, and on the mechanisms of their action. Polysaccharides, which are widely distributed in animals, plants and fungi, were considered to play an important role as dietary radical scavenger for the prevention of oxidative damage in living systems [20,21,22]. *Misgurnus anguillicaudatus* polysaccharide (MAP), a kind of neutral free polysaccharide, was extracted from the mucus of the loach (*Misgurnus anguillicaudatus*) and found to scarvenge reactive oxygen species and to protect DNA chains [9]. The protective effects of MAP on the *in vitro* H_2O_2-induced cytotoxicity in rat hepatocytes and against the *in vivo* mice liver injury caused by t-BHP were examined in the present study. The results showed that MAP could prevent the hepatocytes from the oxidative damage induced by hydroperoxide *in vitro* and by t-BHP *in vivo.*

The previous research works have shown that hydroperoxide could be metabolized into free radicals by cytochrome P450 (in hepatocytes) or hemoglobin (in erythrocytes), which can subsequently initiate lipid peroxidation, affect cell integrity and form covalent bonds with cellular molecules resulting in cell damage [23]. Because it may interact chemically with iron in or released from P450, or other sources of iron like hemoglobin, etc, leading to the formation of hydroxyl radical. H_2O_2 and t-BHP caused production of MDA and leakage of LDH and ALT in hepatocyte cultures [11,18]. They also mediated DNA base injury in mammalian cells [24]. These phenomena are similar to the oxidative stress occurring in cell and/or tissue. Oxidative stress is considered to play a prominent role in the causation of many diseases such as inflammation, aging and cancer [25–29].

As the integrity of cellular membranes is critical for normal cell function, the peroxidative decomposition of membrane lipids is an implication of chemical-induced toxicity. In the present study, H_2O_2 and t-BHP were shown to enhance lipid peroxidation in both cultured cell and animal systems. This could be due to the cytochrome P450-mediated metabolism of hydroperoxides to active hydroxyl or alkoxyl radicals that initiated lipid peroxidation and resulted in liver damage. The results of this study showed that MAP exhibited protective effects on hepatocytes and liver from the oxidative damage caused by hydroperoxides.

It is well established that reduced glutathione (GSH), the most important biomolecule protecting against chemically caused cytotoxicity, can participate in the elimination of reactive intermediates by conjugation and hydroperoxide reduction, or of free radicals by direct quenching.

The relationship of GSH content to the extent of hepar injury has been demoastrated in the experiments that the content of hepatic GSH was altered by toxin treatment. There are two distinct pathways of hydroperoxides metabolism, one is via cytochrome P450, and the other is by GSH-Px [23,30,31]. Our in vivo study showed that t-BHP reduced GSH levels in mouse livers, and a high dose of MAP blocked the phenomenon effectively. However, this mode of action needs further probe.

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