

Hepatoprotective Effects of Paramylon, a β -1, 3-D-Glucan Isolated from *Euglena gracilis* Z, on Acute Liver Injury Induced by Carbon Tetrachloride in Rats

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ABSTRACT. Paramylon is a β -(1-3)-D-glucan isolated from *Euglena gracilis* Z. This study was designed to evaluate the protective effects of paramylon on liver injury induced by carbon tetrachloride (CCl₄) in rats. Wistar stain male rats were orally administered paramylon (500, 1,000 and 2,000 mg/kg body weight) before treatment with a single intraperitoneal dose of 50% CCl₄ (2 ml/kg body weight). The rats were sacrificed 24 hr later, and blood samples were collected for assay of serum biochemical parameters. The livers were excised to evaluate the activity of antioxidant enzymes. Histopathological examination of the livers was also performed. The results showed that the treatment of paramylon prevented elevation of the serum levels of hepatic enzyme markers and inhibited fatty degeneration and hepatic necrosis induced by CCl₄. Pre-administration of paramylon reduced the liver apoptotic index. The treatment of paramylon recovered reductions of activity of hepatic superoxide dismutase, catalase and glutathione peroxidase induced by CCl₄. These results demonstrate that paramylon exhibits protective action on acute hepatic injury induced by CCl₄ via an antioxidative mechanism. To the best of our knowledge, this is the first report of a hepatoprotective effect based on the antioxidative action of paramylon.

KEY WORDS: antioxidation, carbon tetrachloride, hepatoprotection, liver injury, paramylon.

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Paramylon is a β -(1-3)-D-glucan isolated from *Euglena gracilis* Z [7]. The yield of paramylon from *Euglena gracilis* Z amounts to approximately 60-70% of the dried cells. Native paramylon shows cytokine-related immunopotentiating activity [18]. Sulfated derivatives of paramylon and N, N-dimethylaminoethyl paramylon exhibit anti-human immunodeficiency virus (HIV) and antimicrobial effects [17, 29]. To the best of our knowledge, there has been no research reported on the antioxidant effect of paramylon.

β -glucans are structurally complex homopolymers of glucose found in the cell walls of fungi and cereal plants. Their beneficial effects on the immune system and the lack of toxic or adverse effects has focused studies on β -glucan molecules [38]. β -glucans are acknowledged to be one of the immune response modifiers [6]. Studies conducted over the past decade have shown that β -glucans inhibit tumor development [28, 38], enhance defense against bacterial infection [15, 24], activate macrophages [8], induce production of cytokines [10, 34], nitric oxide (NO) and arachidonic acid metabolites [14, 21], increase hematopoiesis [35], exert radioprotective effects [12], are effective against burns [9, 37], improve wound healing by inducing the macrophage release of wound growth factors [40] and lower serum lipids [4, 23]. Among the several mechanisms proposed for the protective effects of β -glucans, the major one is related to

antioxidant capacity of the molecules [1, 20], which has been further proven by Kogan *et al.* [16] using electron paramagnetic response spectroscopy.

Carbon tetrachloride (CCl₄) is an extensively used industrial solvent. It is used as an injury agent for animal experiments, and induces reactive oxygen formation and depletes Glutathione (GSH) of the phase II enzyme. It may reduce antioxidant enzyme and antioxidant substrates to induce oxidative stress. CCl₄ induces liver injury caused by free radical and induces lipid peroxidation that can result in hepatic cell injury. Oxidative stress is an important factor in acute and chronic liver injury. The hepatotoxicity of CCl₄ requires bioactivation by the cytochrome P450 (Cyt P450) phase I system in the liver and yields a reactive metabolic trichloromethyl radical (CCl₃) and proxy trichloromethyl radical (OCCl₃). These free radicals can bind with polyunsaturated fatty acid (PUFA), and form alkoxy (R) and peroxy radicals (ROO) that can generate lipid peroxide, cause damage in the cell membrane, change enzyme activity and finally induce hepatic injury or necrosis [26, 27, 39].

We designed this study to investigate the possible protective effects against acute hepatic injury induced by CCl₄ and its mechanism in rats based on the antioxidative action of paramylon, a β -(1-3)-D-glucan, isolated from *Euglena gracilis* Z.

MATERIALS AND METHODS

Preparation of paramylon: Paramylon isolated from *Euglena gracilis* Z was obtained from euglena Co., Ltd.

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(Tokyo, Japan). The general method for paramylon production is as follows. Cultured *Euglena gracilis* Z cells collected by continuous centrifugation are washed with water. After suspending in water, the cells are smashed with ultrasonic waves and paramylon is collected. To remove the lipid and protein, this rough paramylon is treated at 95°C for 1 hr with 1% sodium dodecyl sulfate solution and then at 50°C for 30 min with 0.1% SDS. With further centrifugation, paramylon is obtained, and after repeated washing with water, acetone and ether, respectively, refined paramylon is acquired.

Animals: 30 Wistar strain male rats weighing 190–210 g, were obtained from the Institute of Animal Reproduction (Ibaraki, Japan). The animals were maintained at a controlled temperature of $22 \pm 2^\circ\text{C}$ with a 12:12-hr light/dark cycle (light cycle, 7:00–19:00), and were given standard chow (CE-2, Clea Japan, Inc., Tokyo, Japan). The use of these animals and the procedures performed on them were approved by the Animal Research Committee of Tottori University.

Animal treatment: The animals were divided into six groups ($n=5$). Group I served as a normal control, and Group II served as a CCl_4 control. Both groups received saline. Group III was given a standard drug, silymarin (Sigma Aldrich Co., St. Louis, MO, U.S.A.), at 200 mg/kg body weight. The animals in groups IV, V and VI, respectively, were administered paramylon orally for 3 days at 500, 1,000 and 2,000 mg/kg body weight. On the 3rd day, all the other animal groups except for group I received CCl_4 (Wako, Osaka, Japan) intraperitoneally (1 mg/kg in olive oil/1:1); all were sacrificed at 24 hr post-injection of CCl_4 , and blood and liver samples were immediately obtained.

Serum and liver biochemical assays: The blood samples were centrifuged at 3,000 rpm for 10 min at 4°C to obtain serum. Serum glutamic pyruvate transaminase (GPT) and glutamic oxaloacetate transaminase (GOT) levels were determined by the optimized ultraviolet method (GPT or GOT assay kit, Wako, Osaka, Japan).

Liver samples were dissected out and washed immediately with ice cold saline to remove as much blood as possible. Liver homogenates (5% w/v) were prepared in cold 50 mM potassium phosphate buffer (pH 7.4). The unbroken cells and cell debris were removed by centrifugation at 2,000 rpm at 4°C for 10 min, and clear supernatant was used for estimation of the activities of hepatic superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Hepatic SOD activity was measured based on hematoxylin autoxidation [22] with a commercial kit (Northwest Life Science Specialties, Vancouver, BC, Canada). Hepatic CAT activity was assayed by the method of Smna *et al.* [33] with a commercial kit (Cell Tech, California, U.S.A.). Hepatic GPx activity was assayed by the method of Pagli *et al.* [25] with a commercial kit (Northwest Life Science Specialties, Vancouver, BC, Canada).

The amount of protein was measured with Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA, U.S.A.) according to the method

described by Bradford *et al.* [5].

Histopathological examinations: Liver tissues were fixed in 10% buffered formaldehyde, processed for histological examination by the conventional methods, and stained with hematoxylin and eosin (HE). The liver pathology was scored by French *et al.* [11] as follows: 0=no visible cell damage; 1=focal hepatocyte damage to <25% of tissue; 2=focal hepatocyte damage to <25–50% of the tissue; 3=extensive, but focal, hepatocyte lesion; 4=global hepatocyte necrosis. Pathologists performing the histopathological examinations were blinded to the study treatments.

Immunohistochemical examinations: For immunohistochemistry, anti-single stranded DNA (ssDNA) rabbit polyclonal antibody (DakoCytomation, Kyoto, Japan) was used as the primary antibody. Tissue sections for detection of ssDNA were incubated in 95°C 0.01 M citrate buffer (pH 6.0) for 5 min to retrieve antigens. All sections were dewaxed, rehydrated, rinsed with 0.05 M tris-buffered saline containing Tween 20 (TBST; pH 7.6), treated with 1% hydrogen peroxide and then rinsed again with TBST. Slides were incubated with primary antibody for 30 min at room temperature and, after rinsing with TBST, were treated with Simple Stain MAX-PO (Multi) (Nichirei, Tokyo, Japan) for 30 min at room temperature. They were then rinsed with TBST before being treated with a 3,3'-diaminobenzidine solution containing 0.01% hydrogen peroxide to facilitate a peroxidase colour reaction. After a further wash with TBST, the slides were counterstained with Mayer's haematoxylin. More than 1000 hepatocytes were counted in several fields ($\times 400$). The apoptotic index was calculated as the percentage of immunoreactive nuclei out of the total number of hepatocytes counted [19].

Statistical analysis: All data are expressed as means \pm SE of 5 rats in each group. The results in each group were compared by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test with GraphPad PRISM (GraphPad Software, San Diego, CA, U.S.A.). $P<0.05$ was considered to be statistically significant.

RESULTS

The effects of pretreatment with paramylon on the CCl_4 -induced elevation of serum GOT and GPT are shown in Table 1. The serum GOT and GPT levels were found to be significantly elevated in rats administered CCl_4 . Treatment with paramylon at 1,000 and 2,000 mg/kg prevented the elevation of the serum levels of GOT and GPT induced by CCl_4 ($p<0.05$; Table 1).

Figure 1 showed the effect of paramylon on CCl_4 -induced liver damage in rats. In the saline + CCl_4 -treated group, there were extensive degenerative and necrotic areas around the central vein of the liver. Pre-administration of paramylon could reduce these pathological changes dose-dependently (Fig. 1). Table 2 summarizes the data relating to liver damage induced by CCl_4 in the pathological histology. Treatment of paramylon reduced the injury score of degeneration and hepatic necrosis (Table 2). Pre-administration

Table 1. Effect of paramylon on serum markers levels in CCl₄-induced hepatic injury in rats

Groups	Test substances	GOT (IU/l)	GPT (IU/l)
I	saline	48.33 ± 1.74	36.96 ± 3.99
II	saline + CCl ₄	1588.60 ± 57.87	726.50 ± 56.19
III	silymarin + CCl ₄	198.26 ± 11.27	138.28 ± 15.67
IV	paramylon (500 mg/kg) + CCl ₄	1339.86 ± 75.22	631.98 ± 48.65
V	paramylon (1000 mg/kg) + CCl ₄	906.87 ± 77.26*†	499.58 ± 35.21*
VI	paramylon (2000 mg/kg) + CCl ₄	654.16 ± 51.52*†	426.63 ± 28.12*†

All data are expressed as means ± SE (n=5) and were compared by ANOVA.

Differences of $p < 0.05$ are considered statistically significant.

* $p < 0.05$, compared with the saline + CCl₄ group.

† $p < 0.05$, compared with the paramylon (500 mg/kg) + CCl₄ group.

Table 2. Histological injury score and apoptotic index of the liver under paramylon in rats treated with CCl₄

Groups	Test substances	Injury of score ^{a)}		Apoptotic index ^{b)} (%)
		Degeneration	Necrosis	
I	saline	0.0	0.0	2.56 ± 0.99
II	saline + CCl ₄	3.8	3.0	13.24 ± 1.13
III	silymarin + CCl ₄	2.0	1.2	5.50 ± 0.46
IV	paramylon (500 mg/kg) + CCl ₄	3.0	3.0	10.35 ± 0.77
V	paramylon (1000 mg/kg) + CCl ₄	2.4	2.4	8.38 ± 0.94*
VI	paramylon (2000 mg/kg) + CCl ₄	2.2	2.0	7.39 ± 0.73*

a) Livers were scored for hepatic injury via light microscopy with score 0 = no visible cell damage; score 1=focal hepatocyte damage on less than 25% of the tissue; score 2=focal hepatocytes damage on 25–50% of the tissue; score 3=extensive, but focal, hepatocyte lesions; score 4=global hepatocyte necrosis.

Hepatic injury scores are expressed as means (n=5).

b) Apoptotic indices are expressed as means ± SE (n=5) and were compared by ANOVA.

Differences of $p < 0.05$ are considered statistically significant.

* $p < 0.05$, compared with the saline + CCl₄ group.

Table 3. Effect of paramylon on hepatic SOD, CAT and GPx activity in CCl₄-induced hepatic injury in rats

Groups	Test substances	SOD (U/mg protein)	CAT (U/mg protein)	GPx (mU/mg protein)
I	saline	10.04 ± 1.19	86.40 ± 7.62	132.40 ± 11.37
II	saline + CCl ₄	2.83 ± 0.50	28.83 ± 2.20	58.83 ± 2.19
III	silymarin + CCl ₄	7.01 ± 0.78	60.83 ± 5.76	94.98 ± 5.58
IV	paramylon (500 mg/kg) + CCl ₄	3.84 ± 0.34	35.84 ± 2.04	63.84 ± 3.48
V	paramylon (1000 mg/kg) + CCl ₄	4.42 ± 0.56	44.42 ± 3.19*	73.02 ± 5.30
VI	paramylon (2000 mg/kg) + CCl ₄	4.98 ± 0.22*	46.98 ± 3.87*	76.98 ± 3.86*

All data are expressed as means ± SE (n=5) and were compared with the ANOVA.

Differences of $p < 0.05$ are considered statistically significant.

* $p < 0.05$, compared with the saline + CCl₄ group.

of paramylon reduced the liver apoptotic index (Fig. 2, Table 2: $p < 0.05$). Histological examination of the liver showed the preventive effects of paramylon on CCl₄-induced hepatotoxicity.

Table 3 shows the effects of pretreatment with paramylon on the CCl₄-induced decrease in the levels of hepatic antioxidant enzyme activity. The levels of hepatic SOD, CAT and GPx activity were significantly decreased in the rats of the saline + CCl₄ group. Treatment with paramylon restored the levels of hepatic SOD, CAT and GPx activity (Table 3).

DISCUSSION

CCl₄ hepatotoxicity depends on its reductive dehalogena-

tion catalyzed by Cyt P450 in the endoplasmic reticulum of hepatic cells leading to the generation of the unstable complex trichloromethyl radical. The superoxide anion (O₂⁻), H₂O₂, and the hydroxyl radical (-OH) are reactive oxygen species (ROS) mainly produced in mitochondria [41]. Cells have a number of mechanisms to protect against the toxic effects of ROS including free radical scavengers and chain reaction terminators such as SOD, CAT and GPx systems [13, 41]. SOD removes O₂⁻ by converting it to H₂O₂ that can be rapidly converted to water by CAT and GPx [13]. Cellular injury occurs when ROS generation exceeds the cellular removal capacity.

In the present study, decreased SOD, CAT and GPx activities occurred in liver injury induced by CCl₄, implying

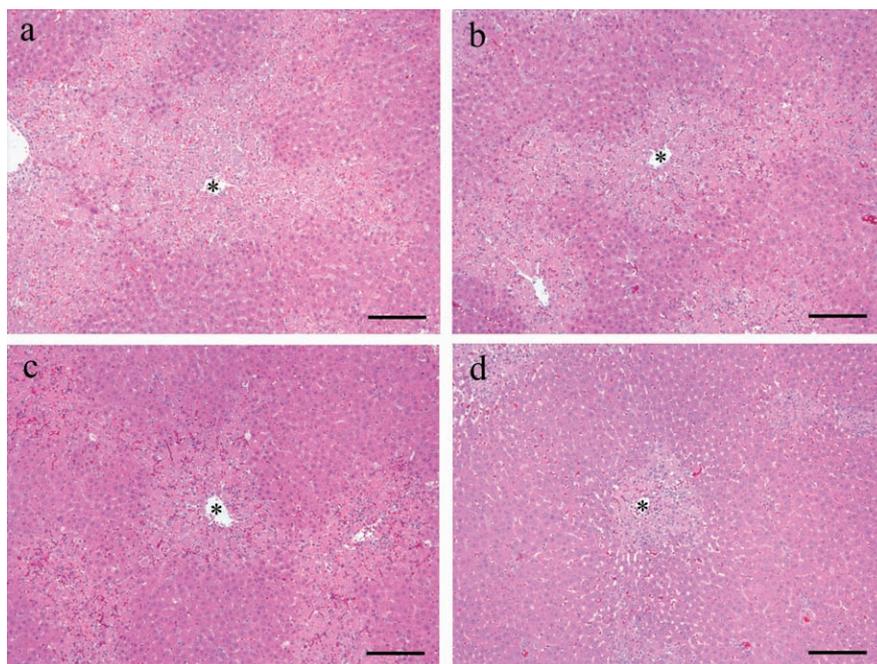


Fig. 1. Effect of paramylon on CCl_4 -induced liver damage of rats. Animals were treated with CCl_4 in the presence and absence of paramylon as follows: (a) saline + CCl_4 , (b) paramylon (500 mg/kg) + CCl_4 , (c) paramylon (1,000 mg/kg) + CCl_4 and (d) paramylon (2,000 mg/kg) + CCl_4 . In the saline + CCl_4 -treated group (a), there were extensive degenerative and necrotic areas around the central vein of the liver. Treatment with paramylon reduced the sizes of these damaged areas dose-dependently (b, c, d). *=central vein. HE stain, bar=150 μm .

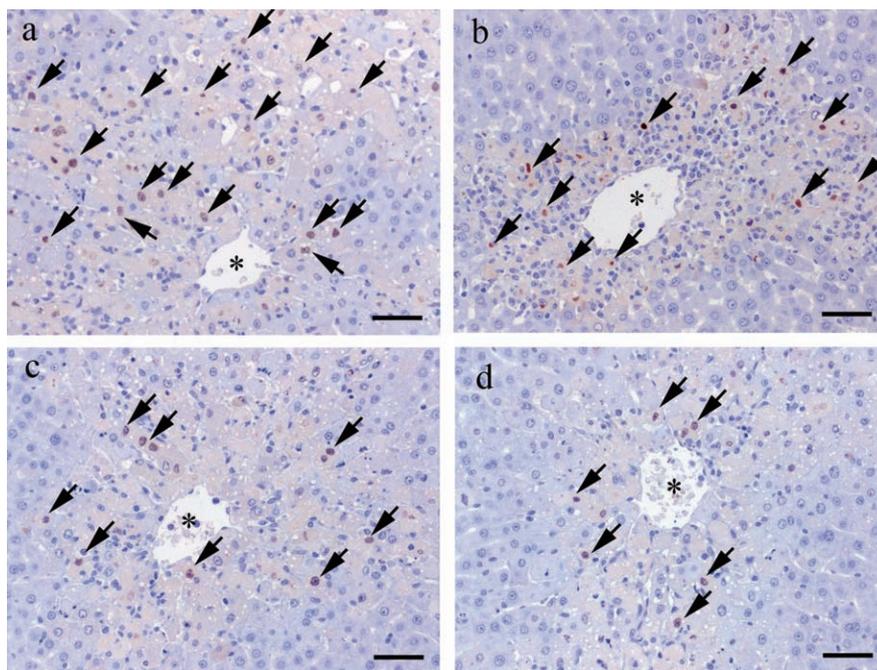


Fig. 2. Effect of paramylon on apoptosis in the liver. (a) Saline + CCl_4 , (b) paramylon (500 mg/kg) + CCl_4 , (c) paramylon (1,000 mg/kg) + CCl_4 and (d) paramylon (2,000 mg/kg) + CCl_4 . In the saline + CCl_4 -treated group (a), there were more ssDNA-positive cells than in the paramylon + CCl_4 groups (b, c, d). *=central vein. Immunohistochemistry with ssDNA, bar=50 μm .

downregulation of numerous enzymatic oxidative reactions in the cytosolic compartments and mitochondria. Furthermore, liver damage via the ROS pathway caused remarkable increases in the GOT and GPT levels in serum and histological changes in hepatic cells. Paramylon exhibited its protective effect on liver injury in response to CCl₄ by elevating SOD, CAT and GPx activities to increase scavenging of ROS in mitochondria, leading to decreased activity of GOT and GPT.

In previous studies, oral administration of β -glucan showed protective effects against oxidative tissue damages induced by sepsis, burn, pressure ulcer, ischemia/reperfusion or drugs such as acetaminophen and methotrexate in rodents through its antioxidant properties [3, 30–32, 36, 37]. The aforementioned studies showed that β -D-glucan increased the activities of antioxidant enzymes, such as SOD, CAT, GPx and GSH, and inhibited lipid peroxidation.

β -glucan receptor activity has been reported for a variety of other leukocytes, including macrophages, neutrophils, eosinophils and NK cells, as well as for nonimmune cells including endothelial cells, alveolar epithelial cells and fibroblasts [6]. Nonopsonic recognition of β -glucans by these cells has been ascribed to multiple receptors [2], and indeed a number of β -glucan receptors have been identified, including complement receptor type 3, lactosylceramide, scavenger receptor and Dectin-1. However, in these receptors, only Dectin-1 has been clearly shown to have a role in mediating the biological response to β -glucans [6]. Thus, in the present study, paramylon exhibited a protective effect against liver injury via β -glucan receptors such as Dectin-1 receptor.

In conclusion, our results demonstrated the protective effect of paramylon against CCl₄-induced liver injury via its antioxidative action. To the best of our knowledge, this is the first report of a hepatoprotective effect based on the antioxidative action of paramylon. Our results support the potential use of paramylon in clinical conditions where oxidative organ failure may be present. Further studies with paramylon receptors and transduction pathways should be conducted to highlight the underlying mechanisms of this protective effect.

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