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Effects of alginic acid on toxicity of cadmium in rat primary hepatocyte cultures

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Abstract

The protective effects of effects of alginic acid, a water soluble dietary fiber containing in a brown marine plants, on the toxicity of Cd in the rat primary hepatocyte cultures were studied. Cytotoxicity was assessed by measuring cell viability, extra cellular lactic dehydrogenase (LDH) activity, and intracellular lipid peroxidation and active oxygen species. Primary hepatocyte cultures were treated with $^{109}CdCl_2$ (5, 10 or 50 μ M Cd and 1.85 KBq of ^{109}Cd/well) for 30 min 4 h. Alginic acid was added to the culture medium to make the final concentration of 100 μ M and incubated for 4.5h in 30 min Cd exposure or 1 h in 4 h Cd exposure. Decreases in the hepatocyte viability caused by all Cd exposure concentrations were significantly prevented by treatment with alginic acid. The treatment with alginic acid for 4.5 h after Cd exposure for 30 min significantly prevented increases in extracellular LDH activity. Increases in the lipid peroxidation in hepatocytes exposed Cd for 30 min or 4 h were prevented significantly by treatment with alginic acid for 4.5 h or 1 h, respectively. Moreover, the increases in the level of active oxygen species caused by Cd exposure for 30 min were significantly prevented by treatment with alginic acid for 1.5 h. These findings suggest that alginic acid protects against the cytotoxicity of Cd in rat primary hepatocyte cultures and that the protective effects of alginic acid presumably result from a decrease in the Cd level, the effective sequestration of the reactive Cd ion, and the direct preventive effect on the active oxygen species in the hepatocytes.

Keywords : Cadmium cytotoxicity; Primary hepatocyte cultures; Alginic acid; Detoxication

1. Introduction

Liver plays an important role in the disposition of cadmium (Cd). The liver accumulates substantial amounts of Cd after both acute and chronic toxicity[1,2], which results in hepatic injury during both types of exposure[3-5]. The liver is a target organ for Cd after acute exposure in laboratory rodents[6] and the resultant hepatic necrosis after Cd exposure may play a role in the lethality after exposure. Repeated exposures of rats to low daily doses of Cd over 6 months resulted in liver injury prior to the onset of renal damage and the effects in liver included

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elevations in plasma activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and decreased structural integrity of hepato cytes[7]. Resently we have shown that the new ditiocarbamates of effective chelators of Cd, N-benzyl-D-glucamine dithiocarbamate (BGD)[8-10] and N-*p*-hydroxymethylbenzyl-D-glucamine dithiocarbamate (HBGD) [11,12] are more effective than other dithiocarbamates in decreasing Cd levels in the liver and kidney in Cd-treated rats and mice without redistribution of the metal to tissues such as the brain, testes and heart. Our studies also indicated that an increase in the plasma AST activity after Cd administration, which is a biochemical evidence of hepatic damage, was prevented by BGD treatment[13] and that BGD treatment prevented Cd-induced decreases in the activities of AST and ALT in the liver and kidney indicating the acute hepatic and renal toxicity[14].

The utility of isolated rat hepatocytes as a model system for screening potential chelators in treatment of Cd detoxication was studied[15]. The effects of diethylenetriaminopentaacetic acid (DTPA), ethylenediaminetetraacetic acid(EDTA), diethyldithiocarbamic acid (DDC), and 2,3-dimercaptopropanol(BAL) in the hepatocyte model screening system correlated well with their reported in vivo effects of Cd were studied in primary cultures of rat hepatocytes incubated with $CdCl_2$ or Cd-diethyldithiocarbamate (Cd(DTC)$_2$), labeled with ^{109}Cd[16]. The lipid- soluble complex Cd(DTC), was rapidly taken up into the cells and a maximal concentration was reached after 4 h incubation. The results from these studies show that DTC can increase the transport of into the cell by complex formation with Cd.

The present study was undertaken to examine the effects of alginic acid, a water soluble dietary fiber containing a cell wall viscosity polysaccharide of brown algae, on the toxicity of Cd in the rat hepatocyte primary cultures.

2. Materials and methods

2.1. Materials

^{109}Cd (specific radioactivity, 1.85 MBq/μg) was obtained from Japan Radioisotope Association. (Tokyo). Alginic acid and $CdCl_2$ was obtained from Nakarai Tesque (Kyoto). 3-(4,5 -Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (St. Louis, MO). All other chemicals were of analytical and reagent grade.

2.2. Preparation and exposure of the rat primary hepatocyte cultures to Cd

The hepatocytes were isolated from male Wistar rats (Kyudo Co., Ltd. Ltd. Kumamoto), weighing 150-200 g, by the method of Berry and Friend[17]. Cell viability ranged from 95-98% as determined by MTT assay Method[18].

Primary cultures of hepatocytes were established by seeding 7 x 10^5 cells onto 6-well plate or 7 x 10^4 cells onto 96-well plate and the plates incubated for 24 h with the growth medium at 37℃ in atmosphere of 5% CO_2 and 95% air. The growth medium was Eagle's minimum essential medium (MEM), pH 7.4, (Nissui Pharm. Co., Ltd., Tokyo) with 5% fetal bovine serum (FBS) (Gibco Lab.,Grand Island, NY) and 0.03% L-glutamine.

After attachment of cells to the plate, the growth medium was removed. The cultures were washed by Hank's balanced salt solution (HBSS) (Sigma Chemical Co., St. Louis, MO) and treated with $CdCl_2$ (2, 5, 10 and 50μM Cd) in

HBSS at 37℃.

Control experiments were performed at the same conditions without $^{109}CdCl_2$, respectively. Data obtained were expressed as a percentage of each control.

2.3. Evaluation of cytotoxicity

Primary hepatocyte cultures were treated with $^{109}CdCl_2$ (2, 5, 10 and 50 μ M Cd and 1.85 KBq of ^{109}Cd/well). Cytotoxicity was assessed by measuring cell viability, extracellular lactate dehydrogenase (LDH) activity, and intracellular lipid peroxidation, glutathione (GSH) and active oxygen species. For the measurement of LDH, medium was collected and analyzed for LDH activity by the procedure of Wroblewski and LaDue[19]using a commer- cially available kit, LDH-UV Test Wako (Wako Pure Chemical Ind., Ltd., Osaka). For the assay of lipid peroxidation, hepatocytes were rinsed three times with phosphate-buffered saline (PBS), pH 7.4, containing 2 mM EGTA, collected in 10 mM Tris-HCl buffer, pH 7.4, and sonicated with SHARP UT-204 for 30 s. An aliquot (0.5 ml) of suspensions was analyzed for lipid peroxidation by thiobarbituric acid (TBA) method of Uchiyama and Mihara[20] using 1,1,3,3,-tetra-ethoxypropane as the standard and expressed as nanomol of TBA reactive substances (TBARS) per mg of protein. For the assay of GSH and active oxygen species, the hepatocytes rinsed as described above were collected in 40 mM Tris-HCl buffer, pH 7.4, sonicated, and centrifuged (2,400 x g, 5 min at 4℃). Each aliquot (0.5 ml) of supernatant was analyzed for GSH and active oxygen species by the method of Kaplowitz et.[21] and LeBel et al.[22] , respectively. The active oxygen species were expressed as 2',7'-dichlorofluorescein (DCF) formed. For the assay of Cd accumulation, at the end of incubation, radioactive medium was removed and cells were washed three times with PBS containing 2 mM EGTA. Cells were collected and added 1 ml of 1 N NaOH. Cd content was determined by an Aloka auto well gamma scintillation counter (model ARC 300). Control experiments were performed at the same conditions without $^{109}CdCl_2$, respectively.

2.4. Effects of alginic acid on cytotoxicity of Cd

Primary hepatocyte cultures were treated with $^{109}CdCl_2$ (2, 5, 10 and 50 μ M Cd and 1.85 KBq of ^{109}Cd/well) for 5 h in the presence or absence of the alginic acid. Alginic acid was added to the culture medium to make the final concentration of 100 μ M at 30 min or 4 h after $^{109}CdCl_2$ was added and the incubation was continued for 4.5 h or 1 h, respectively. Cell viability, extracellular LDH activity, and intracellular lipid peroxidation and GSH were measured as mentioned above.

2.5. Effects of alginic acid on active oxygen species in hepatocytes induced by Cd or CCl_4

Primary hepatocyte cultures were incubated with 5, 10 and 50 μ M $CdCl_2$ or CCl_4 for 30 min. Alginic acid was added to the culture medium to make the final concentration of 100 μ M and incubated for 1.5 h.. Control experiments were performed at the same conditions without $CdCl_2$ or CCl_4 and the chelating agents, respectively. Active oxygenspecies were expressed as DCF formed.

2.6. Statistical analysis

Data were analyzed by a one-way analysis of variance. When the analysis indicated that a

significant difference existed, the treated groups were compared to the controls by Duncan's new multiple range test.

3. Results

3.1 Cytotoxicity of Cd

The effect of Cd on the viability of hepatocytes is shown in Fig. 1. The data obtained were

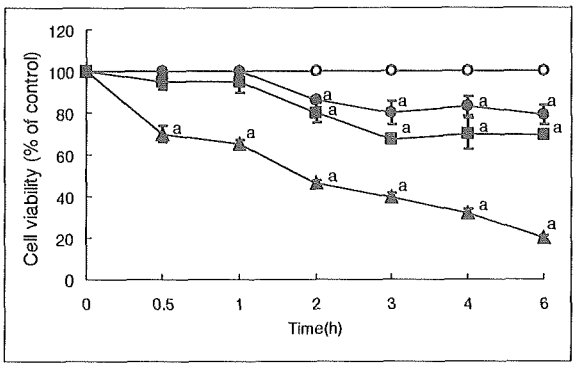


Fig. 1. Effects of Cd on viability of hepatocytes. Hepatocytes were incubated with 2 - 50 μM $CdCl_2$ in Hank's solution at 37℃ for 0.5 - 6.0 h. The data are the mean±S.D. for three independent cell preparations. Control experiments were performed at the same conditions without $CdCl_2$. Data obtained were expressed as a percentage of each control. (○): 2 μM Cd; (●): 5 μM Cd; (■): 10 μM Cd; (▲): 50 μM Cd. [a]Significantly different from control ($P<0.05$)

expressed as a percentage of each control. Cell viability of each control ranged from 95-98% as determined by MTT assay Method[18]. Treatment with 2 μM Cd did not affect the cell viability and the levels of GSH, Ca, Fe, Zn and Cu in the hepatocytes (data not shown). The cell viability was significantly decreased 2 h after treatment with 5 or 10 μM Cd and remarkably decreased by treatment with 50 μM Cd.

The activity of LDH released from the hepatocytes after Cd treatment is shown in Fig. 2A. The extracellular LDH activity significantly increased 6 h, 4 h, and 2 h, respectively after treatment with 5, 10 and 50 μM Cd. Fig. 2B shows the lipid peroxidation in the hepatocytes

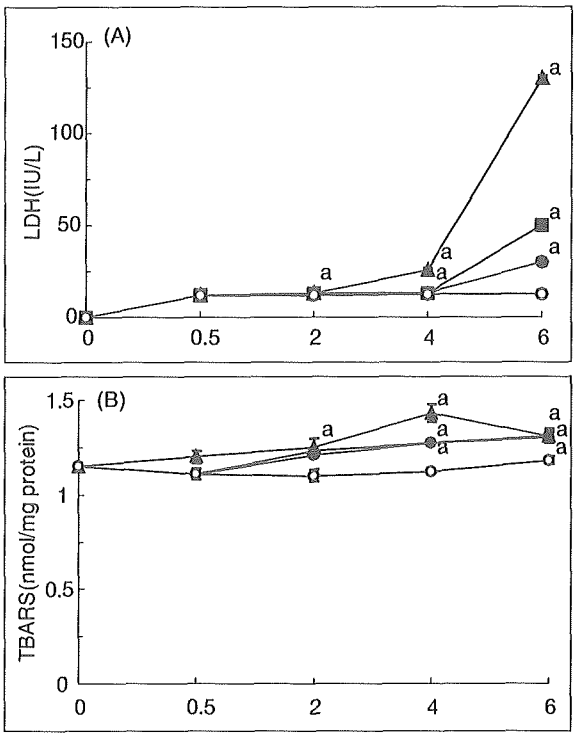


Fig. 2. Effects of Cd on LDH release (A), and lipid peroxidation (B). Hepatocytes were incubated with 5-50 μM $CdCl_2$ in Hank's solution at 37℃ for 0.5-6.0 h. The data are the mean±S.D. for three independent cell preparations. Control experiments were performed at the same conditions without $CdCl_2$. (○): Control; (●): 5 μM Cd; (■): 10 μM Cd; (▲): 50 μM Cd. [a]Significantly different from control ($P<0.05$)

after Cd treatment. The lipid peroxidation significantly increased 4 h after treatment with 5 or 10 μM Cd and 2 h after treatment with 50 μM Cd. Changes in active oxygen species in the hepatocytes after Cd treatment are shown in Fig. 2C. Treatment with 5, 10 and 50 μM Cd increased linearly the levels of active oxygen species until 2 h after Cd treatment, then decreased gradually, and returned to the control levels 6 h after Cd treatment

The accumulation of ^{109}Cd in the hepatocytes after incubation with 2-50 μM ^{109}Cd is shown in Fig. 3. The accumulation of ^{109}Cd increased with times and reached nearly to plateau at about 4 h. At 6 h, the hepatocytes accumulated 0.60, 2.39, 4.00 and 12.60 nmol Cd/mg protein after incubation with 2, 5, 10 and 50 μM Cd,

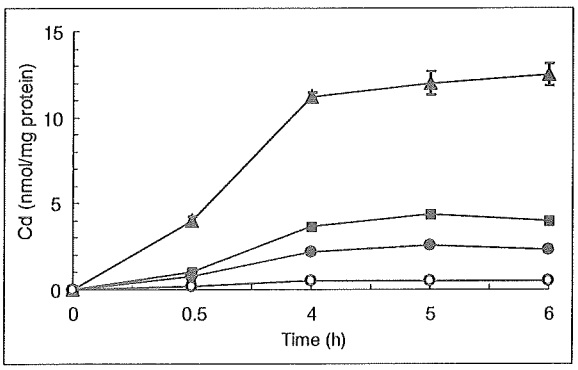


Fig. 3. Time courses of Cd accumulation in hepatocytes. Hepatocytes were incubated with 5-50 μ M $^{109}CdCl_2$ in Hank's solution at 37℃ for 0.5-6.0 h. Control experiments were performed at the same conditions without $^{109}CdCl_2$. (○): 2 μ M Cd; (●): 5 μ M Cd; (■): 10 μ M Cd; (▲): 50 μ M Cd.

respectively. Control experiments were performed at the same conditions without $^{109}CdCl_2$. However, the accumulation of ^{109}Cd in the hepatocytes was not detected at any incubation times.

3.2. Effect of alginic acid on cytotoxicity of Cd

Changes in the viability of hepatocytes treated with alginic acid after Cd (5, 10 and 50 μ M Cd) exposure for 30 min or 4 h are shown in Fig. 4A or B, respectively. Decreases in the hepatocyte viability caused by all Cd exposure concentrations were significantly prevented by treatment with alginic acid (100 μ M). The preventive effects of the alginic acid on the decreased cell viability were considerably less in Cd exposure for 4 h than in that for 30 min. The cell viability was expressed as a percentage of each control. Control experiments were performed at the same conditions without CdCl2 and the chelating agents. The cell viability of the controls ranged from 95-98% as determined with MTT assay Method.

The effect of alginic acid on LDH released by the hepatocytes after Cd exposure are shown in

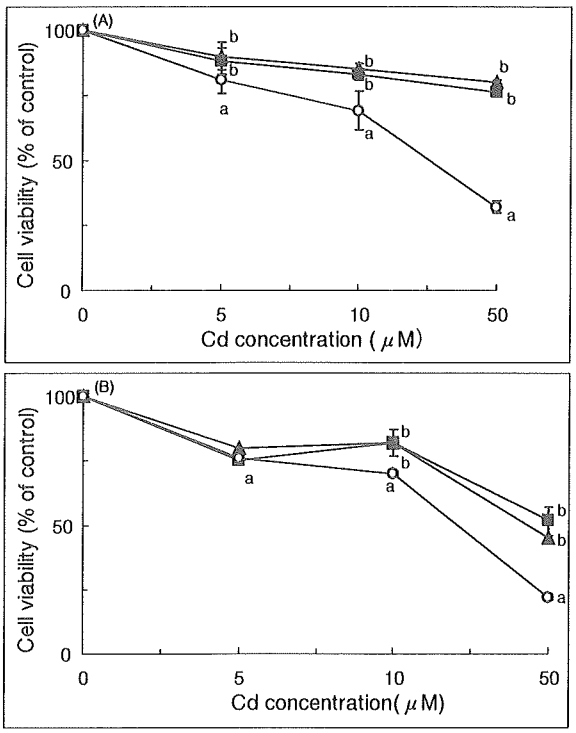


Fig. 4. Effects of alginic acids on viability of hepatocytes after Cd treatment for 0.5 h (A) or 4 h (B). After hepatocytes were incubated with 5, 10 or 50 μ M CdCl2 for 30 min of 4 h, they were treated with 50 and 100 μ M alginic acids for 4.5 h or 1 h. Control experiments were performed at the same conditions without $CdCl_2$. and the alginic acids. Data obtained were expressed as a percentage of each control. The data are the mean±S.D. for three independent cell preparations. (○): Cd alone; (▲): Cd + 50 μ M alginic acid; (■): Cd + 100 μ M alginic acid. [a]Significantly different from control ($P<0.05$). [b]Significantly different from Cd alone ($P<0.05$).

Fig. 5. The treatment with the chelating agents for 4.5 h after Cd (5, 10 and 50 μ M) exposure for 30 min significantly prevented increases in extracellular LDH activity. The treatment with the chelating agents for 1 h after Cd exposure for 4 h prevented increases in LDH activity after exposure with Cd at 5 and 10 μ M, but did not prevent those after 50 μ M Cd exposure.

The effects of BGD and HBGD on the lipid peroxidation in hepatocytes after Cd treatment are shown in Fig. 6. Increases in the lipid peroxidation in hepatocytes exposed to Cd (5, 10

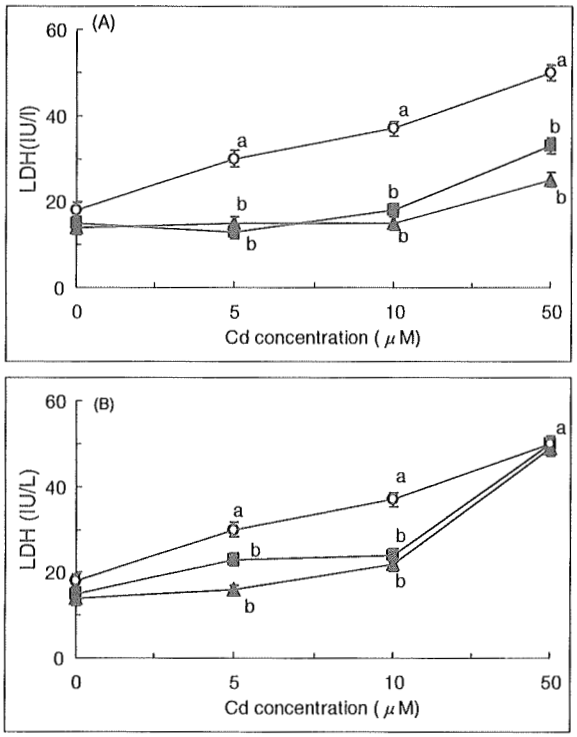
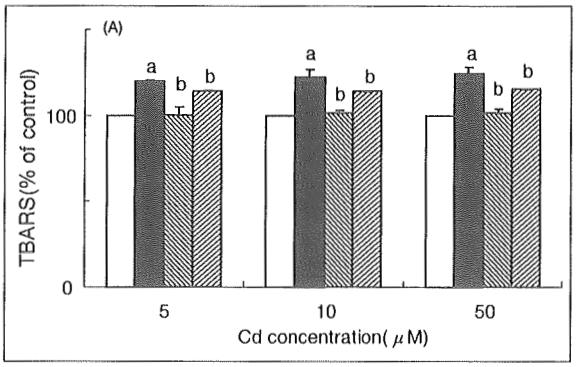


Fig. 5. Effects of alginic acids on LDH leakage in hepatocytes after Cd treat ment for 0.5 h (A) or 4 h (B). After hepatocytes were incubated with 5, 10 or 50 μM $CdCl_2$ for 0.5 h or 4 h, they were treated with 50 and 100 μM alginic acids for 4.5 h or 1 h. The data are the mean± S.D. for three independent cell preparations. (○): Cd alone; (■): Cd + 50 μM alginic acid; (▲): Cd + 100 μM alginic acid. [a]Significantly different from control ($P<0.05$). [b]Significantly different from Cd alone ($P<0.05$).

and 50 μM) for 30 min or 4 h were prevented significantly by treatment with alginic acid for 4.5h or 1 h, respectively. As shown in Fig. 7A, also, increases in the level of active oxygen species caused by Cd exposure for 30 min were significantly prevented by treatment with the



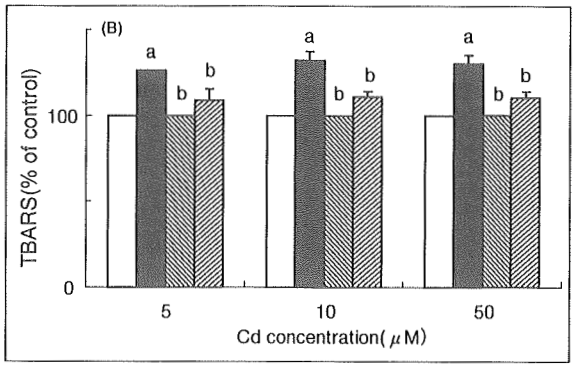
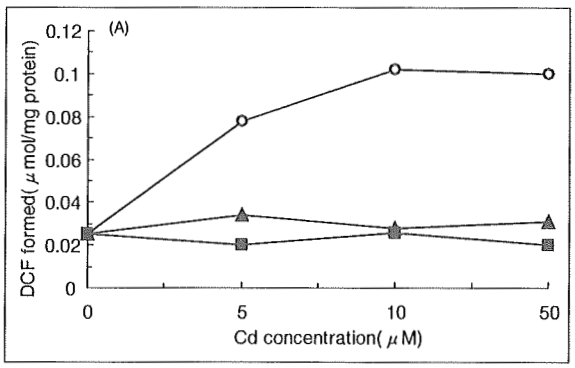


Fig. 6. Effects of alginic acids on lipid peroxidation in hepatocytes after Cd treat ment for
0.5 h(A) or 4 h (B). After hepatocytes were incubated with 5, 10 or 50 μM $CdCl_2$ for 30 min or 4 h, they were treated with 50 and 100 μM alginic acids for 4.5 h or 1 h. The data are the mean± S.D. for three independent cell preparations. (□): Control; (■): Cd alone; (▨): Cd+50 μM alginic acid; (▨): Cd + 100 μM alginic acid. [a]Significantly different from control ($P<0.05$). [b]Significantly different from Cd alone ($P<0.05$).

alginic acid for 1.5 h. In order to further examine this respect, the effects of the alginic acid on active oxygen species in the hepatocytes induced by treatment with carbon tetrachloride (CCl_4) were investigated (Fig. 7B). Treatment resulted in increases in the active oxygen species in the



hepatocytes, which were significantly prevented by the chelating agents.

Cd concentrations in the hepatocytes in these experiments were determined. In Cd (30 min)-alginic acid (4.5 h) treatment, treatment with alginic acid significantly decreased Cd concentrations in the hepatocytes after exposure with Cd at 5, 10 and 50 μM. In Cd (4 h)- alginic acid (1 h) treatment, with alginic acid decreased

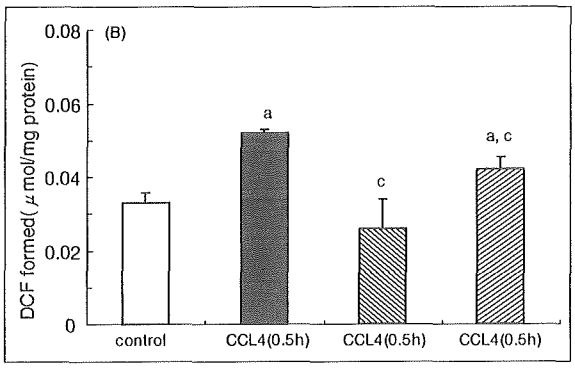


Fig. 7. Effects of alginic acids on active oxygen species in hepatocytes induced by Cd (A) or CCl_4 (B). After hepatocytes were incubated with 5, 10 or 50 μ M $CdCl_2$ or 10 μ M CCl_4 for30 min, they were treated with 50 and 100 μ M alginic acids for 1.5 h. Control experiments were performed at the same conditions without $CdCl_2$ (A) or CCl_4 (B) and the alginic acids, respectively. Active oxygen species were expressed as 2' ,7' -dichlorofluorescein (DCF) formed. The data are the mean±S.D. for three independent cell preparations. (A) (○): Control; (▲): Cd+50 μ M alginic acid; (■)Cd+50 μ M alginic acid. (B) (□):Control; (■);CCl_4 alone; (▨):CCl_4+50 μ M alginic acid;(▨):CCl_4+100 μ M alginicacid.[a]Significantly different from control ($P<0.05$). [b]Significantly different from Cd alone ($P<0.05$). [b]Significantly different from CCl_4 alone ($P<0.05$).

the hepatocyte Cd concentrations after exposure to 5 and 10 μ M Cd and did not significantly decreased it with 50 μ M Cd.

5. Discussion

Extracellular LDH activity, which is known to be sensitive index for metal toxicity[23,24], was found to increase with increasing Cd exposure concentration over the range 2-50 μ M Cd. This increase indicates metal-induced damage to the cell membrane and leakage of soluble cell components into the medium. The toxicity of Cd to hepatocytes was decreased in the cell viability. In hepatocytes, the correlation between the loss of cell viability and the release of LDH is well-established[25].

The lipid peroxidation in hepatocytes increased 2-4 h after Cd (5, 10 and 50 μ M) exposure. In the hepatocytes after Cd exposure, increases in active oxygen species appeared prior to increases in lipid peroxidation. Active oxygen species, if present in excess, are thought to be initiators of peroxidative cell damage[26-28]. These findings suggest a role for active oxygen species in the increase in lipid peroxidation produced by Cd exposure. Several mechanisms for Cd cytotoxicity have been proposed including lipid peroxidation, interference with mitochondrial function, and the interaction with cellular thiol ligands[29-32]. GSH generally play a protective role against the toxicity of many xenobiotics, including metals, by reacting either directly with the toxicant or in enzyme-catalyzed reactions that allow detoxication.

The effect of alginic acid on the hepatocyte toxicity of Cd indicated that alginic acid prevented the decrease in the viability of hepatocytes by Cd exposure. This is supported by the preventive effects of the alginic acid on the increase in lipid peroxidation and the LDH leakage in the hepatocytes, which participate to the toxicity of hepatocytes after Cd exposure. The protective effect of the alginic acid against the hepatocyte toxicity was greater in the treatment after Cd exposure for 30 min than for 4 h. This shows the unsatisfactory protection of the alginic acid against more toxicity of hepatocytes proceeding with the longer exposure with Cd. Three results of the protective effects of alginic acid on the Cd detoxication in cultured hepatocytes were correlated with our reported in vivo effects of chelating agents[7-10].

Moreover, alginic acid decreased the Cd level and the active oxygen species in hepatocytes after Cd exposure, and also prevented the increase in active oxygen species in the hepatocytes after CCl_4 treatment. These results indicate that the

direct preventive effects of alginic acid on the active oxygen species in hepatocytes are one of the causes of the protective effects of the alginic acid against the hepatocyte toxicity of Cd.

In conclusion, the present study reveals that alginic acid protect against the cytotoxicity of Cd in rat primary hepatocyte cultures and that the protective effects of alginic acid presumably result from a decrease in the Cd level, the effective sequestration of the reactive Cd ion, and the direct preventive effect on the active oxygen species in the hepatocytes.

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初代培養ラット肝細胞におけるカドミウム毒性に対するアルギン酸の抑制効果

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要　旨

カドミウム（Cd）は代表的な環境汚染金属であり、我が国では、岐阜県神岡鉱山（亜鉛・鉛・銀・Cd 等）の排水が流入した富山県神通川中流域で集中的に発生した骨軟化症を主体とするイタイイタイ病の発症原因物質がCdと考えられている。Cdの生産量は1910年より毎年増加しており、メッキ、顔料、塩化ビニール安定剤、電池、合金等幅広く使用されているが、これらのCdはほとんど回収不能であり、環境汚染の原因となっている。Cdによる急性中毒は、呼吸器からのCd微粒子の吸入により発生し、その主症状は胸痛、嘔吐、めまい、呼吸困難、肺炎、肺水腫などである。また、イタイイタイ病のように微量のCdでも、長期間摂取すると体内に蓄積し（全身に分布、その大部分は肝臓と腎臓）、肺気腫による呼吸困難や肝臓・腎機能障害によるビタミンDの肝・腎臓での活性化が阻害され、その結果、カルシウム、マグネシウム等の骨塩代謝異常が起こる。これらCdによる急性及び慢性中毒の治療には、体内に蓄積したCdを体外へ排泄させる水溶性の食物繊維等の機能性食品の使用が、生体に対する安全性が高く、有効と思われる。

本研究では、初代培養ラット肝細胞に対するCdの毒性並びに細胞組織に蓄積したCdに対して、褐藻類（ワカメ、ヒジキ、ノリ等）の細胞壁粘質に多く含まれる水溶性食物繊維アルギン酸のもつ毒性防御効果並びにCd排出促進作用機構について検討した。

キーワード：カドミウムの毒性，培養肝細胞，水溶性食物繊維，アルギン酸，毒性防御