IN VITRO TOXICITY TEST OF POISONOUS MUSHROOM EXTRACTS WITH ISOLATED RAT HEPATOCYTES

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Abstract—Effects of poisonous mushroom extracts on isolated rat hepatocytes were studied. Though no significant decrease in the cell viability was observed during the incubation of hepatocytes with the extracts at a concentration of 5%(v/v) of Amanita abrupta, A. gymnopis, and A. virosa caused marked decreases in the intracellular glutathione content in sharp contrast to the extracts of A. volvata and A. flavipes.

Comparative toxicity tests were carried out for the effects of the extract of A. abrupta, dl-propargylglycine, and α-amanitin. The extract of A. abrupta at a concentration of 1%(v/v) caused a marked decrease in the glycogen content, a noticeable elevation in the phosphorylase α activity, and a slight acceleration of lipid peroxidation in the hepatocytes. Although dl-propargylglycine decreased the intracellular glutathione content progressively with the incubation time, a significant effect of the chemical on lipid peroxidation and the glycogen content was observed only after prolonged incubation at a concentration of 5 mM. On the other hand, α-amanitin exerted a little effect on the hepatocytes at 1 μM. These results have indicated that the intoxication by the extract of A. abrupta on the hepatocytes might not due to independently each component, dl-propargylglycine and α-amanitin, but combined effect of these components or unidentified substances.

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**Key words**: *Amanita abrupta*, glutathione, glycogen, dl-propargylglycine, 
a-amanitin, isolated hepatocyte.

**INTRODUCTION**

Various kinds of mushroom poisoning occur occasionally, and the number of the 
victims in Japan has been estimated to be a few hundreds annually. For their clinical 
treatment, it is essential to identify the species of the mushrooms. However, the 
mushrooms responsible for these poisonings have usually been consumed or disposed 
of when the patients are found, and the species are only estimated from their clinical 
symptoms. Therefore, the biochemical changes in blood and urine have become 
useful information for diagnostic purposes, but efficient data have been not presented 
to serve the purposes.

We have studied on biochemical changes in the blood and livers of mice induced 
by the extracts from various species of poisonous mushrooms (Yamaura et al., 1981, 
1982, 1984, 1986; Yamaura, 1988). Recently, *in vitro* toxicity testing such as that 
with isolated hepatocytes has become noticeable as the method replacing *in vivo* tests 
(Moldéus et al., 1978). We attempted to qualify a species of poisoning mushroom 
using any residual samples and investigated the effects of aqueous extracts from toxic 
mushrooms on isolated rat hepatocytes. Comparative toxicity tests of the *Amanita abrupta* 
extract, dl-propargylglycine (PPG), and *a*-amanitin were also carried out 
with a view to the elucidation of intoxication mechanisms and the evaluation of *in vitro* 
toxicity testing.

**MATERIALS AND METHODS**

*Extracts of Toxic Mushroom*: Fruit bodies of *Amanita abrupta* (Tamashironitake), *A. virosa* (Dokutsurutake), *A. volvata* (Fukurotsurutake), *A. gymnopus* 
(Kabutengutake), and *A. flavipes* (Koganetengutake) were collected in the Nagano 
district, Japan, in 1987. The fruit body stored at −20°C was cut into small pieces 
and boiled for 15 min in 3-fold weight of distilled water. The aqueous extract of the 
mushroom was obtained by filtration after cooling to room temperature. The 
extrait was stored at −20°C until use.

*Chemicals*: *a*-Amanitin and glucose 1-phosphate (G-1-P) were purchased from 
Boehringer (Mannheim, F.R.G.), dl-Propargylglycine (PPG), 4-(2-hydroxyethyl)-1-
piperazineethanesulfonic acid (HEPES), and ethylene glycol-bis-(β-
ami-noethylether)-N,N,N',N'-tetraacetic acid (EGTA) from Sigma (St. Louis, U.S. 
A.), adenosine-5'-monophosphate (AMP) and β-nicotinamide-adenine dinucleotide, 
reduced form (NADH) from Oriental Yeast (Tokyo), and glycogen from Wako Pure 
Chemicals (Osaka) were used. All other reagents used were of analytical grade.

*Preparation of Isolated Hepatocytes*: Male Sprague-Dawley rats (5 weeks old, 
180–200 g) were purchased from Shimizu Laboratory Supplies (Kyoto). They were
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allowed free access to food (MF, Oriental Yeast, Tokyo) and water for a week prior to use. Hepatocytes were isolated by the collagenase perfusion method (Moldéus et al., 1978) with modifications described as follows. All buffer solutions used for the preparation of hepatocytes had been gassed with 95% oxygen-5% carbon dioxide. Under anesthesia with sodium pentobarbital, the liver was cannulated via the portal vein. The perfusion was started in situ with a Ca²⁺-free Hanks bicarbonate buffer, pH 7.4, containing 12.6 mM HEPES, 200 u/ml penicillin K, and 0.6 mM EGTA. After the liver was freed from the body, the perfusion was continued for 5 min at 37°C. Then the buffer was replaced by a Hanks bicarbonate buffer, pH 7.4, containing 12.6 mM HEPES, 200 u/ml penicillin K, 0.08% collagenase (Clostridium histolyticum, Boehringer), and 3.6 mM CaCl₂. The perfusion was continued further for 8-10 min at 37°C. After the digestive perfusion, the liver cells were dispersed into a Krebs-Henseleit buffer, pH 7.4, containing 12.6 mM HEPES, 200 u/ml penicillin K, and 10 mM or 100 mM glucose with gentle shaking. The hepatocytes were washed twice with the same buffer, and the suspension was kept in an ice cold bath. The cell viability was estimated by the Trypan Blue exclusion test and confirmed to be more than 93% before use.

Incubation of Hepatocytes: The incubation mixture contained the hepatocytes (final concentration: 2 x 10⁶ viable cells/ml) and the mushroom extract or chemical in a modified Krebs-Henseleit buffer, pH 7.4, containing 12.6 mM HEPES, 200 u/ml penicillin K, and 10 mM or 100 mM glucose. The suspension in an Erlenmeyer flask was incubated at 37°C with gentle shaking (60 strokes/min) and in an atmosphere of 95% O₂-5% CO₂. Aliquots of the suspension were taken for biochemical analysis at various intervals.

Biochemical Analysis: Cell viability was estimated by monitoring the lactate dehydrogenase activity leaked into the incubation medium (Moldéus et al., 1978).

Intracellular reduced glutathione (GSH) content was assayed colorimetrically (Ellman, 1959). The cells harvested by centrifugation were treated with a trichloroacetic acid (TCA) solution and the aqueous supernatant was used for the assay.

Lipid peroxidation was monitored by the formation of thiobarbituric acid reactive materials (Tappel and Zalkin, 1959). The result was expressed in terms of the amount of the authentic malondialdehyde showing the same absorbance.

Intracellular glycogen content was determined by the method of Várhonyi et al. (1980) with some modification. Cell pellets obtained from an aliquot (1.5 ml) of the incubation mixture were suspended in 1.0 ml of a 30% potassium hydroxide solution, and the suspension was heated in a boiling water bath for 30 min. After cooling, 2.5 ml abs. ethanol was added and the mixture was kept overnight at −70°C to precipitate glycogen. The precipitate obtained by centrifugation was washed with 2 ml of 96% ethanol followed by 60% ethanol. To the residual pellets, 1.0 ml of 2N sulfuric acid was added, and the mixture was incubated in a boiling water bath for 60 min. The glucose content in the hydrolysate was determined by the o-toluidine 

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method. The glycogen content was obtained by multiplying the glucose content by a factor of 0.925.

The phosphorylase a activity was determined by the method of Hue et al. (1979) with some modification. The hepatocytes obtained by centrifugation from 0.5 ml of the cell suspension was immediately frozen in liquid nitrogen. The sample was thawed and homogenized in 0.3 ml of a 50 mM glycyglycine buffer, pH 7.4, containing 100 mM sodium fluoride, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.5% glycogen, and 0.5 mM caffeine. The enzymic reaction was started by the addition of 0.2 ml of the homogenate to an equal volume of a 50 mM glycyglycine buffer, pH 6.1, containing 15 mM G-1-P, 2% glycogen, 0.3 M sodium fluoride, and 1 mM caffeine. After the incubation at 37°C for 30 min, the reaction was stopped by the addition of 0.4 ml of ice cold 5% TCA. The mixture was centrifuged at 10,000×g for 5 min. To 0.5 ml of the supernatant, 1.0 ml of a molybdate reagent and 1.5 ml of a 1-amino 2-naphthol-4-sulfonic acid reagent were added for the determination of inorganic phosphate. The glycogen phosphorylase activity was expressed as the phosphate formed per 10^6 cells per min.

RESULTS

1. Effects of Mushroom Extracts on Hepatocytes:

Isolated rat hepatocytes were incubated at 37°C in the modified Krebs-Henseleit buffer containing 10 mM glucose. Though the cell viability slightly decreased during incubation both in the absence and in the presence of poisonous mushroom extracts at a concentration of 5%(v/v), there was no significant difference between the two groups at each time (Table 1).

Table 1. Effects of poisonous mushroom extracts on cell viability of isolated rat hepatocytes.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cell viability (%)</th>
<th>Incubation time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>96.8±0.4</td>
<td>95.6±0.4</td>
</tr>
<tr>
<td>A. abrupta ext.</td>
<td>94.2±0.9</td>
<td>93.6±0.6</td>
</tr>
<tr>
<td>A. virosoa ext.</td>
<td>95.6±0.4</td>
<td>94.6±0.6</td>
</tr>
<tr>
<td>A. volvata ext.</td>
<td>95.7±0.6</td>
<td>94.4±0.5</td>
</tr>
<tr>
<td>A. gymnopus ext.</td>
<td>95.2±0.4</td>
<td>94.4±0.2</td>
</tr>
<tr>
<td>A. flavipes ext.</td>
<td>96.0±0.4</td>
<td>94.9±0.4</td>
</tr>
</tbody>
</table>

Hepatocytes were incubated at 37°C in Hanks bicarbonate buffer, pH 7.4, containing 10 mM glucose. Values are mean ± S. D. (n=3).

Final concentration of the mushroom extract in the incubation medium was 5%(v/v).

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On the other hand, the intracellular GSH content was affected by the addition of the mushroom extract. Extracts of *A. abrupta*, *A. virosa*, and *A. gymnopus* reduced the GSH content to 46%, 59%, and 68%, respectively, after 4 h incubation (Table 2). But no significant effects of the extracts from *A. volvata* and *A. flavipes* were observed.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Incubation time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>36.0±0.6</td>
</tr>
<tr>
<td><em>A. abrupta</em> ext.</td>
<td>35.9±1.2</td>
</tr>
<tr>
<td><em>A. virosa</em> ext.</td>
<td>33.1±2.1</td>
</tr>
<tr>
<td><em>A. volvata</em> ext.</td>
<td>34.8±0.3</td>
</tr>
<tr>
<td><em>A. gymnopus</em> ext.</td>
<td>29.8±0.3*</td>
</tr>
<tr>
<td><em>A. flavipes</em> ext.</td>
<td>34.4±1.0</td>
</tr>
</tbody>
</table>

Table 2. Effects of poisonous mushroom extracts on intracellular glutathione content in isolated rat hepatocytes.

Hepatocytes were incubated at 37°C in Hanks bicarbonate buffer, pH 7.4, containing 10 mM glucose. Values are mean ± S. D. (n=3). Final concentration of the mushroom extract in the incubation medium was 5%(v/v).

* Significant difference from control at each time ; P<0.01

2. Comparative Toxicity Tests of the Extract of *A. abrupta*, dl-Propargyglycine, and α-Amanitin:

Effects of the extract of *A. abrupta* which had been observed most significant on the hepatocytes were compared with those of PPG and α-amanitin. In this experiment, the incubation medium containing a high content (100 mM) of glucose was used, because high concentrations of carbohydrate are needed for maintaining intracellular glycogen in hepatocytes (Fig. 1) as has been reported by Krack et al. (1980). In the presence of 100 mM glucose, decrease in the cell viability was slight throughout the incubation time (viability : from 94.7±0.3% at zero time to 88.4 ± 1.8% after 5 h incubation) as well as that in the presence of 10 mM glucose. Also, no significant changes in the GSH content and lipid hydroperoxide formation were observed when 10 mM glucose had been replaced by 100 mM glucose.
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![Graph showing intracellular glycogen content over incubation time.](image)

**Fig. 1.** Effects of extracellular glucose on glycogen contents in isolated rat hepatocytes.
Hepatocytes were incubated at 37°C in a Krebs-Henseleit buffer, pH 7.4, containing 10 mM (□), 50 mM (△), or 100 mM (○) glucose.
Significant difference from zero time incubation: *, P<0.05; **, P<0.01

The concentrations of PPG (1 and 5 mM) and α-amanitin (1 and 5 μM) in the incubation medium were adopted in consideration of the toxic dose in vivo (Yamaura et al., 1986; Wieland, 1968). In the following experiments, the extract of *A. abrupta* was used at the final concentration of 1%(v/v), because the preliminary experiment had showed no linear relationship between the dose of the mushroom extract and the decreases of intracellular GSH content at the higher concentrations (more than 5%).

There were no significant differences in the cell viability between the control incubation and the incubations with 1% *A. abrupta* extract, 1 mM PPG, 1 or 5 μM α-amanitin at any time up to 5 h except for the 5 h with 5 mM PPG. The cell viability of the hepatocytes incubated for 5 h with PPG was decreased to 85% of the control. The initial content of intracellular GSH in the hepatocytes was slightly lower than that of the data in Table 2 depending on the individual differences of rats used for the preparation of the hepatocytes. In the presence of the extract of *A. abrupta* (1%), the intracellular GSH content decreased at a similar rate in the incubation either with 10 mM or 100 mM glucose. On the other hand, both 1 mM and 5 mM PPG caused marked decreases in the GSH content during incubation. But only a slight decrease in GSH was observed when 1 μM or 5 μM α-amanitin was added (Fig. 2)
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Fig. 2. Effects of Amanita abrupta extract, dl-propargylglycine, and α-amanitin on the intracellular glutathione content in isolated rat hepatocytes.

Hepatocytes were incubated at 37°C in a Krebs-Henseleit buffer, pH 7.4, containing 100 mM glucose in the absence (□) or in the presence of A. abrupta extract (1%, ◻), dl-propargylglycine (1 mM, □; 5 mM, ◻), or α-amanitin (1 μM, ◼; 5 μM, ◼). Significant difference from the control incubation at each time: *, P<0.05; **, P<0.01.

The lipid peroxidation reaction in the isolated hepatocytes was monitored by the thiobarbituric acid method. The extract of A. abrupta slightly accelerated lipid peroxidation after 1, 3, and 5 h of incubation. On the other hand, PPG caused the acceleration of lipid peroxidation only after 5 h incubation, and α-amanitin had no effect on the cell lipid peroxidation (Fig. 3).

Though the intracellular glycogen content was held at a constant level during the 5 h incubation in the buffer, the content decreased remarkably during incubation in the presence of the extract of A. abrupta. PPG and α-amanitin caused significant decreases in the glycogen content only after 5 h incubation (Fig. 4). On the other hand, the phosphorylase α activity in the hepatocytes was twice as much as in the control when the extract of A. abrupta was added. But there was no noticeable difference in enzymic activity between the control group and the 5 mM PPG or 1 μM α-amanitin group (Fig. 5).
Fig. 3. Effects of Amanita abrupta extract, dl-propargylglycine, and α-amanitin on the lipid peroxidation in isolated rat hepatocytes.
Hepatocytes were incubated 37°C in a Krebs-Henseleit buffer, pH 7.4, containing 100 mM glucose in the absence (□) or in the presence of A. abrupta extract (1%, □□□), dl-propargylglycine (5 mM, □□□), or α-amanitin (1 μM, □□□). Significant difference from the control incubation at each time: *, P<0.05; **, P<0.01.

Fig. 4. Effects of Amanita abrupta extract, dl-propargylglycine, and α-amanitin on the intracellular glycogen content in isolated rat hepatocytes.
Hepatocytes were incubated at 37°C in a Krebs-Henseleit buffer, pH 7.4, containing 100 mM glucose in the absence (□) or in the presence of A. abrupta extract (1%, □□□), dl-propargylglycine (5 mM, □□□), or α-amanitin (1 mM, □□□).
Significant difference from the control incubation at each time: *, P<0.05; **, P<0.01.
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![Graph showing phosphorlase activity over incubation time.](image)

**Fig. 5.** Effects of *Amanita abrupta* extract, dl-propargylglycine, and α-amanin on the intracellular phosphorlase α activity in isolated rat hepatocytes. Hepatocytes were incubated at 37°C in a Krebs-Henseleit buffer, pH 7.4, containing 100 mM glucose in the absence (□) or in the presence of *A. abrupta* extract (1%, ■), dl-propargylglycine (5 mM, □), or α-amanitin (1 μM, △). Significant difference from the control incubation at each time: *, P<0.05; **, P<0.01

**DISCUSSION**

Toxicity tests on isolated rat hepatocytes were carried out in the presence of aqueous extracts of poisonous mushrooms, *Amanita* sp. It is well known that hepatotoxic substances (e.g. acetaminophen (Moore et al., 1985), bromobenzene (Casini et al., 1987) and carbon tetrachloride (Long and Moore, 1988) etc.) cause various biochemical changes such as intracellular mobilization of calcium, activation of phosphorlase, glycogen degradation, depletion of GSH, and acceleration of lipid peroxidation. Therefore, we investigated the effects of extracts from toxic mushrooms on these parameters in isolated rat hepatocytes. The cell viability was also monitored for checking a total condition of cells.

It is difficult to assign the appropriate dose *in vitro* from the dose *in vivo*, because the real concentration of toxins in tissues after application to body was unable to be estimated. We tried preliminarily several doses of mushroom extracts for the experiments with isolated hepatocytes and chose the dose, 1% and 5%, causing apparent biochemical responses without severe cell damage. Though the cell
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viability did not decrease during the incubation for 4 h, the intracellular GSH content decreased significantly in the presence of extracts obtained from A. abrupta, A. virosa, and A. gymnopus.

Further experiments were conducted on the extract of A. abrupta in comparison with PPG and a-amanitin. PPG isolated as a toxic component from A. abrupta (Yamaura et al., 1986) as well as from A. solitaria (Chilton and Tsou, 1973) and A. pseudoporphryia (Hatanaka et al., 1985) causes a marked decrease in the intracellular GSH content. The decrease is accounted for by the inhibition of enzymes in the biosynthesis of GSH (Marcotte and Walsh, 1975; Uren et al., 1978; Johnston et al., 1979; Beatty and Reed, 1986). The acceleration of lipid peroxidation after 5 h incubation with PPG may be caused secondarily by the depletion of GSH, an endogenous antioxidant.

PPG showed no significant effects on the glycogen content and phosphorylase a activity in the hepatocytes during the incubation for 3 h. On the other hand, the extract of A. abrupta caused a marked decrease in the glycogen content and an increase in the phosphorylase a activity, concomitantly with a decrease in the GSH content and an increase in the lipid peroxidation. The marked decrease in the glycogen content by the addition of the extract was also observed in vivo experiments with mice (Yamaura et al., 1982, 1984). These results have suggested that the extract of A. abrupta enhances the hepatic glycolysis and/or inhibits the glycogenogenesis leading to an extensive disorder in carbohydrate metabolism. The intoxication mechanism is also supported by the results in in vivo experiments that the blood glucose level and the activities of glucose 6-phosphatase and glucose 6-phosphate dehydrogenase were decreased by the administration of the extract to mice (Yamaura et al., 1984).

a-Amanitin, a toxic peptide of A. phalloides, did not show any effect on the hepatocytes during 3 h incubation. The lack of response by the hepatocytes to a-amanitin within short-time incubation seems to require a long lag time for the appearance of the cell toxicity as the result of protein synthesis inhibition (Wieland, 1968; Gómez-Lechón et al., 1988). The interpretation is consistent with the fact that the lethal interval by a-amanitin in vivo is over 15 h and that the toxic symptoms caused by A. phalloides appear several hours after the ingestion in man (Wieland, 1968).

Though the poisonous mushroom, A. abrupta, causes a cholera-like symptoms in man (Yamaura, 1988) similar to those by A. phalloides, effects of the extracts on the hepatocytes were different from each other. It has been reported that the extract of A. abrupta contains a low concentration of PPG but not a-amanitin (Yamaura et al., 1984, 1986). The major toxic effects of the A. abrupta extract on the hepatocytes could not be interpreted by the action of PPG alone, because the pronounced effects of PPG are the decrease in the intracellular GSH content and the delayed increase in the lipid peroxide formation. The extract of A. abrupta showed a remarkable decreasing effects in the glycogen content, but effects on the GSH content and the
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lipid peroxide formation were less than that of PPG. These results reveal that the toxic effects exerted by the extract of A. abrupta on the hepatocytes might not due to independently each component, PPG or α-amantin, but combined effect of these components or unidentified substances. It has been also concluded that the toxicity test on isolated hepatocytes provides useful information for the study of the mechanism of intoxication caused by poisonous mushrooms.

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