The Protective Effect of Green Tea Extract on Alpha-amanitin Induced Hepatotoxicity

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Purpose: Alpha-amanitin induces potent oxidative stress and apoptosis, and may play a significant role in the pathogenesis of hepatotoxicity. This study examined the mechanisms of α-amanitin-induced apoptosis in vitro, and whether green tea extract (GTE) offers protection against hepatic damage caused by α-amanitin (AMA) induced apoptosis in vivo.

Methods: The effects of GTE and SIL on the cell viability of cultured murine hepatocytes induced by AMA were evaluated using an MTT assay. Apoptosis was assessed by an analysis of DNA fragmentation and caspase-3. In the in vivo protocol, mice were divided into the following four groups: control group (0.9% saline injection), AMA group (α-amanitin 0.6 mg/kg), AMA+SIL group (α-amanitin and silibinin 50 mg/kg), and AMA+GTE group (α-amanitin and green tea extract 25 mg/kg). After 48 hours of treatment, the hepatic aminotransferase and the extent of hepatonecrosis of each subject was evaluated.

Results: In the hepatocytes exposed to AMA and the tested antidotes, the cell viability was significantly lower than the AMA only group. An analysis of DNA fragmentation showed distinctive cleavage of hepatocyte nuclear DNA in the cells exposed to AMA. In addition, the AMA and GTE or SIL groups showed more relief of the cleavage of the nuclear DNA ladder. Similarly, values of caspase-3 in the AMA+GTE and AMA+SIL groups were significantly lower than in the AMA group. The serum AST and ALT levels were significantly higher in the AMA group than in the controls and significantly lower in the AMA+GTE group. In addition, AMA+GTE induced a significant decrease in hepatonecrosis compared to the controls when a histologic grading scale was used.

Conclusion: GTE is effective against AMA-induced hepatotoxicity with its apoptosis regulatory properties under in vitro and in vivo conditions.

Key Words: Alpha-amanitin, Green tea extract, Liver toxicity, Antidotes, Apoptosis

INTRODUCTION

Mushroom poisoning is an increasingly common medical emergency in many countries because outdoor leisure activities increase these days. The overwhelming majority of lethal mushroom poisonings
are attributable to the genus Amanita. Of the three common Amanita species—A. phalloides, A. verna, and A. virosa—A. phalloides has been held accountable for more than 90% of fatalities.\(^1\) Alpha-amanitin (α-amanitin) is the most potent of the toxins occurring in poisonous mushrooms in the genus Amanita. These differ from the other amatoxins in that they are heat resistant, alcohol and lipid soluble and indigestible in gastric and small intestinal enzymes.\(^2\) In addition, they can be absorbed rapidly by both gastric and duodenal tissues. Alpha-amanitin does not cause a direct cytolytic effect but blocks a RNA polymerase II. This results in inhibition of transcription of DNA and protein synthesis processes and leads to cell death.\(^3,4\) We reported the effect that these findings support that α-amanitin generates free radicals, which may contribute to its severe hepatotoxicity.\(^5\) Alpha-amanitin is also a strong apoptosis inductor.\(^6-8\) Experiments performed on canine hepatocyte cultures suggest that apoptosis may play a significant role in pathogenesis of hepatic damage in course of amanitin intoxication.\(^9\) Green tea catechins are involved in many biological activities and have antioxidative, antiviral, and antitumor properties.\(^10\) And anti-proliferation and apoptosis controlling effects of green tea polyphenols were reported by many previous studies. This study aims to examine the protective effect of green tea on the disturbances in apoptosis related factors produced due to α-amanitin exposure that subsequently induces liver cell damage in both in vitro and in vivo models and to compare this with silibinin, a known antidote.

**MATERIALS AND METHODS**

1. *In vitro* studies

1) Chemicals and materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin, streptomycin, silibinin (SIL) and α-amanitin (AMA) were purchased from Sigma Chemical (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco’s Modified Eagle Medium (DMEM), trypan blue, trypsin and Hank’s buffer were obtained from Hyclone Laboratories, INC. (Logan, UT, USA). All other reagents used were of analytical grade.

2) Preparation of extract

The green tea extract (GTE) was prepared from green tea (Thea sinensis L., Theaceae) cultivated in Bosung area in Chonnam province, Republic of Korea. The GTE was prepared according to Maity et al.\(^11\) by soaking 15 gram of green tea powder in 100 ml of boiling distilled water for 5 minutes. The GTE contained epigallocatechin gallate (337 mg/l), epigallocatechin (268 mg/l), epicatechin (90 mg/l), epicatechingallate (60 mg/l), and coffeic acid (35 mg/l) as determined by the HPLC method\(^12\).

3) Cell culture

The protocol of the experiment was approved by Animal Care and Use ethical Committee of Chosun University (CIACUC2016-A0038). Hepatocytes were isolated from white male laboratory mice with weight 20-30 gram by collagenase perfusion.\(^13\) The viability of the isolated hepatocytes was over 90% as determined by trypan blue exclusion test. The cells were cultured overnight in a humidified atmosphere at 37°C. This medium was renewed the next day with Hank’s buffer with or without AMA, GTE and SIL. AMA (60 μmol/L), GTE (2 μM), SIL (500 μmol/L) were dosed at different final concentrations by previous study.\(^14,15\) The incubation buffer was changed to Hank’s buffer because this did not interfere with other factors in this experiment. The green tea extract and tea polyphenols were dissolved in ethanol (final concentration of ethanol was 0.25%), before being added to the incubation medium. The control consisted of the medium, ethanol and DMSO. Viability and apoptosis evaluation of cultured cells were performed after 24 hours of exposure to α-amanitin and/or tested antidotes.

4) Analytical methods

Retained functional integrity and viability of cultured hepatocytes were assessed using the MTT assay. Reduction in yellow salt MTT by mitochondrial dehydrogenases in viable cells to a purple formazan precipitate was determined by measuring the absorbance.
at 570 nm on a plate reader (Bio-TEK Instruments, Winooski, VT, USA). Detection of apoptosis in murine hepatocyte cultures was performed by analysis of DNA fragmentation (DNA laddering). For analysis of DNA fragmentation, hepatocyte DNAs was extracted and purified using the ApopLadderEx™ Kit (Takara Bio Inc., Otsu, Shiga, Japan). For analysis of DNA fragmentation by agarose gel electrophoresis, total DNA was extracted and purified using the ApopLadderEx™ Kit. The individual DNA extracts were loaded into the wells of a 1.5% agarose gel containing 1 μg/ml of ethidium bromide and the bands were visualized by the GelDocXR (BioRad, USA) using the QuantityOne4.6.1 software. Intensity and mechanisms of apoptosis processes were evaluated by determination of caspase-3 activity (Caspase-3 Colorimetric Assay Kit, BioVision Research Products, USA).

2. In vivo studies

1) Animals and laboratory
After getting approval for the experimental protocol as already described, male BALB/c mice weighing 20-30 gram were used (n=40). These were housed in a 12 hours light/12 hours dark cycle in the mouse accommodation room. Mice were fed with standard chow and water and were fasted for 12 hours before the experiment. All efforts were made to minimize animal suffering.

2) Experimental protocol
The mice were randomly assigned to one of four groups (n=10 for the control group; CTL, n=10 for the α-amanitin treated group; AMA, n=10 for the AMA and silibinin treated group; AMA+SIL, and n=10 for the AMA and GTE treated group; AMA+GTE). After dissolving it in distilled water, α-amanitin was administered to all animals except those in the CTL group in doses of 0.6 mg/kg intraperitoneally (i.p.) (in 0.2 mL distilled water) because it represents 50% lethal dose value (LD50) of α-amanitin in mice according to previous study. During the experimental procedure, GTE (25 mg/kg) and silibinin (50 mg/kg) at 12-hours intervals were given for 48 hours, while normal saline (NS) was given every 12 hours as previous study protocol16. NS was also administered between the GTE treatments in order to reduce the potential stress factor associated with the number of injections and to ensure standardization among the mice in terms of injury. All injections were performed intraperitoneally (i.p.) to lower abdominal quadrant.

3) Data collection and processing
At the end of the 48-hours experimental protocol, the mice were killed by cervical dislocation under ether anesthesia, and blood was collected by cardiac puncture. Immediately afterwards, the right hepatic lobe was fixed in 10% formalin buffer and embedded in paraffin for examination under light microscopy (Olympus BX-50, Japan).

4) Biochemical analysis
Blood specimens (0.3 ml) were centrifuged to separate the sera, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) values were then measured using the methods recommended by the International Federation of Clinical Chemistry (IFCC).

5) Histopathological study
Five-micron sections were taken from liver tissues and stained with hematoxylin-eosin. Specimens were assessed by independent board-certified pathologist under a blind fashion in terms of congestion, necrosis, cytoplasmic vacuolization, eosinophilia, nuclear pyknosis and inflammatory cell density. A Hepatic Histological Damage Score (HHDS) was used during evaluation 0: no or minimal damage, 1: mild damage, 2: moderate damage, and 3: severe damage.

3. Statistical analysis
Differences between MTT values and biochemical parameters were analyzed by the one-way ANOVA with Tukey test, whereas caspase-3 values and histopathological parameters were analyzed by the Kruskal-Wallis test. All data were expressed as the form of mean (± SEM) and analyzed by SPSS software version 19.0 (SPSS Inc., Chicago, IL, USA). p<0.05 was regarded as sta-
RESULTS

In vitro the cytotoxicity of αamanitin and the protective effect of GTE were evaluated by the MTT assay in murine hepatocytes. As shown in Fig. 1, the cell viability decreased remarkably (p<0.001), after incubation with αamanitin. In hepatocytes exposed simultaneously to AMA and tested antidotes (AMA+GTE and AMA+SIL groups), cell viability was significantly lower compared to the control, but remained significantly higher compared to the AMA group.

Analysis of DNA fragmentation by agarose gel electrophoresis showed changes in the characteristic of apoptosis with a distinctive cleavage of hepatocyte nuclear DNA after 24 hours of exposition to AMA. But in AMA+GTE and AMA+SIL groups showed the reduction of cleavage. In control, GTE and SIL groups no cleavage of hepatocyte nuclear DNA typical for apoptosis was revealed (Fig. 2).

Similarly, values of apoptosis marker (caspase-3), in AMA+GTE and AMA+SIL groups were higher compared to the control, but significantly lower compared to the AMA group (Fig. 3).

In vivo study, aminotransferase values increased significantly in the AMA group compared to the CTL group (p<0.01, respectively). However, the AST and ALT values in the AMA+SIL group were lower than those of the AMA group. AMA+GTE group showed a marked decrease in the serum AST and ALT levels as compared with the AMA and AMA+SIL groups. However, they were still higher than those of the CTL group. The biochemical analysis results of the liver function tests of all groups were given in Table 1.

In the AMA group, light microscopy revealed liver cell injury, periportal mononuclear cell (MNC) infiltration, focal necrosis, and also diffuse liver cell necrosis and sinusoidal dilatation in specimens from some mice (Fig. 4B). Less liver cell injury, periportal MNC infiltration, focal necrosis and sinusoidal dilatation were observed in the AMA+SIL and AMA+GTE group.
As shown in Fig. 4E, significantly lower hepatic injury scores compared to those in the AMA group (3.4±0.6) were observed in the CTL group (0.2±0.05), the AMA+SIL group (1.8±0.6), and in the AMA+GTE group (1.5±0.5) (p<0.01 respectively).

**DISCUSSION**

This study investigated the efficacy of green tea extract in an experimental in vitro and in vivo model of hepatotoxicity induced in mice with AMA and compared it with that of silibinin, used as an antidote in the treatment of poisoning. In vitro part of present experiment, both GTE and SIL efficiently protected murine hepatocytes against AMA-induced viability loss and cell death. While hepatocyte viabilities in AMA+GTE and AMA+SIL groups were lower compared to the control, it was significantly higher than in AMA group. Similar results were obtained, assessing values of apoptosis marker (caspase-3). Thus, probably protective effect of GTE and SIL against AMA-induced apoptosis and viability loss resulted from an antidote-associated inhibition of AMA uptake by murine hepatocytes.

In vivo part of this study, we primarily determined the hepatotoxic dose of AMA and GTE and SIL solvent on the liver. We employed an increase in aminotransferase levels as a supporting biochemical parameter in addition to histopathological analysis in confirming AMA-induced hepatotoxicity. AMA+GTE group resulted in a marked decrease in the serum AST and ALT levels as compared with the AMA (p<0.001 for AST and ALT) and AMA+SIL groups (p<0.002 for AST and ALT). And histopathologic finding of AMA+antidotes groups the Hepatic Histological Damage Score (HHDS) was significantly lower compared to the AMA group, but remained significantly higher compared to the CTL group.

AMA has been shown to cause toxicity in hepatocytes cell culture via necrosis and apoptosis. AMA directly interacts with the enzyme RNA polymerase II in eukaryotic cells and inhibits the transcription, causing a progressive decrease in mRNA, deficient protein synthesis, and cell death. For this reason, metabolically active tissues dependent on high rates of protein synthesis, such as the cells of the gastrointestinal tract, hepatocytes, and the proximal tubules of kidney, are disproportionately affected. But, recent in vitro and in vivo studies have suggested oxidative stress might contribute to powerful amatoxin hepatotoxicity in studies. And among other potential toxic mechanisms, it has been proposed that AMA acts in synergy with endogenous cytokines (e.g., tumor

**Table 1. Comparison of biochemical liver function test results.**

<table>
<thead>
<tr>
<th>Biochemical Parameters</th>
<th>AST</th>
<th>ALT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL group</td>
<td>61.3±4.2</td>
<td>26.7±2.7</td>
</tr>
<tr>
<td>AMA group</td>
<td>3723±40*</td>
<td>4378±321*</td>
</tr>
<tr>
<td>AMA+SIL group</td>
<td>1659±129*,#</td>
<td>2199±215*,#</td>
</tr>
<tr>
<td>AMA+GTE group</td>
<td>1068±60*,**</td>
<td>1079±19*,**</td>
</tr>
</tbody>
</table>

AST, ALT: IU/L. Values are expressed as means±SEM. n=10 all groups. Statistical analysis was done by One way ANOVA followed by Bonferroni test.

* p<0.01 difference from CTL;
# p<0.01 difference from the AMA group;
** p<0.01 difference from AMA and AMA+SIL groups.
necrosis factor) and that this might cause cell damage through the induction of apoptosis\(^{20}\). Apoptosis is a form of programmed cell death that occurs in multicellular organisms. Biochemical events lead to characteristic cell changes and death. These changes include

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**Fig. 4.** Represents the micrographs related to histopathologic evaluation (×100 enlargement after hematoxylin-eosin staining) of the liver tissues. Section (A) shows normal liver architecture. Section (B) shows severe hepatocellular necrosis after AMA administration, which is mainly centrilobular in nature. Sections (C) and (D) show a marked decrease in the severity of hepatocellular necrosis. (E): The degree of liver necrosis was further characterized by using Hepatic Histological Damage Score (HHDS) analysis. Each value represents the mean±SEM.

* \(p<0.001\) AMA vs. CTL; AMA+SIL; AMA+GTE,

** \(p<0.001\) AMA+SIL vs. CTL; AMA+GTE,

*** \(p<0.05\) AMA+GTE vs. AMA+SIL.

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zeiosis, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA decay. Because apoptosis cannot stop once it has begun, it is a highly regulated process. Apoptosis can be initiated through one of
two pathways. In the intrinsic pathway the cell kills itself because it senses cell stress, while in the extrinsic pathway the cell kills itself because of signals from other cells. Weak external signals may also activate the intrinsic pathway of apoptosis. Both pathways induce cell death by activating caspases, which are proteases, or enzymes that degrade proteins. The two pathways both activate initiator caspases, which then activate executioner caspases, which then kill the cell by degrading proteins indiscriminately. Caspases play the central role in the transduction of apoptotic signals. Caspases are enzymes regulating numerous intracellular processes and play a crucial role in programmed cell death. Caspase-3 activity is established as an apoptotic marker\(^{21,22}\). AMA is a toxin that inhibits global transcription by different mechanisms and induces programmed cell death.

Green tea made from *Camellia sinensis* (Theaceae family) is a widely consumed beverage which provides a dietary source of biologically active compounds considered to be beneficial to human health. Green tea extract contains polyphenols, tannin and caffeine, and catechins have been most widely studied to prove the pharmacological action of green tea. They have demonstrated significant antioxidant, anticarcinogenic, anti-inflammatory, thermogenic, probiotic, and antimicrobial properties\(^{23-25}\).

Although the researches concerning GTE are still on the road accompanied with quite a few controversies, GTE is more likely to be beneficial to health. The results of the present analysis, combined with the previously published report from Dekant et al.\(^{26}\) suggest that the composition of green tea preparations that most closely reflects that of a traditional infusion is safe\(^{27}\). But, preparations based on concentrated extracts, containing high levels of individual constituents, such as EGCG, and consumed in solid dosage form, may require health-based guidance values to assure their safe use. Because the catechins of green tea are synergistic with each other, it is more effective than taking EGCG alone.

There are various limitations to our experimentation, particularly their applicability to clinical practice. We could not measure the effects of each component of GTE catechins separately. We were only able to support the thesis that GTE prevents AMA-related hepatotoxicity through its apoptosis controlling effect by means of biochemical and histopathological data, and we did not measure various anti-inflammatory and apoptosis parameters. In addition, the pharmacodynamics of orally ingested α-amanitin may differ from injected intraperitoneally. Also, intravenous administration of silibinin is ideal, but it was administered intraperitoneally in this experiment.

CONCLUSION

Summing up, AMA-induced apoptosis in murine hepatocyte cultures is caspase-3-dependent. Murine hepatocyte cultures exposed simultaneously to AMA and tested antidotes (GTE or SIL) showed significantly higher cell viability and significantly lower values of apoptosis markers compared to the cultures exposed to AMA, only. And in murine model, green tea extract was effective in limiting hepatic injury after α-amanitin poisoning by decreases of aminotransferases and pathologic degrees of hepatonecrosis.

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ACKNOWLEDGEMENTS

This study was supported by research fund from Chosun University, 2018.

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