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A Comparison of the Effectiveness of Silibinin and Resveratrol in Preventing Alpha-Amanitin-Induced Hepatotoxicity

Aynur Sahin[1], Mualla Aylin Arici[2], Yeliz Yilmaz[3,4], Sule Kalkan[5], Nergiz Durmus[2], Bekir Ugur Ergur[6], Ilkay Yakut Aksu[7], Neşe Atabey[4,8] and Yesim Tuncok[5]

[1]Department of Emergency Medicine, Karadeniz Technical University School of Medicine, Trabzon, Turkey, [2]Department of Medical Pharmacology, Dokuz Eylul University School of Medicine, Izmir, Turkey, [3]Department of Medical Biology and Genetics, Dokuz Eylul University Institute of Health Sciences, Izmir, Turkey, [4]Dokuz Eylul University Izmir Biomedicine and Genome Institute (iBG-izmir), Izmir, Turkey, [5]Division of Clinical Toxicology, Department of Medical Pharmacology, Dokuz Eylul University School of Medicine, Izmir, Turkey, [6]Department of Histology and Embriology, Dokuz Eylul University School of Medicine, Izmir, Turkey, [7]Department of Physiology, Dokuz Eylul University School of Medicine, Izmir, Turkey and [8]Department of Medical Biology and Genetics, Dokuz Eylul University School of Medicine, Izmir, Turkey

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Abstract: Amanita phalloides species mushrooms containing alpha-amanitin (α-AMA) are responsible for the majority of fatal mushroom intoxications and can lead to severe poisonings resulting in hepatotoxicity and acute hepatic failure. Existing antidotes, such as silibinin, are not sufficiently effective in the prevention and/or resolution of α-AMA-induced hepatotoxicity. We investigated the effects of resveratrol on α-AMA-induced hepatotoxicity and compared with silibinin, a known antidote using *in vivo* and *in vitro* toxicity models. In the *in vivo* protocol, resveratrol (30 mg/kg) was given simultaneously with α-AMA (α-AMA + SR) or 12 (α-AMA + 12R) or 24 (α-AMA + 24R) hr after α-AMA administration. Silibinin (5 mg/kg) (α-AMA + Sil) and normal saline (α-AMA + NS) were given simultaneously with α-AMA. We found that liver transaminase levels in α-AMA + SR and α-AMA + 12R groups and histomorphologic injury score in the α-AMA + SR, α-AMA + 12R, α-AMA + 24R and α-AMA + Sil groups were significantly lower than that of the α-AMA + NS group. Resveratrol decreased mononuclear cell infiltration, necrosis and active caspase-3 immunopositivity in the liver. In the *in vitro* protocol, the effects of resveratrol and silibinin were evaluated in a reduction in cell viability induced by α-AMA in THLE-2 and THLE-3 hepatocytes. Neither resveratrol nor silibinin was found to be effective in increasing cell viability decreased by α-AMA + NS. As a conclusion, resveratrol was found to be effective in α-AMA-induced hepatotoxicity with its anti-inflammatory properties in *in vivo* conditions. It is a promising compound with the potential for use in the treatment of hepatotoxicity associated with *Amanita phalloides* type mushroom poisonings.

Morbidity and mortality caused by mushroom intoxications is an important global health problem. *Amanita phalloides* mushrooms containing alpha-amanitin (α-AMA) are responsible for the majority of fatal mushroom poisonings [1]. α-AMA is the primary toxin responsible for hepatotoxicity [2]. After being absorbed by the gastrointestinal system (GIS) and entering the systemic circulation, α-AMA passes into the hepatic sinusoidal membrane, its main target, with the organic anion transporter peptide (OATP)1B3. It then blocks mRNA synthesis and DNA transcription by inhibiting RNA polymerase II in the nucleus [3]. Cell death therefore occurs as a result of deterioration of protein synthesis in cells with high turnover, such as hepatocytes, GIS epithelial and hepatic tubule cells [4,5].

α-AMA-induced mushroom intoxication consists of four clinical stages. Unfortunately, the clinical signs become obvious 24–72 hr after mushroom consumption [4].

The treatment of α-AMA-containing mushroom intoxication consists of gastric lavage and activated charcoal administration in the first hour after ingestion and the administration of silibinin as an antidote [6]. As the toxin enters the enterohepatic circulation, repeated doses of activated charcoal are useful for treatment [7]. However, poisonings resulting in liver failure and mortality are still observed. Levels of clinical evidence for the efficacy of extracorporeal methods, such as haemodialysis, haemoperfusion and plasmapheresis, for the purpose of toxin removal are not high [8–10].

Resveratrol is a compound with a polyphenolic structure containing non-flavonoid stilbenes. It is naturally present in approximately 72 plants, particularly red grapes but also in peanuts, pine and legumes. With its antioxidant and anti-inflammatory effects, there have been numerous studies on the prevention and treatment of hepatotoxicity due to various drugs and toxins about resveratrol. It has been shown to exhibit protective properties in hepatotoxicity developing in association with various agents, such as paracetamol, carbon tetrachloride (CCl_4) and alcohol [11–15]. In addition to the preclinical *in vitro* and *in vivo* studies, recent studies including systematic reviews have demonstrated that resveratrol provides liver protection against cholestatic, drug- and alcohol-induced injury[16,17].

Objective.

The purpose of this study was to investigate the early and late-term effectiveness of resveratrol in both *in vitro* and

Author for correspondence: Mualla Aylin Arici, Division of Clinical Pahramacology, Department of Pharmacology, Dokuz Eylul University School of Medicine, Balcova, 35340 Izmir, Turkey (e-mail draylinarici@gmail.com).

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in vivo models of α-AMA-induced hepatotoxicity and to compare this with silibinin, a known antidote.

Methods

In vitro studies.

Cell culture. Human normal hepatocyte cell lines THLE-2 and THLE-3 were purchased from American Type Culture Collection (ATCC) and maintained in accordance with the supplier's instructions. Following cell adherence to multiwell plates, the medium was replaced with BEGM complete medium including α-AMA (Sigma-Aldrich®, St Louis, Missouri, USA). The effects of resveratrol (Sigma-Aldrich®) and silibinin (Sigma-Aldrich®) were tested following 24-hr incubation of α-AMA. Dimethyl sulfoxide (DMSO) was used as a control for resveratrol and silibinin treatments.

Sulforhodamine B (SRB) assay. Sulforhodamine B (SRB) is an assay widely used to detect drug cytotoxicity and cell viability based on the quantitative staining of cellular proteins by the SRB [18]. Hence, this assay was used to determine cellular cytotoxicity in this study. THLE-2 and THLE-3 cells were plated to a density of 2×10^3 and 3×10^3 cells/well in pre-coated 96-well plates, respectively. After 24 hr, cells were attached and medium was replaced with treatment medium. The effect of α-AMA on cell viability was tested after incubation for 24, 48 and 72 hr, followed by 24-hr incubation of α-AMA. The effects of resveratrol and silibinin were also tested for 24, 48 and 72 hr by SRB assay [19]. Inhibitory concentration 50 (IC_{50}) values were calculated using GraphPad Prism 6.

xCELLigence real-time cell analysis. Real-time effects of the tested compounds on cells were evaluated using 96-well E-plates on xCELLigence system (Acea Biosciences, San Diego, California, USA) as previously described [20]. Cells were plated and treated with α-AMA for 24 hr. Cell index values representing impedances were measured every 15 min. and used to plot cell viability curves. Each condition was tested at least four times. Statistical analysis was performed using GraphPad Prism 6, using two-way ANOVA for group comparisons. Measurements at 24, 48 and 72 hr were used to plot for regression and slope analysis, and group comparisons were carried out using one-way ANOVA.

In vivo study design.

Approval for the experimental protocol was obtained from the Dokuz Eylul University Medical Faculty (DEUMF) Animal Experiments Ethical Committee (No:44/2014). Male BALB/c mice weighing 20–30 g were used (n = 107). These were housed in a 12-hr light/12-hr dark cycle in the DEUMF Laboratory Animals Department mouse accommodation room. Mice were fed with standard chow and water and were fasted for 12 hr before the experiment.

Preliminary study.

- Basal liver function test values were determined in mice subjected to no procedures (n = 3).
- To find the hepatotoxic dose of α-AMA, the lethal dose 50 (LD_{50}) (0.6 mg/kg, n = 4) for α-AMA (Sigma Chemical Company, St.Louis, MO, USA) as described in the previous literature was administered to four mice. As no mortality occurred in the mice at the end of 48 hr, logarithmic dosage increments were applied to the mice (1.08 mg/kg, n = 10; 1.4 mg/kg, n = 10 and 1.92 mg/kg, n = 8). No LD_{50} was again observed at these three doses, and the lowest of these four doses (1.4 mg/kg) determined to produce significant serum aminotransferase elevation and histomorphological score increases was selected for use in the main experimental protocol.
- The hepatotoxic effect of DMSO, the solvent of silibinin and resveratrol, was also assessed.

Experimental protocol. The mice were randomly assigned to one of five groups: α-AMA + NS (simultaneously administration of normal saline with α-AMA, served as a control for treatment), α-AMA + SR (simultaneously administration of resveratrol with α-AMA), α-AMA + 12R (resveratrol was given 12 hr (h) after administration of α-AMA), α-AMA + 24R (resveratrol was given 24 hr after administration of α-AMA) and α-AMA + Sil (simultaneously administration of silibinin with α-AMA) (fig. 1).

During the experimental procedure, resveratrol (30 mg/kg; Sigma Chemical Company) was given at 12-hr intervals and silibinin (5 mg/kg; Sigma Chemical Company) at 6-hr intervals for 48 hr (fig. 1), while normal saline (NS) was given every 6 hr [21,22]. NS was also administered between the resveratrol 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.

Data collection and processing. At the end of the 48-hr experimental protocol, the mice were killed by cervical dislocation under ether anaesthesia, and blood was collected by cardiac puncture. Immediately afterwards, 2/3 of the right hepatic lobes were fixed in 10% formalin buffer and embedded in paraffin for examination under light microscopy (Olympus BX-50, Japan). The remaining 1/3 of the liver specimens were fixed in phosphate-buffered 2.5% glutaraldehyde for electron microscope examination (Carl Zeiss Libra 120 EFTEM, Germany). Specimens from the left part of the liver were placed into cryotubes and stored at −80°C for oxidative stress parameter and antioxidant enzyme measurements.

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) (Hitachi 902 analyser) [23,24].

Antioxidant enzyme measurement. Malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH) levels were measured using the spectrophotometric method (Oxis International, USA). Homogenate and supernatant SOD activity were determined using Bioxytech SD-525 (Oxis International, USA), MDA levels using Bioxytech MDA-586 (Oxis International, USA) and protein concentrations using a total protein kit (Randox Labs., Crumlin, UK).

Histomorphological analysis.

1 *Haematoxylin–eosin staining:* Five-micron sections were taken from liver tissues and stained with haematoxylin–eosin. Specimens were assessed blind under a light microscope 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.
2 *The TUNEL method:* DNA fragmentation was shown using a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate DNA nick-end labelling (TUNEL) kit (In Situ Cell Death Detection Kit, Roche, Manheim, Germany)[25].
3 *Active caspase-3 immunohistochemical staining method:* After being kept in a stove at 60°C for 24 hr, sections were stained using the caspase-3 immunohistochemical staining method. Mouse-specific

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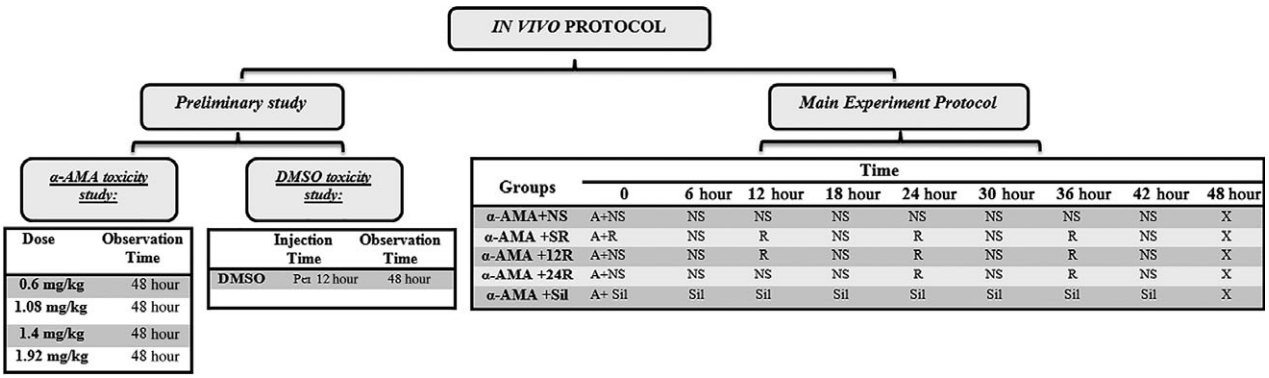


Fig. 1. Experimental protocol (α-AMA + NS: Simultaneous α-amanitin and normal saline administration, AMA + SR: Simultaneous α-amanitin and resveratrol administration, α-AMA + 12R: α-amanitin and resveratrol 12 h after administration of α-AMA, α-AMA + 24R: α-amanitin and resveratrol 24 hr after administration of α-AMA and α-AMA + Sil: Simultaneous α-amanitin and silibinin administration).

anti-caspase-3 (Neomarkers, Fremont, CA) was used to show active caspase-3 immunoreactivity [26].

Electron microscopy examination. Liver tissues (~1 mm^3) were fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 48 hr at 4°C. The tissues were washed in the same buffer overnight after the primary fixation. The tissues were post-fixed with 1% osmium tetroxide in sodium phosphate buffer for 1 hr at 4°C. The post-fixed tissues were then washed in the same buffer and dehydrated by a graded series of ethanol starting at 50% for each step for 10 min., and finally with propylene oxide. The tissue specimens were embedded in Araldite. Ultrathin sections were cut from the blocks using an ultramicrotome (Leica, Deerfield, IL, USA) and mounted on copper grids. These were double-stained with uranyl acetate and lead citrate before being examined under a transmission electron microscope (Libra 120; Carl Zeiss, Germany) and digitally photographed [27].

Primary data analysis. ANOVA was used in the statistical analysis of biochemical measurements and enzyme activities, and the Tukey-Kramer multiple comparisons test was applied to identify the group representing the source of variation. The nonparametric Mann–Whitney U-test and Kruskal–Wallis test (GraphPad Software, San Diego California, USA) were used in the statistical analysis of histomorphological examinations. All data were expressed as mean ± standard deviation. $p < 0.05$ was regarded as statistically significant.

Results

In vitro results.

To determine the cytotoxic effect of α-AMA in liver cells, cell lines generated from normal liver cells, THLE-2 and THLE-3, were used. Cells were incubated with 0.1, 1, 5 and 10 μM α-AMA. The effect of α-AMA on cell viability and growth inhibitory 50 (GI_{50}) doses of α-AMA for each cell line was then determined using the SRB assay (fig. 2A). The results for 24-hr incubation with α-AMA were therefore used to determine GI_{50} doses. Values of 8.695 μM and 9.20 μM α-AMA were determined as the GI_{50} doses for the THLE-2 and THLE-3 cell lines, respectively. Therefore, we decided to continue our investigation with 24-hr incubation of 8 μM α-AMA to mimic the effects of α-AMA poisoning in cell culture.

To identify an effective dose of resveratrol against cytotoxicity generated by α-AMA, THLE-2 and THLE-3 cells were incubated with 1, 5 and 10 μM resveratrol. The effect of resveratrol and silibinin on viability was determined using the SRB assay (fig. 2B). No significant effect of either resveratrol or silibinin was observed for 24- or 48-hr incubations. However, 72-hr incubations of 1 μM and 5 μM resveratrol and 80 μM silibinin treatments increased THLE-2 cell viability compared to cells treated only with α-AMA. All doses of resveratrol and silibinin were found to be effective compared to cells treated only with α-AMA for THLE-3 cells. Compared to THLE-2 and THLE-3 cells treated with α-AMA only, 1 μM resveratrol treatment gave better results for both cells. We therefore decided to use this dose to observe its effect in live cell analysis over a longer incubation time.

For xCELLigence live cell analysis, α-AMA treated cells were incubated with resveratrol, silibinin or DMSO. α-AMA significantly reduced the viability of both THLE-2 and THLE-3 cells ($p < 0.0001$) (fig. S1). In terms of cell index measurements, neither resveratrol nor silibinin was able to prevent the effects of α-AMA on cell viability and proliferation. No statistically significant difference was observed between the effects of resveratrol and silibinin on cell viability and proliferation when cells were treated with α-AMA.

In vivo study results.

Preliminary study results. Sufficient hepatotoxicity was not observed in mice using the LD_{50} (0.6 mg/kg) for α-AMA reported in previous studies. When the dose of α-AMA was increased logarithmically, AST and ALT values at 1.4 mg/kg and 1.92 mg/kg increased significantly in the α-AMA group compared to the control group ($p < 0.05$). Hepatotoxicity in the histomorphological damage score increased in a dose-dependent manner. A dose of 1.4 mg/kg α-AMA causing a statistically significant elevation in liver function tests and leading to sufficient histomorphological injury was therefore used to induce hepatotoxicity in the experiments. The DMSO used as a solvent caused no biochemically or histomorphologically significant damage to the liver (table 1).

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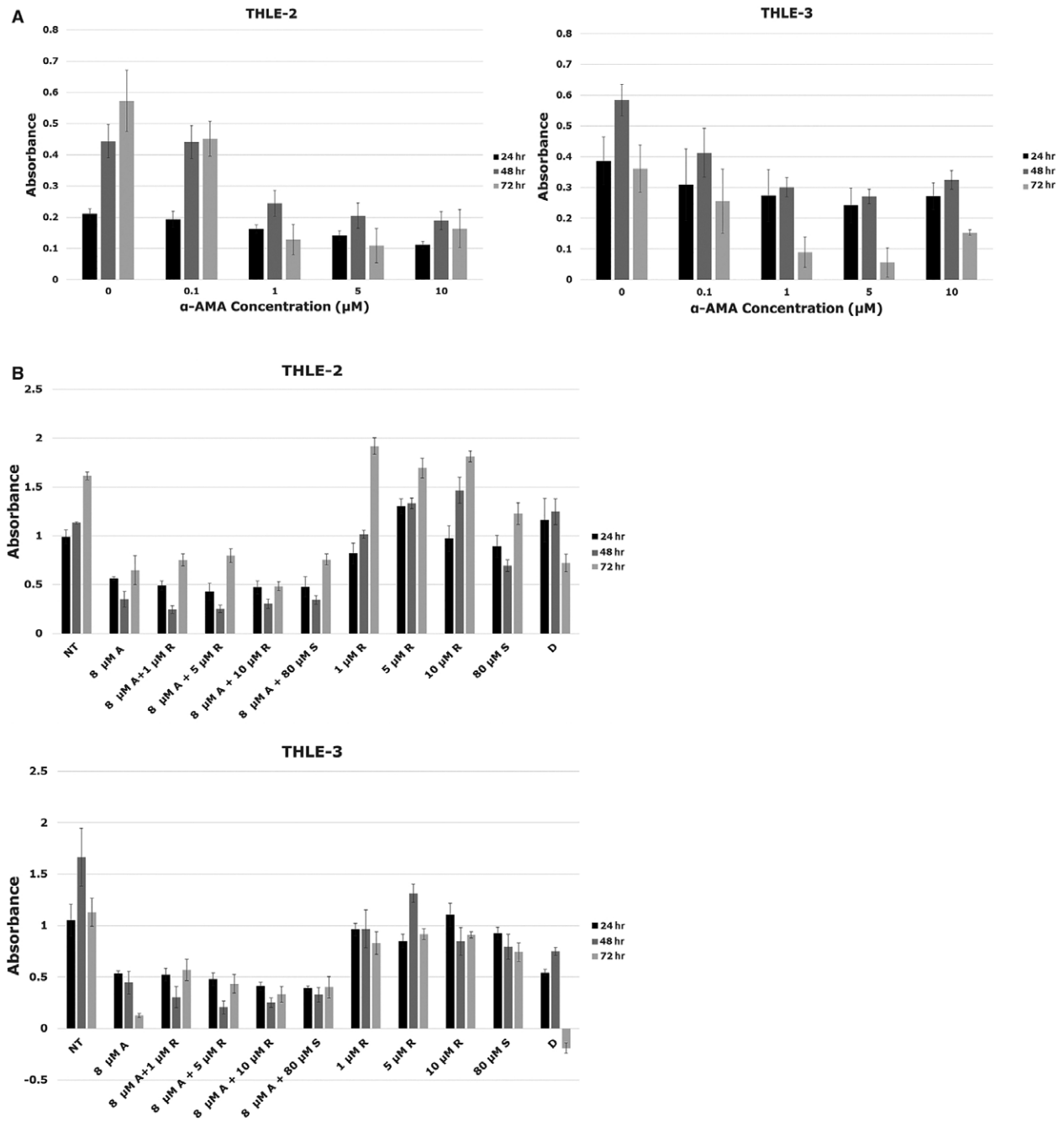


Fig. 2. (A) The role of resveratrol and silibinin on cell viability of α-AMA intoxicated liver cells, THLE-2 and THLE-3. (A): IC50 concentrations of α-AMA for THLE-2 and THLE-3 cells were determined by incubating cells with 0.1 μM, 1 μM, 5 μM and 10 μM α-AMA for 24, 48 and 72 hr and performing SRB assay. The absorbances were measured at 565 nm and background at 690 nm; absorbances of the medium with no cells were removed as blank. (B) Cells were treated with 8 μM α-AMA for 24 hr and then with 1 μM, 5 μM and 10 μM resveratrol and 80 μM silibinin for 24, 48 and 72 hr. SRB assay was performed to evaluate cell viability. The DMSO group was used as a control (A: α-AMA, R: resveratrol, S: Silibinin, D: DMSO).

Main experiment protocol results.

Biochemical measurements. Aminotransferase values decreased significantly in the α-AMA + SR and α-AMA + 12R groups compared to the α-AMA + NS group ($p < 0.01$, $p < 0.05$ and $p < 0.01$, $p < 0.05$, respectively). However, the values measured in the α-AMA + Sil group did not differ significantly from those of the α-AMA + NS group (fig. 3).

Histomorphological evaluation of the liver. In the α-AMA + NS group, light microscopy revealed liver cell injury, periportal mononuclear cell (MNC) infiltration and

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focal necrosis, and also diffuse liver cell necrosis and sinusoidal dilatation in specimens from some mice. Less liver cell injury, periportal MNC infiltration, focal necrosis and sinusoidal dilatation were observed in the resveratrol group. Less histological improvement was determined in the α-AMA + 24R group than in the α-AMA + SR and α-AMA + 12R groups. Histomorphological examination of the group receiving silibinin revealed greater liver cell injury, periportal MNC infiltration, focal necrosis and sinusoidal dilatation compared to the experimental groups receiving resveratrol (fig. 4A[1]).

Significantly lower hepatic injury scores compared to those in the α-AMA + NS group (2.6 ± 0.2) were observed in the α-AMA + SR group (1.4 ± 0.2), the α-AMA + 12R group in which resveratrol was administered 12 hr after α-AMA administration (1.3 ± 0.2), the α-AMA + 24R group in which resveratrol was administered after 24 hr (1.4 ± 0.2) and in the α-AMA + Sil group (1.8 ± 0.2) ($p < 0.01$, $p < 0.01$, $p < 0.01$ and $p < 0.05$, respectively).

Antioxidant enzymes. No statistically significant difference was determined among the groups in terms of MDA [α-AMA + NS 0.440 ± 0.05 *versus* 0.474 ± 0.04, 0.438 ± 0.04, 0.446 ± 0.04 and 0.453 ± 0.05 for α-AMA + SR, α-AMA + 12R, α-AMA + 24R and α-AMA + Sil, respectively, $p > 0.05$ for all], SOD [α-AMA+NS 86.8 ± 13.2 *versus* 43.5 ± 18.1, 54.6 ± 11.2, 36.5 ± 10.9 and 42.7 ± 7.105 for α-AMA + SR, α-AMA + 12R, α-AMA + 24R and α-AMA + Sil, respectively, $p > 0.05$ for all] and glutathione levels [α-AMA + NS 521.8 ± 34.6 *versus* 403.2 ± 55.6, 477.8 ± 40.8, 532.6 ± 34.6 and 578.0 ± 16.2 for α-AMA + SR, α-AMA + 12R, α-AMA + 24R and α-AMA + Sil, respectively, $p > 0.05$ for all].

Immunohistochemical examinations. At active caspase-3 immunohistochemical staining, immune-positive staining in liver tissue was statistically significantly greater in the α-AMA + NS group compared to the α-AMA + SR and α-AMA + 12R groups ($p < 0.05$) (figs 4A[2] and 5). At TUNEL staining, more TUNEL-positive cells were observed in the control group. The numbers of TUNEL-positive cells decreased in the experimental groups receiving resveratrol, while no difference in TUNEL positivity was determined between the α-AMA + NS group and the experimental group receiving silibinin (fig. 4A[3]).

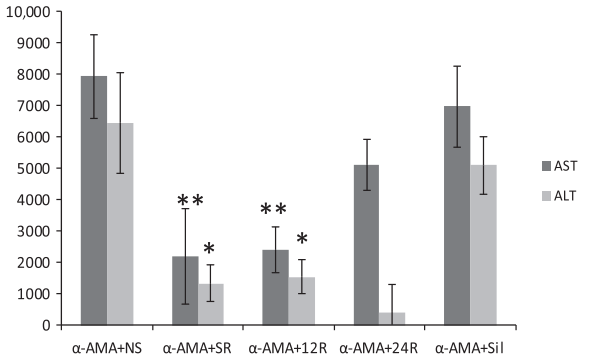


Fig. 3. Serum aminotransferase levels in experimental groups (AST, ALT U/L) (Experimental protocol (α-AMA + NS: Simultaneous α-amanitin and normal saline administration, AMA + SR: Simultaneous α-amanitin and resveratrol administration, α-AMA + 12R: α-amanitin and resveratrol 12 hr after administration of α-AMA, α-AMA + 24R: α-amanitin and resveratrol 24 hr after administration of α-AMA and α-AMA + Sil: Simultaneous α-amanitin and silibinin administration). (*), (**);$p < 0.05$, $p < 0.01$ showing significance compared the control group.

Electron microscope examination. Diffuse mitochondrial swelling, disintegration and dilation in agranular endoplasmic reticulum and hepatocyte injury were observed in the α-AMA + NS group. This injury resolved in the experimental groups receiving resveratrol, but no improvement was observed in the group receiving silibinin (fig. 4A[4]).

Discussion

This study investigated the efficacy of resveratrol in an experimental *in vitro* and *in vivo* model of hepatotoxicity induced in mice with α-AMA and compared it with that of silibinin, used as an antidote in the treatment of poisoning.

α-AMA has been shown to cause toxicity in hepatocytes cell culture [28–31]. In our *in vitro* experiments, we used normal cell lines THLE-2 and THLE-3 and tested resveratrol and silibinin in terms of reversing the effects of α-AMA on cell viability. α-AMA-induced liver cell death is successfully mimicked in hepatocyte cell culture, and neither resveratrol nor

Table 1.
The effect of α-AMA logarithmically increased and DMSO on biochemical parameters and histomorphological damage scores (α-AMA: α-amanitin, DMSO: Dimethyl sulfoxide, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase).

Groups	Number of mice that measurements were made	AST (U/l)	ALT (U/l)	Histomorphological damage score
Control (n = 5)	3	75.3 ± 9.8	68.0 ± 9.0	0
0.6 mg/kg α-AMA (n = 4)	4	656.8 ± 243.5	624.5 ± 214.6	1.5 ± 0.3
1.08 mg/kg α-AMA (n = 10)	8	3523.0 ± 1513.0	2712.0 ± 988.6	1.6 ± 0.3
1.4 mg/kg α-AMA (n = 10)	7	5267.0 ± 1201.0*	4513.0 ± 1171.0*	1.7 ± 0.3
1.92 mg/kg α-AMA (n = 8)	6	9834.0 ± 2759.0*	7700.0 ± 2014.0*	2.4 ± 0.3
DMSO (n = 5)	3	943.0 ± 224.0	758.3 ± 355.1	0.7 ± 0.3

*$p < 0.05$, compared to control group.

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silibinin therapy was associated with any negative effects on hepatocytes when incubated alone. Although silibinin was shown to exhibit protective and proliferative effects on hepatocytes in previous studies, we observed no significant proliferative effect using normal liver cell lines. Judging from our *in vitro* study results, both resveratrol and silibinin could not show a protective effect on hepatocytes from α-AMA toxicity. Also, these treatments were not effective in terms of the proliferation trends of control cells. In SRB assays, although 72-hr exposure of α-AMA intoxicated cells to resveratrol and silibinin elicited promising results, we determined no significant preventive effect on cell damage at real-time cell analysis. α-AMA treatment prior to plating cells on xCELLigence plates may have adversely affected cell adherence to plates. Similarly, Magdalan *et al.* previously showed that α-AMA exposure to hepatocyte results in an inhibition of cell proliferation and attachment [32]. Impedance-based real-time cell analysis may not therefore accurately reflect the results of cells with impaired adhesion but that are still viable, and may give results inconsistent with end-point assays. Higher cell viability measurements have also been reported in the literature in end-point assays compared to real-time cell analysis [33].

In the *in vivo* part of this study, we primarily determined the hepatotoxic dose of α-AMA and assessed the effect of DMSO, a resveratrol and silibinin solvent, on the liver [34]. We employed an increase in aminotransferase levels as a supporting biochemical parameter in addition to histomorphological analysis in confirming α-AMA-induced hepatotoxicity

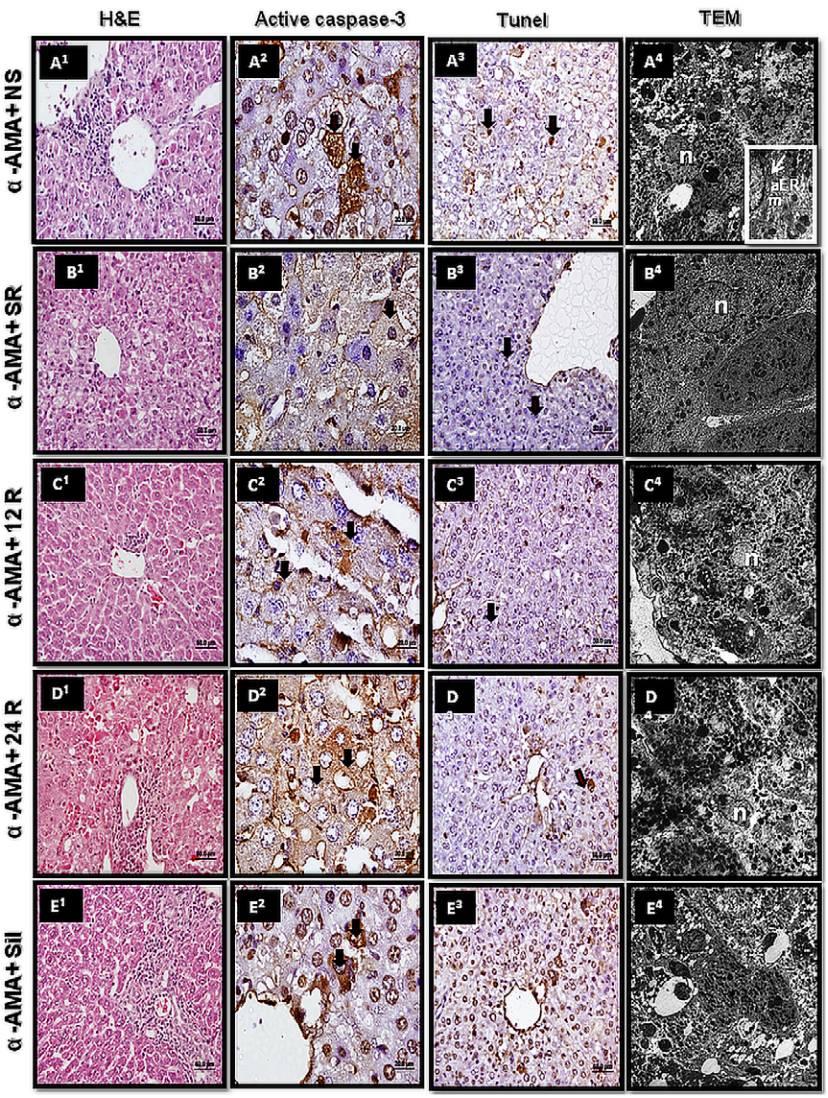


Fig. 4. Represents the micrographs related to histochemical, immunohistochemical and electron microscopic methods used for the histomorphometric evaluation of the liver tissues. A^1,B^1,C^1,D^1 micrographs from H&E staining, A^2,B^2,C^2,D^2 micrographs from active caspase-3 immunohistochemical staining, A^3,B^3,C^3,D^3 micrographs from TUNEL staining, and A^4,B^4,C^4,D^4 micrographs from transmission electron microscope imaging. The experimental groups; Control group (A^1,A^2,A^3,A^4), AMA + SR group (B^1,B^2,B^3,B^4), AMA + 12R group (C^1,C^2,C^3,C^4), AMA + 24R group (D^1,D^2,D^3,D^4) and AMA + Sil group (E^1,E^2,E^3,E^4). (➡) indicates periportal mononuclear cell infiltration,(★): hepatocyte injury, (▼): sinusoidal dilatation, (▶): perinuclear and cytoplasmic active caspase-3 immune-positive and TUNEL-positive hepatocytes.

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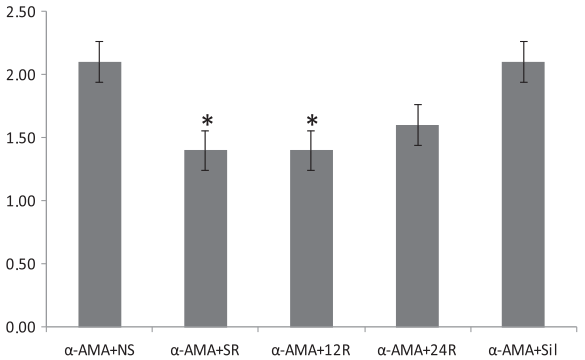


Fig. 5. Mean active caspase-3 immunohistochemical scores in the experimental groups (Experimental protocol (α-AMA + NS: Simultaneous α-amanitin and normal saline administration, AMA + SR: Simultaneous α-amanitin and resveratrol administration, α-AMA + 12R: α-amanitin and resveratrol 12 hr after administration of α-AMA, α-AMA + 24R: α-amanitin and resveratrol 24 hr after administration of α-AMA and α-AMA + Sil: Simultaneous α-amanitin and silibinin administration). (*),$p < 0.05$, indicating significance compared to the α-AMA + NS group.

[35]. We used the lowest dose of α-AMA, 1.4 mg/kg, resulting in a significant increase in both biochemical and histomorphological damage to induce α-AMA hepatotoxicity. As the 0.6 mg/kg α-AMA dose described in the literature failed to elicit a sufficient number of animal deaths, it could not be used as a LD_{50} dose. Ergin *et al.* observed no mortality in mice at 48 hr with any of the increasing doses of α-AMA administered, showing that the LD_{50} can change under varying conditions [36].

In our preliminary study, DMSO used as a resveratrol and silibinin solvent in our experimental groups produced a moderate increase in aminotransferases levels. In addition, the increases in aminotransferase levels and histomorphological injury scores were not statistically significant. We therefore used DMSO to dissolve resveratrol and silibinin in our experimental groups.

The hepatoprotective and therapeutic effects of resveratrol have been shown in various *in vivo* hepatotoxicity models using paracetamol, CCl_4, naphthalene and alcohol [12,21,35,37–39]. Although the most important mechanism underlying the antioxidant, anti-inflammatory and anticancer properties shown in studies up to now is the strong protective effect against the oxidative stress, the relationship with many signalling pathways at the molecular level is one of the most researched topics today [13,14]. Our findings show that the earlier that resveratrol begins being administered in a α-AMA-induced hepatotoxicity model, the better its healing effect, and that it significantly improved serum aminotransferase levels and liver damage scores compared to the α-AMA + NS group. Histomorphological analysis of the experimental groups receiving resveratrol revealed less periportal MNC infiltration, focal necrosis and sinusoidal dilatation, all findings indicating liver cell damage, compared to the control group. In necrosis, classic cell death, lysosomal enzymes resulting from the swelling and breakdown of cells due to hypoxia or various toxic agents initiate an inflammatory process. Resveratrol has been shown to have an anti-inflammatory effect in liver fibrosis developing in association with CCl_4, in non-alcoholic liver disease and in fatty liver in rats [11,40,41]. Based on these findings, we conclude that resveratrol healed α-AMA-related hepatotoxicity through its anti-inflammatory effect.

Resveratrol caused no significant change in oxidative stress parameters in the treatment groups compared to the α-AMA + NS group. A possible explanation is that the protocol of this study might have involved a rather aggressive stress and the antioxidant capacity of these agents did not provide a significant antioxidant effect on this condition. Although the SOD and glutathione levels tended to decrease in the groups treated with resveratrol compared to α-AMA group, we did not find statistical significance. Studies have reported that the hepatotoxicity healing effects of resveratrol depend on its antioxidant effects. However, in these models, resveratrol was applied either before the administration of a toxic substance or else in a long-term manner for therapeutic purposes [40,42]. As ours was a 48-hr protocol, the total resveratrol dosage we administered was lower than the dosages applied in other toxicity models in the literature. Therefore, in our experimental model, we think that resveratrol may have an antioxidant effect on α-AMA-induced hepatotoxicity. The changes in antioxidant parameters at the tissue level may be seen in a protocol better detected with longer protocols, exceeding 48 hr.

In this study, both immunohistochemical staining and electron microscopy demonstrated that α-AMA causes apoptosis and that the application of resveratrol in the early period prevents it. Apoptosis is a process that causes the activation of genes and enzymes in association with various physiological and pathological agents. Resveratrol has been shown to prevent apoptosis in the liver of rats with streptozotocin-induced diabetes and in a CCl_4 hepatotoxicity model [43,44]. Exposure to α-AMA for 12 hr in primary dog hepatocyte cell cultures has been reported to cause a decrease in cell vitality proportional to the concentration of α-AMA and length of exposure. Although marked apoptosis was reported after 6-hr exposure in particular, apoptosis and necrosis in dog hepatocyte cell cultures were shown to occur concurrently in proportion to the length of exposure to and concentration of α-AMA [30].

Silibinin is still used as an antidote in *Amanita phalloides type* mushroom poisonings, although the clinical evidence for its effectiveness is not strong. No improving effect of silibinin on biochemical and histomorphological parameters was observed in the α-AMA hepatotoxicity model established in this study. Tong *et al.* also showed that, like cimetidine, benzyl penicillin and thioctic acid, silibinin was not effective in treating α-AMA-related hepatotoxicity in mice [22]. However, Vogel *et al.* showed that the administration of 50 mg/kg silibinin 5–24 hr following toxicity in a α-AMA hepatotoxicity induced in beagles reduced necrosis in the liver by regulating biochemical enzymes, and observed no mortality in the group receiving silibinin [45].

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One study performed with the aim of developing an antidotal treatment against α-AMA in recent years is Garcia *et al.* experimental research into the use of polymyxin B for therapeutic purposes [46]. That study involved both *in silico* and *in vivo* protocols and concluded that polymyxin B prevented α-AMA-related organ failure in mice and increased survival rates. The α-AMA dose employed in that study was approximately one-fourth of the dose we used in our experiment. This indicates that the protective and healing effect we observed with resveratrol in the *in vivo* protocol despite the high dose of toxin administered, will be more powerful in the case of exposure to lower doses of toxin. Polymyxin B was only applied 4 hr after exposure to α-AMA in the *in vivo* protocol in this study. Late period effects are not investigated. However, when applied after 12 hr in our experimental protocol, resveratrol prevented a statistically significant α-AMA-related increase in both aminotransferases and in histomorphological damage scores.

Although we observed no significant reversing effects of resveratrol or silibinin on α-AMA intoxication under *in vitro* conditions, resveratrol was effective when tested in *in vivo* model. We may hypothesize that, in addition to hepatocytes, the effects of resveratrol and silibinin are related to the hepatocyte microenvironment in the liver and the organism's immune system, which cannot be mimicked in two-dimensional cell culture models and exhibit contradictory results between human and animal studies [45,47]. Therefore, as most of the studies in the literature have used silibinin prior to or simultaneously with administration of α-AMA to cells, better-reversing effects on intoxication might well be observed when compared to our *in vitro* studies. Additionally, silibinin has been shown to prevent hepatocyte membrane transport by competitive inhibition in rat hepatocytes, and differences in the human hepatocyte membrane structure might result in a different sensitivity to silibinin. This is the first study to use normal liver cell lines, THLE-2 and THLE-3, to test α-AMA cytotoxicity. Epithelial-like hepatocarcinoma cell lines are generally employed to test α-AMA cytotoxicity. Further studies including 3D co-culture models, such as lab-on-a-chip systems which better mimic the organ structure and microenvironment, may be useful for testing drug efficiency and for the discovery of new drugs in the future [47].

Conclusion

In our study involving an experimental *in vivo* hepatotoxicity model in mice, α-AMA caused an increase in serum aminotransferases levels, raised histomorphological injury scores and caused hepatic necrosis and apoptosis. Early administration of resveratrol prevents and heals α-AMA-related hepatotoxicity through its anti-inflammatory effect and the prevention of α-AMA-induced apoptosis. Resveratrol is a promising compound with the potential for use in the treatment of hepatotoxicity associated with *Amanita phalloides* mushroom poisoning. However, clinical studies investigating its development as a new treatment alternative for *Amanita phalloides* poisoning are needed.

Limitations

There are various limitations to experimental studies, particularly their applicability to clinical practice. The principal limitations of this study are as follows:

- The hepatocyte microenvironment could not be exactly replicated in our cell culture model using normal liver cell lines, THLE-2 and THLE-3.
- Physical characteristics of mice, and also impaired haemodynamics due to toxicity, made it difficult to obtain blood through cardiac puncture. Blood could not therefore be collected from all mice.
- As the number of experimental animals was limited, we were only able to investigate the efficacy of resveratrol at a dose of 30 mg/kg, and we were unable to assess its effectiveness at higher doses.
- We were only able to support the thesis that resveratrol prevents α-AMA-related hepatotoxicity through its anti-inflammatory effect by means of histomorphological data, and we did not measure anti-inflammatory parameters.

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Declaration of Interest

There is no conflict of interest. This study was supported by the Scientific and Technological Research Council of Turkey (114S927). This study was partly presented as a poster at the EPHAR 2016 Congress held in Istanbul, Turkey.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Real-time cell analysis was performed using the xCELLigence system when 24 hr α-AMA treated cells were incubated with 1 μM resveratrol or 80 μM silibinin for 96 hr. Impedance measurements were taken every 15 min.

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