FOLIA HISTOCHEMICA
ET CYTOBIOLOGICA
Vol. 48, No. 1, 2010
pp. 58-62

α-Amanitin induced apoptosis in primary cultured dog hepatocytes

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Abstract: Amatoxin poisoning is caused by mushroom species belonging to the genera *Amanita*, *Galerina* and *Lepiota* with the majority of lethal mushroom exposures attributable to *Amanita phalloides*. High mortality rate in intoxications with these mushrooms is principally a result of the acute liver failure following significant hepatocyte damage due to hepatocellular uptake of amatoxins. A wide variety of amatoxins have been isolated; however, α-amanitin (α-AMA) appears to be the primary toxin. Studies *in vitro* and *in vivo* suggest that α-AMA does not only cause hepatocyte necrosis, but also may lead to apoptotic cell death. The objective of this study was to evaluate the complex hepatocyte apoptosis in α-AMA cytotoxicity. All experiments were performed on primary cultured canine hepatocytes. The cells were incubated for 12 h with α-AMA at a final concentration of 1, 5, 10 and 20 μM. Viability test (MTT assay), apoptosis evaluation (TUNEL reaction, detection of DNA laddering and electron microscopy) were performed at 6 and 12 h of exposure to α-AMA. There was a clear correlation between hepatocyte viability, concentration of α-AMA and time of exposure to this toxin. The decline in cultured dog hepatocyte viability during the exposure to α-AMA is most likely preceded by enhanced cellular apoptosis. Our results demonstrate that apoptosis might contribute to pathogenesis of the severe liver injury in the course of amanitin intoxication, particularly during the early phase of poisoning.

Key words: α-amanitin, apoptosis, dog hepatocytes, hepatocyte cultures

Introduction

Among severe mushroom intoxications, the amatoxin poisoning is of primary importance because it accounts for about 90% of fatality. Amatoxin poisoning is caused by mushroom species belonging to the genera *Amanita*, *Galerina* and *Lepiota* with the majority of lethal mushroom exposures attributable to *Amanita phalloides* [1]. High mortality rate in intoxications with these mushrooms is principally a result of the acute liver failure following significant hepatocyte damage due to hepatocellular uptake of amatoxins [2-5]. Amatoxins are heat-stable octapeptides. A wide variety of amatoxins have been isolated; however, α-amanitin (α-AMA) appears to be the primary toxin [2]. α-AMA uptake by hepatocytes is mediated by the organic anion-transporting polypeptide located in the plasma membrane. It then binds to the RPB1 subunit of RNA polymerase II (pol II), thereby blocking the synthesis of proteins including intracellular enzymes, leading to cell death [6,7]. Experimental studies *in vitro* and *in vivo* suggest that α-AMA does not only cause hepatocyte necrosis, but also may lead to apoptotic cell death [8,9]. Moreover, addition of α-AMA to exeprimental hepatocyte cultures induces both necrosis and apoptosis and morphological signs of these two types of cell death occur concomitantly [9]. However the relationship between hepatocyte apoptosis and necrosis remains still undefined. It is also unclear

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Folia Histochem Cytobiol. 2010:48(1): 58 (58-62)
10.2478/v10042-010-0010-6

whether and to what extent hepatocyte loss throughout apoptosis causes liver damage in the course of α-AMA toxicity.

The aim of this study was to evaluate the complex hepatocyte apoptosis effects based on exposition time and dose-related α-AMA cytotoxicity. All experiments were performed on a canine hepatocyte model since clinical course and symptoms of amanitin intoxication in dogs are almost identical to those seen in humans [10,11].

Materials and Methods

Chemicals and materials. Media and reagents used for hepatocyte isolation, including Hank's balanced salt solution (HBSS), Leibovitz (L-15) medium, EBSS (Earle's balanced salt solution), Waymouth's 752 medium, phosphate-buffered saline (PBS), ethylene glycolbis(aminoethylether)-tetraacetic acid (EGTA), glucose, gentamicin/amphotericin B solution, media supplements, fetal bovine serum (FBS), collagenase type I and α-amanitin were purchased from Sigma Poland Chem. Corp. Collagen-coated 96-well plates and 8-well-chambered slides for hepatocyte culture were purchased from Becton Dickinson (USA). MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] kit was from Sigma Poland Chem.

Hepatocyte isolation and culture. All experiments were performed after approval by the Local Ethics Commission for Experiments on Animals at Institute of Immunology and Experimental Therapy in Wroclaw (license no. 54/2007). The liver was obtained from a 6-year old beagle dog (male, weighing 18 kg). Briefly, after initial heparinization (200 IU/ kg, *i.v.* injection) the animal underwent a full midline incision under xylazine (2 mg/kg, *i.m.*) and ketamine (10 mg/kg, *i.v.*) general anesthesia. The liver was completely perfused through the portal vein and then removed from the abdomen. Hepatocytes were isolated from the left lateral lobe by a modified two-step perfusion as described previously [9]. The viability and yield of isolated hepatocytes were estimated by trypan blue staining. The cells were resuspended in L-15 plating medium supplemented with 10% FBS, gentamicin and amphotericin B, and then dispensed into 96-well collagen-coated plates and 8-well-chambered slides. The cultures were incubated at 37°C in a humidified atmosphere of 95% air with 5% CO_2. After 4 h of initial incubation, the plating medium was substituted with defined culture medium (combination of EBSS and Waymouth's 752/1, supplemented with 10% FBS). After the next 12 h incubation the medium was exchanged and primary hepatocyte cultures were maintained for 12 h with α-AMA at a final concentration of 1, 5, 10 and 20 μM (experimental groups 1 μM, 5 μM, 10 μM and 20 μM respectively). Control hepatocyte cultures received medium without α-AMA. Viability test and apoptosis evaluation of cultured cells was performed at 6 and 12 h of exposure to α-AMA.

Analytical methods. The overall functional integrity and viability of cultured hepatocytes were assessed using the MTT assay. Reduction of a 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 (Elx 800 Universal Microplate Reader, Bio-Tek Instruments, USA). Apoptosis was evaluated using TUNEL method, electron microscopy and detection of DNA laddering by agarose gel electrophoresis.

TUNEL method. Isolated hepatocytes cultured on 8-well-chambered slides (Becton Dickinson, USA) were washed in PBS, fixed in cold acetone-methanol (1:1) for 10 min. at 4°C and then air-dried. Apoptosis was detected by TUNEL technique, using the ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit (MP Biomedicals, USA). Percentage of apoptotic nuclei was evaluated by scoring the brownish-labeled cell nuclei (positive cells) in selected hot-spots under × 400 magnification (Olympus BX 41 light microscope with visual mode AnalySis 3.2 software for computer-assisted image analysis).

Electron microscopy. Cultured hepatocytes were harvested from plates by gentle scraping, suspended in HBSS with 10% FBS, spun for 2 min at 60 g and then fixed for 24 h with 2% glutaraldehyde in 0.1 M sodium cacodylate bufer at 4°C. After fixation the specimens were rinsed several times with cacodylate bufer (4 × 15 min) followed by post fixation with 2% osmium tetroxide in cacodylate bufer for 1 h, and then dehydrated through a series of graded ethyl alcohols. The fixed cells were pelleted and embedded in EPON resin. Ultrathin sections were stained and examined by a JEOL JEM 1011 (Japan) transmission electron microscopy.

Detection of DNA laddering. Hepatocyte DNAs were extracted and purified using the ApopLadder Ex™ Kit (Takara Bio Inc., Otsu, Shiga, Japan). For analysis of DNA fragmentation by agarose gel electrophoresis, total DNA was extracted and purified using the ApopLadder Ex™ Kit (Takara Bio Inc., Otsu, Shiga, Japan). The sample DNA concentration was measured by using NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). The individual DNA extracts were loaded into the wells of a 1.5% agarose gel containing 1μg/ml of ethidium bromide and the bands were visualized by Gel-Doc XR, (BioRad, USA) using QuantityOne 4.6.1 software.

Statistical analysis. Differences between values (MTT, TUNEL) were analyzed by one-way ANOVA with Tukey test using the Statistica 7.1 software (Stat Soft, Poland), and $p<0.05$ was considered statistically significant.

Results

There was a significant decline of cell viability after 6 h exposure to α-AMA at concentrations 10 μM and 20 μM, while 12 h exposure caused significant decrease in cell viability in groups dosed with 5, 10, and 20 μM of α-AMA, respectively (Figs. 1, 2). Application of TUNEL technique revealed significantly increased apoptosis in all experimental groups of cells exposed to α-AMA for 6 and 12 h ($p<0.05$ *vs*. control group). A remarkable increase in number of apoptotic cells in groups 5 μM, 10 μM, and 20 μM compared with group 1 μM was also observed ($p < 0.05$). However, there was no statistical difference between groups 5 μM, 10 μM and 20 μM (Figs. 3, 4).

Microscopic examination of representative hepatocyte groups exposed to α-AMA for 6 and 12 h revealed apoptotic nuclei (Fig. 5) and dead cells displaying typical apoptotic features including condensation of chromatin, with parts of cytoplasm separated and fragmented in numerous bodies (Fig. 6).

Analysis of DNA fragmentation by agarose gel electrophoresis showed changes characteristic of apoptosis with a distinctive cleavage of hepatocyte nuclear DNA after 6 and 12 h of exposition to α-AMA in concentrations of 1 μM, 10 μM, and 20 μM (Fig. 7).

Folia Histochem Cytobiol. 2010:48(1): 59 (58-62)
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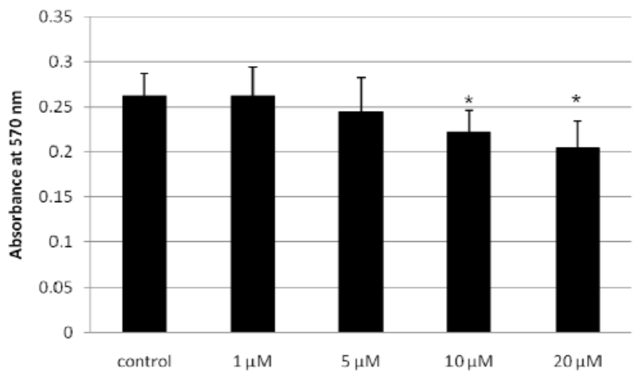

Fig. 1. MTT activity in control and experimental groups after 6 h exposure to α-AMA at concentration 1, 5, 10 and 20 µM. The number of viable hepatocytes (one representative hepatocyte preparation) is proportional to the MTT reaction product, as determined by the optical density. Each value represents the mean ±SD, n=16, *p<0.05 *vs*. control.

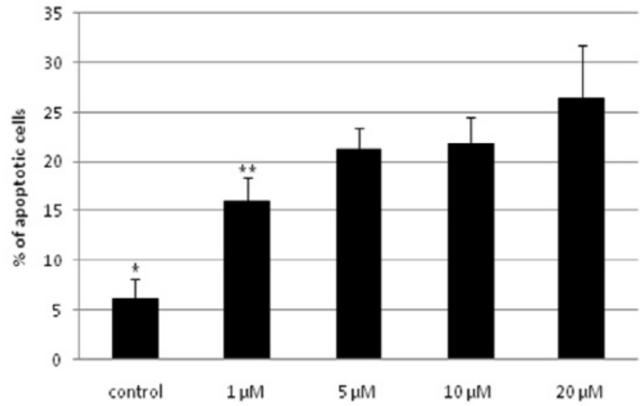

Fig. 3. Apoptosis in control and experimental groups after 6 h exposure to α-AMA at concentration 1, 5, 10 and 20 µM. Each value represents the mean ±SD, n=16; *p<0.05 comparison to all groups; **p<0.05 comparison to all groups.

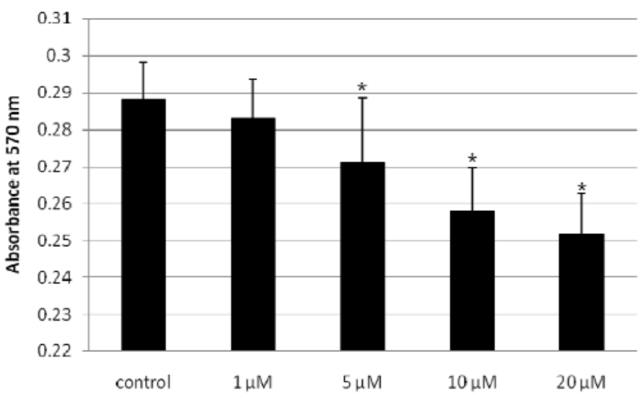

Fig. 2. MTT activity in control and experimental groups after 12 h exposure to α-AMA at concentration 1, 5, 10 and 20 µM. The number of viable hepatocytes (one representative hepatocyte preparation) is proportional to the MTT reaction product, as determined by the optical density. Each value represents the mean ±SD, n=16, *p<0.05 *vs*. control.

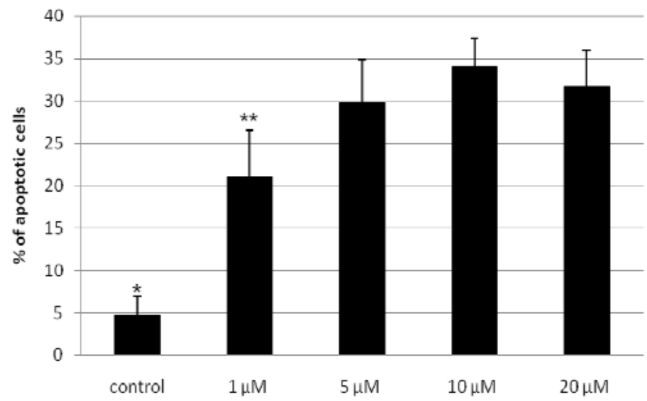

Fig. 4. Apoptosis in control and experimental groups after 12 h exposure to α-AMA at concentration 1, 5, 10 and 20 µM. Each value represents the mean ±SD, n=16; *p<0.05 comparison to all groups; **p<0.05 comparison to all groups.

Discussion

It is generally believed that in liver many compounds can even cause hepatocyte apoptosis and necrosis simultaneously [12]. However, apoptosis may be the major event in chemical-induced hepatocyte injury and therefore the detection of apoptotic effects of amatoxin is of high importance. Cultured canine hepatocytes may represent a relevant *in vitro* experimental system for the evaluation of hepatotoxic effects of α-AMA. In this experiment dog primary hepatocytes were exposed to different concentrations of α-AMA for 12 h, since it is known that longer exposition causes death of vast majority of cells, which can significantly complicate the overall analysis of the studied cellular pathological processes [9]. In the present experiment, there was a clear correlation between hepatocyte viability, concentration of α-AMA and time of exposure to this toxin. Furthermore, the observations of the present study provide evidence for apoptosis of cultured dog hepatocytes exposed to different concentrations of α-AMA. Confirmation of this process was based on the morphological data by electron microscopy, including chromatin condensation and formation of apoptotic bodies; TUNEL assay, and the presence of DNA laddering by agarose gel electrophoresis. As noted in Results, the TUNEL staining revealed that significant increase in incidence of apoptosis occurred already after 6 h of exposure to all tested concentrations of α-AMA. Moreover, progressive apoptotic cell death was concentration dependant but only up to 5 µM of α-AMA. Additional exposure of hepatocytes to this toxin at higher concentrations was not leading to any intensification of apoptosis degree, as determined by the TUNEL reaction. Although this assay represents a criterion by which apoptosis could be identified, a TUNEL-positive reac-

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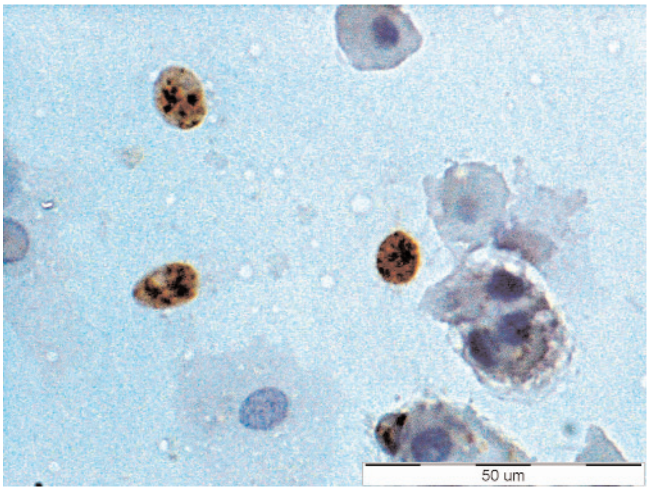

Fig. 5. TUNEL technique detected apoptotic nuclei (brown cell nuclei) in cultured hepatocytes after 6 h incubation with α-AMA at concentration 10 μM (original magnification × 400).

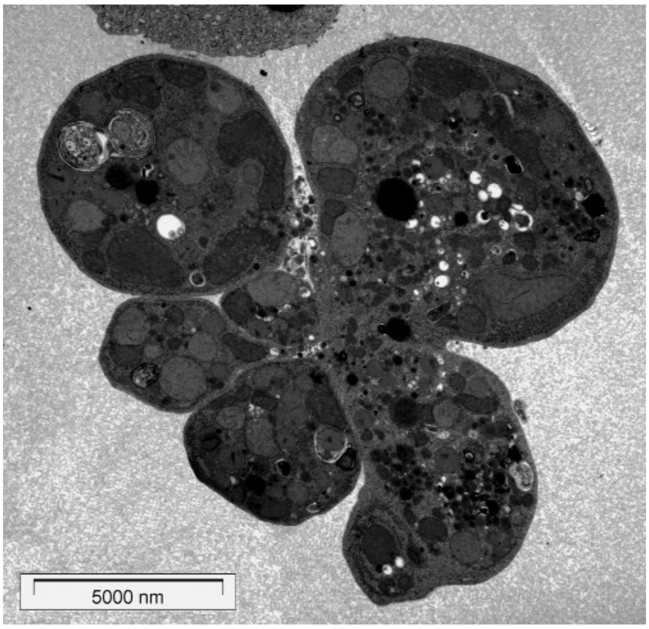

Fig. 6. Electronomicrograph of cultured hepatocyte fragmented into apoptotic bodies. Each is surrounded by cell membrane and contains fragmented, condensed nucleus and cytoplasm with intact organelles (original magnification × 7500).

tion can appear in both apoptosis and necrosis [13,14]. Furthermore, a TUNEL assay provides information that overlaps with other more distinctive and widely used apoptotic markers, and may not fully fit for throughput screening purposes [12].

In apoptotic cells specific DNA cleavage becomes evident as a typical ladder pattern due to multiple DNA fragments. Confirmation of this process in the present study was based on agarose gel electrophoresis which revealed that exposure of cultured hepatocytes to all tested concentrations for 6 and 12 h leads to a distinctive ladder pattern consisting of DNA fragments. However, the manifestation of decreased fragmentation of DNA in cells exposed to 20 μM α-AMA for 12 h may indicate that in this group hepatocytes underwent necrosis with irregular destruction of genomic DNA.

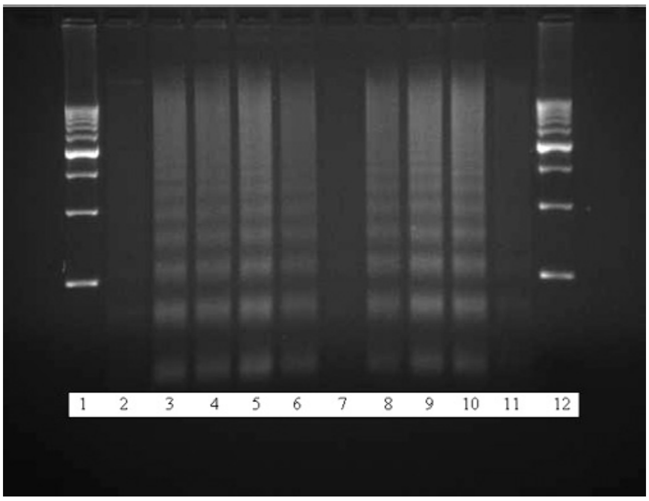

Fig. 7. Analysis of fragmented DNA by electrophoresis on 1,5% agarose gel after 6 h exposure to α-AMA (lanes 1-6) and after 12 h exposure to α-AMA (lanes 7-12). Marker 500 kbp (lanes 1 and 12); control (lanes 2 and 7); 1 μM of α-AMA (lanes 3 and 8); 5 μM of α-AMA (lanes 4 and 9); 10 μM of α-AMA (lanes 5 and 10); 20 μM of α-AMA (lanes 6 and 11).

Severe intoxications by amanitin-producing mushrooms induce liver injury, which can result in acute failure of this organ. Clinical data indicate that progressive necrosis is the morphological hallmark of acute liver injury. Nevertheless, little is known about pathological processes undergoing during the early stages of amanitin intoxication. In humans intoxicated with amanitin-producing mushrooms the onset of clinical symptoms such as gastroenteritis occurs generally 10-14 hr after mushroom ingestion. Therefore, patients with amanitin poisoning typically seek medical care several hours after ingestion, when a large portion of toxins had been absorbed from the gastrointestinal tract and uptaked into the liver [1-3]. Thus, the knowledge of functional and morphological hepatocyte derangement during the early phase of poisoning is incomplete and establishment of a liver screening in such patients by performing liver biopsy is impossible. Therefore, clinical analysis revealing morphological damage of hepatocytes during the course of amanitin poisoning is based mostly on autopsy data, several days after mushroom ingestion. Our results demonstrate that apoptosis might contribute to pathogenesis of the severe liver injury in the course of amanitin intoxication, particularly during the early phase of poisoning. In this context, α-AMA can be considered as a strong inducer of apoptosis although the mechanism of this induction process still remains unclear. It is

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assumed that α-AMA binds to the RPB1 subunit of RNA pol II, thereby blocking the synthesis of proteins [6,7]. A prolonged blockage of pol II-dependent transcription results in cell death by apoptosis [15]. Pol II is responsible for the transcription of most protein-coding genes, including proapoptotic genes. Thus, new trancription of proapoptotic genes would not be expected to contribute to apoptosis induced by pol II inhibition or degradation. According to Arima *et al.* [16] α-AMA elicits p53 accumulation and apoptosis in normal fibroblasts and HCT116 human colon carcinoma cells without the induction of apparent DNA damage. Trancriptional blockade by α-AMA results in the activation of stress-activated kinases that are distinct from those activated by DNA-damaging agents and that phosphorylate and therby stabilize p53. The p53 molecules that accumulate in response to transcriptional blockade translocate to the mitochondria, which may activate the apoptotic program. Moreover apoptosis in HCT116 cells is blocked in the presence of a caspase inhibitor [16]. Therefore, induction of apoptosis by amanitin is a complex process and understanding of the cellular processes that mediate liver injury *in vivo* is of biomedical and clinical relevance.

The decline in cultured dog hepatocyte viability during the exposure to α-AMA is most likely preceded by enhanced cellular apoptosis. Our results demonstrate that apoptosis might contribute to pathogenesis of the severe liver injury in the course of amanitin intoxication, particularly during the early phase of poisoning. Further studies are required to elucidate this hypothesis through directed *in vitro*/*in vivo* experiments.

Acknowledgements: This work is a part of a project granted by Polish Ministry of Science and Higher Education (grant no. N 401 2809 33).

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Submitted: 30 June, 2009
Accepted after reviews: 15 February, 2010

10.2478/v10042-010-0010-6