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Benzylpenicyllin and acetylcysteine protection from α-amanitin-induced apoptosis in human hepatocyte cultures

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ABSTRACT

High mortality rate in *Amanita phalloides* (death cap) intoxications is a result of the acute liver failure following hepatocyte damage due to hepatocellular uptake of amatoxins. α-Amanitin (α-AMA), the major amatoxin, blocks a RNA polymerase II, which results in inhibition of transcription of DNA and protein synthesis processes and leads to hepatocyte death. α-AMA is also a strong apoptosis inductor and may play a significant role in pathogenesis of hepatic damage in course of amanitin intoxication. The aim of this study was to examine mechanisms of α-AMA-induced apoptosis in human hepatocytes, as well as in determining if commonly clinically used antidotes benzylpenicillin (BPCN) and N-acetylcysteine (ACC) are able to protect human hepatocytes against α-AMA-induced apoptosis. The experiment was performed on cultured human hepatocytes. Viability of cultured hepatocytes was assessed using the MTT assay, whereas apoptosis processes were evaluated by the electron microscopy, detection of DNA laddering, determination of caspase-3 activity, and measuring annexin V, p53 and Bcl-2 protein concentration. Cytotoxicity and apoptosis evaluation were performed after 24 h of exposure to α-AMA and/or tested antidotes.Both ACC and BPCN were well tolerated by human hepatocyte cultures, and exposure to those substances did not reduce cell viability nor induce apoptosis. Exposure of hepatocytes to α-AMA at concentration 2 μM resulted in derangement of cell cultures, apoptosis and significant reduction in cell viability. α-AMA-induced apoptosis in human heptocyte cultures is p53- and caspase-3-dependent. Human hepatocyte cultures are exposed simultaneously to α-AMA and tested antidotes (BPCN or ACC) showed significantly higher cell viability and significantly lower values of apoptosis markers compared to the cultures exposed to α-AMA only.

1. Introduction

Most mushroom-related fatalities are associated with the amanitins containing species, especially with Death Cap (*Amanita phalloides*) (Ellenhorn, 1997; Enjalbert et al., 2002; Schneider, 2001). Amanitins are bicyclic octapeptide derivatives. They are thermostable and are not removed by boiling and discarding water or by any form of cooking or drying. Amanitins are liver toxins. Therefore, amanitin poisoning is characterized by liver necrosis, in many cases with acute hepatic failure with subsequent complications, including hepatic coma, coagulation disorders and renal failure (Ellenhorn, 1997; Enjalbert et al., 2002; Escudié et al., 2007; Jan et al., 2008; Schneider, 2001). α-Amanitin (α-AMA), the major toxin of Death Cap, does not cause a direct cytolytic effect but blocks a RNA polymerase II (pol II) subunit RPB1. This results in inhibition of transcription of DNA and protein synthesis processes and leads to cell death (Busnell et al., 2002; Nguyen et al., 1996; Schneider, 2001). α-AMA is also a strong apoptosis inductor (Andera and Wasylyk, 1997; Arima et al., 2005; Gartel, 2008), but the mechanisms of α-AMA-induced apoptosis are poorly understood. Experiments performed on canine hepatocyte cultures suggest that apoptosis may play a significant role in pathogenesis of hepatic damage in course of amanitin intoxication (Magdalan et al., 2009c).

The purpose of this study was to examine mechanisms of α-AMA-induced apoptosis in human hepatocytes, as well as in determining if commonly clinically used antidotes: benzylpenicillin (BPCN) and N-acetylcysteine (ACC) are able to protect human hepatocytes against α-AMA-induced apoptosis.

2. Materials and methods

2.1. Chemicals and materials

Media, supplements and reagents used for hepatocyte culture, α-AMA and all tested antidotes were obtained from the Sigma

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Poland Chem (α-Amanitin cat. no A2263, Benzylpenicillin/Penicillin G potassium salt cat. no P7794, N-Acetyl-L-cysteine cat. no A9165). Human hepatocyte 6-well culture plates (cat. no CC-2691A) and 96-well culture plates (cat. no CC-2698A) were obtained from Lonza, Belgium.

2.2. Hepatocyte culture

Approval from Local Ethics Commission at Wrocław Medical University, (Wrocław, Poland), license no. 481/2006, was obtained for all experiments. After 2 h of initial incubation, the shipping medium was substituted with defined culture medium (combination of EBSS and Waymouth's 752/1, supplemented with 10% FBS). Following another 12 h of incubation, the medium was exchanged and primary hepatocyte cultures were maintained for 24 h with α-AMA and/or tested antidotes (except control). α-AMA and antidotes (ACC, BPCN) were dosed at different final concentrations (Table 1). In AMA+ACC and AMA+BPCN groups, hepatocytes were simultaneously exposed to α-AMA and tested antidotes.

α-AMA was used at concentration causing reduction in cell viability in human hepatocyte cultures (Magdalan et al. in press; Magdalan et al. 2009b). There are no reports on standard dosage regimen of ACC in mushroom poisoning. Therefore, we tested ACC in concentrations corresponding to its plasma levels obtained after the recommended dosage during the treatment in acetaminophen toxicity (Prescott et al., 1989). BPCN concentration corresponded to its plasma levels obtained after the dosage recommended in therapy of the toadstool death cap poisoning, i.e. 300.000–1.000.000 U/kg/day *i.v.* (Heilmeyer et al., 1969; Schneider, 2001).

Viability and apoptosis evaluation of cultured cells were performed after 24 h of exposure to α-AMA and/or tested antidotes.

2.3. 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 (Elx 800 Universal Microplate Reader, Bio-Tek Instruments, USA).

Detection of apoptosis in human hepatocyte cultures was performed by analysis of DNA fragmentation (DNA laddering). For analysis of DNA fragmentation, hepatocyte DNAs were extracted and purified using the ApopLadder Ex^{TM} 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^{TM} Kit (Takara Bio Inc., Otsu, Shiga, Japan). The sample DNA concentration was measured using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). 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 Gel-Doc XR, (BioRad, USA) using the QuantityOne 4.6.1 software.

Table 1
Final concentrations of α-AMA, ACC and BPCN administered to human hepatocyte cultures.

Group	α-AMA. (μM)	ACC. (mM)	BPCN. (mM)
Control (C)			
AMA	2.0		
ACC		1.0	
BPCN			1.0
AMA+ACC	2.0	1.0	
AMA+BPCN	2.0		1.0

Intensity and mechanisms of apoptosis processes were evaluated by determination of caspase-3 activity (Caspase-3 Colorimetric Assay Kit, BioVision Research Products, cat. no BV-K106-200), p53 protein concentration (Human p53 ELISA Kit, BioVenndor, cat. no RBMS256R), Bcl-2 protein concentration (Human Bcl-2 ElLISA Kit, BioVenndor, cat. no RBMS244/3R.) and annexin V concentration (Human Annexin V ELISA Kit, BioVenndor, cat. no RBMS252R). Annexin V and p53 protein concentrations were determined in cell culture supernatant, and Bcl-2 protein concentration and caspase-3 activity were determined in hepatocyte lysates using a plate reader (Microplate Reader, TECAN M200, Switzerland).

The total protein concentration in hepatocyte homogenates was determined by the Total Protein Assay Kit (Sigma Poland Chem. cat. no TP0200). Concentrations of Annexin V and Bcl-2 protein were expressed as ng per mg of the total protein, whereas concentration of p53 protein was expressed as unit per mg of the total protein.

2.4. Microscopy

For electron microscopy analysis, cultured hepatocytes were harvested from the 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 buffer at 4 °C. After fixation, the specimens were rinsed several times with cacodylate buffer (4 × 15 min) followed by post-fixation with 2% osmium tetroxide in cacodylate buffer 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.

2.5. Statistical analysis

Differences between MTT values were analyzed by the one-way ANOVA with Tukey test, whereas annexin V, caspase-3, p53 protein and Bcl-2 protein values were analyzed by the Kruskal–Wallis test.

3. Results

Analysis of DNA fragmentation by agarose gel electrophores showed changes in the characteristic of apoptosis with a distinctive cleavage of hepatocyte nuclear DNA after 24 h of exposition to α-AMA in AMA, AMA+ACC and AMA+BPCN groups. In control, ACC and BPCN groups no cleavage of hepatocyte nuclear DNA typical for apoptosis was revealed (Fig. 1).

In hepatocytes exposed to α-AMA at 2 μM concentration (AMA group), cell viability was significantly lower compared to the control, and apoptosis markers (annexin V and caspase-3) and p53 protein values were significantly higher (Figs. 2–5). Lower concentration of the anti-apoptotic Bcl-2 protein compared to the control was also found in the AMA group (Fig. 6).

In hepatocytes exposed only to tested antidotes (ACC and BPCN groups), cell viability and values of annexin, caspase-3, p53 protein and the anti-apoptotic Bcl-2 protein were the same as in the control group (Figs. 2–6).

In hepatocytes exposed simultaneously to α-AMA and tested antidotes (AMA+ACC and AMA+BPCN groups), cell viability was significantly lower compared to the control, but remained significantly higher compared to the AMA group (Fig. 2).