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Evaluation of the genotoxicity of alpha-amanitin in mice bone marrow cells

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ABSTRACT

Alpha-amanitin is a known cytotoxic substance found in some mushroom species including *Amanita phalloides*. Its main mechanism of action is to block the transcription, which can lead to cell death. Lack of reports on the genotoxicity of this toxin was an inspiration for undertaking this experiment. Genotoxic effect of α-amanitin on balb/c mice bone marrow cells was tested using: comet assay and chromosomal aberration test. The tested substance was given once by intraperitoneal administration to animals at doses: 0.1 mg/kg, 0.15 mg/kg and 0.25 mg/kg (LD_{50}) body weight with 48 h exposure. The comet assay demonstrated a statistically significant increase in DNA damage for all the investigated α-amanitin doses compared to the negative control ($p < 0.0001$). The exposure to 0.15 and 0.25 mg/kg doses of α-amanitin also generated a statistically significant increase in the frequency of chromosomal aberrations in bone marrow cells of mice compared to the negative control ($p < 0.05$). The genotoxic effect induced by α-amanitin in mammalian cells can result in genome instability and its functional consequences.

1. Introduction

Many biologically active substances existing in nature have long been known for their toxic properties. α-Amanitin found in species of the *Amanita* genus of mushrooms (*A. phalloides*, *A. virosa* and *A. verna*) as well as in the species of *Lepiota* genus and *Galerina* genus mushrooms belong to them (Berger and Guss, 2005; Garcia et al., 2015). Chemically α-amanitin belongs to the group of bicyclic octapeptides (Hallen et al., 2007; Garcia et al., 2015). It is characterized by high thermal stability, good solubility in water and resistance to digestive enzymes and acids. In humans α-amanitin is very well absorbed from the gastrointestinal tract causing often irreversible multiorgan damage, particularly to liver and kidneys (Berger and Guss, 2005; Kopala and Gomółka E., 2014; Garcia et al., 2015). The lethal dose of α-amanitin for humans has been estimated to be 0.1 mg/kg body weight (bw) (Karlson-Stiber and Persson, 2003).

Disorder of the chromatin organization is the first change caused by α-amanitin in the cell nucleus. There also comes to the nucleolar dispersion and the formed granule clusters are dispersed within the whole nucleus (Haff and Ward, 1996; Bonnet and Basson, 2002). At the molecular level, α-amanitin blocks RNA polymerase II and thus inhibits the transcription and subsequent synthesis of proteins which consequently leads to cell death. The exact mechanism of interaction between α-amanitin and RNA polymerase II still remains the subject of further studies. Kaplan et al. (2008) indicated that inhibition of transcription resulted from the direct α-amanitin interference with the histidine residue of the trigger loop (TL) at position 1085. The authors present evidence that the TL is a key element for both transcription and its regulation. Constraint of this structural element of Rpb1 subunit - by formation of a complex with α-amanitin - leads in turn to the transcription process inhibition.

Biochemical investigations indicate that α-amanitin may also have an effect on the increase of reactive oxygen species (ROS) pool in cells (Zheleva et al., 2007; Magdalan et al., 2008; Ergin et al., 2015). Furthermore, accumulation of this toxin in cells also leads to the formation of unstable phenoxyl radical, the presence of which may promote free radical chain reactions (Zheleva et al., 2007; Zheleva, 2013). This radical can act prooxidatively contributing to the formation of oxidative DNA damage.

In recent years, α-amanitin has also become an object of interest in anticancer therapy based on the effect of natural toxic substances. Its strong affinity to RNA polymerase II is the starting point

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for a new branch of the treatment the targeted cancer therapy (Moldenhauer et al., 2012; Moshnikova et al., 2013; Kume et al., 2016).

Despite increasing knowledge on the cytotoxic effect of α-amanitin on mammalian cells, there is still lack of reports on the genotoxicity of this toxin. The aim of the study was to evaluate the genotoxicity of α-amanitin in mice bone marrow cells with the use of comet assay and chromosomal aberration test.

2. Material and methods

2.1. Animals

Adult male balb/c mice, 6–8 weeks old were used for the experiment. The animals were housed in standard light/dark cycle conditions, at 19–23 °C, humidity 40–70% and provided with standard food and water *ad libitum*. Prior to entering the study they were acclimated for a week (Preston et al., 1987). The animals received humane care and the study protocol was approved by the Local Ethical Committee on Animal Experiments: No 55/ŁB 439/2008.

2.2. Chemicals

α-Amanitin ($C_{39}H_{54}N_{10}O_{14}$) (Sigma, USA, Cat. No. A2263); Cyclophosphamide monohydrate ($C_7H_{15}C_{12}N_2O_2P \cdot H_2O$) (Sigma, USA, Cat. No. C0768); Colchicine ($C_{22}H_{25}NO_6$) (Sigma, USA, Cat. No. C9754); Trypan blue (Sigma, USA, Cat. No. T8154); Agarose NMP (Normal Melting Point), LMP (Low Melting Point) (Sigma, USA, Cat. No. A9539, A9414), Giemsa stain (AQUA-MED); EDTA (CHEMPUR, Cat. No. 118798103); Eagle medium (Cytogen, Cat. No. CE5-825); Triton X-100 (Sigma, Cat. No. T9284); Trizma base (Sigma, Cat. No. T1503); N-laurosarcosine (Sigma, Cat. No. L5125), Propidium iodide (Sigma, Cat. No. P4170).

2.3. Experimental procedure

Mice were divided into 5 groups: I, II, II – experimental groups; C0 and C – control groups. Each group included 10 animals. The tested substances were administered intraperitoneally. The experiment was proceeded by pilot studies in which experimentally determined LD_{50} dose for α-amanitin was 0.25 mg/kg body weight (bw). α-Amanitin was administered at the following doses: Group I – 0.1 mg/kg bw; II – 0.15 mg/kg bw; III – 0.25 mg/kg (LD_{50}) bw. Control group C0 animals received solvent – 0.9% normal saline solution and control group C - a reference compound (cyclophosphamide) at a dose 50 mg/kg bw (Bowen et al., 2011; Kirkland et al., 2016). The animals were sacrificed 48 h after injection by cervical dislocation. The bone marrow was dissected from femoral bones and subjected to comet assay and structural chromosome aberration test.

2.4. Comet assay in bone marrow in vivo

Alkaline version of the comet assay was used (pH > 13) basing on Singh et al. (1988) method. After isolation the bone marrow was suspended in Eagle's medium and centrifuged at 1000 rpm for 10 min at 4 °C. Then, after supernatant removal, the precipitate was diluted in fresh Eagle's medium.

Cell viability was determined by trypan blue exclusion assay before each comet assay: trypan blue (0.2%) was added to the cell suspension and after approximately 1 min incubation the suspension was spotted onto glass slides. The percentage of live cells was determined analyzing a sample of 300 cells each time (lens 10x) using light microscope.

For the comet assay: the cell suspension was mixed with 0.75% LMP agarose dissolved in PBS (pH 7.4), heated to 37 °C. The obtained mixture was placed on heated glass slides coated with a thin layer of 0.8% NMP agarose and covered with a coverslip. The slides were then placed on ice for about 10 min to solidify the agarose. The next stages were performed at limited light access. After coverslip removal the slides were placed for 1 h in a lysis buffer of pH > 13 and temp. 4 °C (buffer composition: 2.5 M NaCl, 100 mM EDTA, 10 mM TrisHCl, 1% N-laurosarcosine sodium, 1% Triton X-100). One hour afterwards the slides were submerged in electrophoresis buffer (pH = 13; buffer composition: 300 mM NaOH, 1 mM EDTA) for 40 min to allow unwinding of the DNA and then subjected to electrophoresis (25 V; 0.86 V/cm; 300 mA) for 25 min. In subsequent stages the slides were washed for 15 min in neutralization buffer (0.4 M Tris) of pH = 7.5, rinsed in water, stained with propidium iodide 2.5 μg/ml and coverslipped. The analysis of the slides was performed using a fluorescent microscope Axioskop Opton (Germany) with lens 20x. A total of 50 randomly selected nonoverlapping single cells per mouse were estimated. The

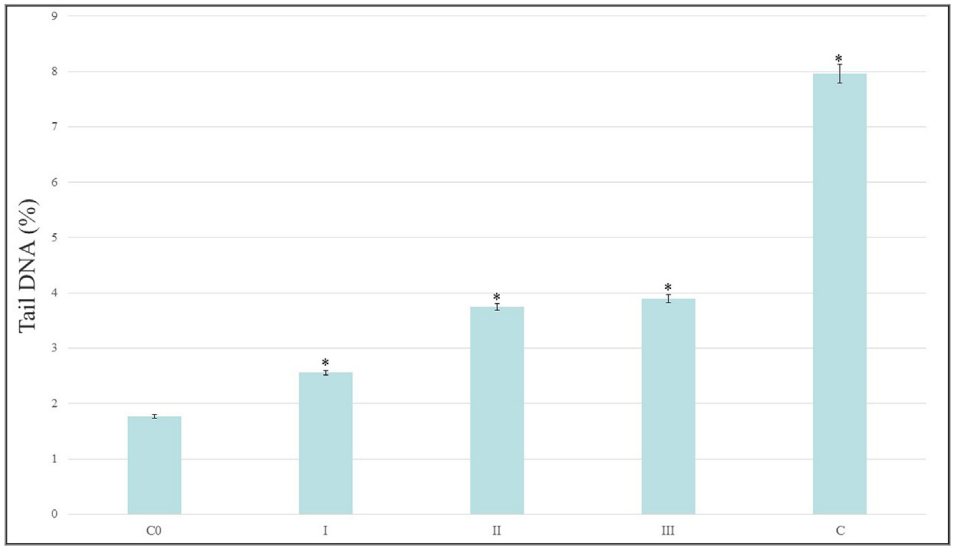

Fig. 1. Assessment of DNA damage after α-amanitin exposure to alkaline (pH > 13) comet assay. Mean percentage DNA content in the tail of the comet (±SEM) for tested doses of α-amanitin. *p < 0.0001 as compared with negative control.