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SEVERE MUSHROOM TOXIN ALPHA AMANITIN CAUSES GENERATION OF REACTIVE OXYGEN SPECIES IN LIVER TISSUES OF MICE – A COMPARATIVE STUDY BY TWO DIFFERENT INSTRUMENTAL METHODS

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Abstract
Alpha amanitin is a natural hepatotoxin isolated from deadly poisonous *Amanita phalloides* mushroom and causes dramatic toxic consequences mainly within the liver and kidneys.
By our formerly *ex vivo* studies using a spectrophotometrical method we demonstrated that alpha amanitin can induce increased levels of lipid peroxidation (LPO) products in livers of mice treated by the toxin compairing to those of the control mice. We assumed that during its metabolism alpha amanitin probably is involved in free radical reactions which were the reason for developing of lipid peroxidation process in livers of mice. The aim of the present study was to evaluate by two different unstrumental methods: Electron Paramagnetic Resonance (EPR) direct and indirect spectroscopy and visible spectrophotometry, the effect of alpha amanitin on livers of mice treated by the toxin. Twenty hours after toxin treatment, ROS production marked by EPR spectra signals of spin-adducts in the mice livers could be detected by indirect EPR spectroscopy. It was found twice higher levels of ROS production in the livers of poisonous mice comparing to those of the control mice. By direct EPR spectroscopy higher levels of ascorbate free radicals were found in liver homogenates of treated mice in comparison with those of the control mice, which confirmed that an oxidative process was developed in livers of poisonous mice. At the same time the levels of lipid peroxidation products measured by visible spectrophotometry as malondialdehyde reactive products in livers of treated mice were slightly increased in comparison with those of the controls.

Keywords: alpha amanitin, lipid peroxidation, EPR spin trapping technique, ascorbate free radicals

INTRODUCTION

Alpha amanitin **(Figure 1)** is a natural hepatotoxin isolated from deadly poisonous *Amanita phalloides* mushroom and causes dramatic toxic consequences mainly within the liver and kidneys (1). The basic molecular mechanism of its toxicity was reported to be due to inhibition of RNA polymerase II of eukaryotic cells (2).

By previous *ex vivo* studies using a visible spectrophotometrical method we demonstrated higher levels of lipid peroxidation products in liver homogenates of mice treated by alpha amanitin comparing to those of controls (3). Because there is no straight correlation between alpha amanitin *in vivo* LD50 and inhibitory constant (Ki) towards RNA polymerase III *in vitro* determined (1), we supposed additional toxic mechanism in relation of generation of toxic reactive oxygen species (ROS) to be involved and contribute to alpha amanitin liver toxicity (4). The best way for confirmation of short-lived free radicals either *in vitro* or at *in vivo* conditions is to be used proper EPR methods. EPR spectroscopy is a direct and definitive technique for direct detection of stable free radicals and for indirect detection of unstable free radical species using chemical compounds

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termed spin-trapping agents. Spin traps react with short–lived free radicals to form a more stable free radical products (spin-adducts) that can be detected and studied by EPR spectroscopy (5, 6). One of the most often methods used for *ex vivo* evaluation of oxidative stress is thiobarbituric acid assay (TBAS) that measures the level of malondialdyhide reactive (MDA) materials.

The aim of the present comparative research is to evaluate the level of oxidative stress caused in livers of mice treated by the mushroom hepatotoxin alpha amnitin, using two different instrumental methods: EPR direct and indirect spectroscopy and visible spectrophotometry.

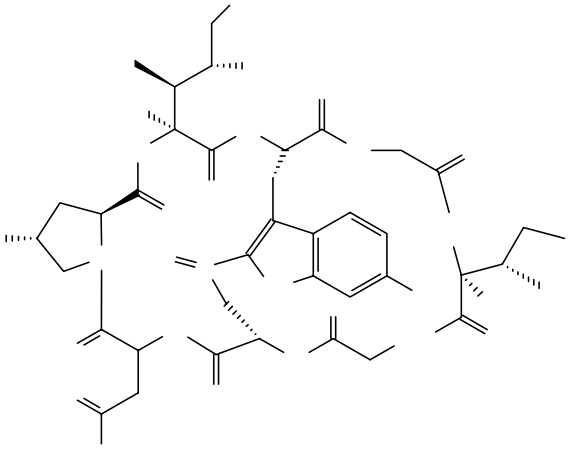

Figure 1. Chemical structure of alpha amanitin

MATERIALS AND METHODS

Chemicals

Alpha amanitin and spin-trap n-tert-butyl-alpha-phenylnitrone (PBN), were purchased by Sigma Chemical Co, St. Louis, USA All other chemicals used in this study were analytical grade.

Animals and treatment

White laboratory mice with weight 20-40 g were used. The mice were housed in polycarbonate cages in controlled conditions (12 h light/dark cycles), temperature of 18–23^{o}C and humidity of 40–70%, with free access to tap water and standard laboratory chow. Experiments were carried out in accordance with European directive 86/609/EEC of 24.11.1986 for protection of animals used in scientific and experimental purposes. Mice were divided in two groups (7 mice in each group) and inoculated ip with alpha amanitin in saline. Before treatment every mouse was weighed out and inoculated ip with a volume of the toxin that corresponded to a dose of 1mg/kg (subletal). Control group was inoculated with solvent only. After 20 hours of the treatment all animals in the tested and control group were exsanguinated under light ether anesthezia and the livers were collected and washed in cool saline.

Electron paramagnetic resonance (EPR) studies

All EPR measurements were performed at room temperature on a X-band EMX^{micro}, spectrometer Bruker, Germany, equipped with standard Resonator. All EPR experiments were carried out in triplicate and repeated thrice. Spectral processing was performed using Bruker WIN-EPR and Simphonia software.

1. EPR ex vivo ROS production in liver tissues of tested and control mice

Preparation of homogenates and EPR study of ROS production was performed according to Shi et al., 2005 with some modifications (7). Briefly, about 0.1 g of liver tissue was homogenized for 2 min after addition 1.0 ml of 50 mM solution of the spin-trapping agent PBN dissolved in DMSO. After centrifugation, 0.4 ml supernatant of homogenized tissue was taken in quartz tube and stored in liquid nitrogen for EPR measurement. EPR spectra were recorded at room temperature. EPR settings were as follows: 3503.74 G center field, 20.49 mW microwave power, 0.50 G modulation amplitude, 100 G sweep width, a

receiver gain $1x10^6$, 327.68 ms time constant, 81.92 s sweep time.

2. EPR ex vivo evaluation the level of ascorbic acid radicals in liver tissues of tested and control mice

Homogenates from mice livers were prepared in DMSO in a ratio of 1:3. After centrifugation the supernatants were collected and immediately transferred into a quartz tubes and placed in EPR cavity. EPR settings were as follows: 3505.000 G center field, 20.37 mW microwave power, 5.00 G modulation amplitude, 30 G sweep width, a receiver gain $1x10^4$, 327.68 ms time constant, 81.92 s sweep time, 5 scans per sample.

Thiobarbituric acid (TBAS) method

Total amount of lipid peroxidation products in the supernatant was estimated using TBAS method, which measures MDA reactive products (8). Briefly, 1 ml of liver supernatant, 1 ml of saline and 1 ml of 25% trichloroacetic acid (TCA) were mixed and centrifuged at 2000 rpm for 20 min. Protein free supernatant (1 ml) was mixed with 0.25 ml of 1% TBA and boiled for 1 h at 95°C. After cooling, the intensity of the pink color of the obtained fraction product was read at 532 nm. Amount of MDA reactive products was expressed in µM/g protein.

Statistical analysis

Statistical analysis was performed with Statistica 6.1, StaSoft, Inc. and results were expressed as means ± standard error (SE). Statistical significance was determined by the Student's t-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Results from EPR *ex vivo* study on the levels of ROS production and the level of ascorbate free radicals in liver tissue of mice treated by the toxin and control mice are presented on **Figure 2, Figure 3, Figure 4 and Figure 5.** Twenty hours after toxin treatment, ROS production marked by EPR spectra signals of the studied mice liver homogenates could be detected. EPR spectra of mice liver free radicals trapped by PBN exhibited six-lines **(Figure 2)**.

By WIN-EPR and Simphonia software programmes the following hyperfine splitting constants of the spin adducts registered were calculated: a^N = 13.88 G and a^H = 2.35 G. Based on the values of the splitting constants, the spin adducts were identified as PBN/$\dot{O}CH_3$ radicals (9). To confirm that the radicals trapped by PBN originated, only from the livers of mice, additional control samples containing alpha amanitin plus DMSO solution of PBN or only DMSO solution of PBN, were also studied but no PBN spin adducts were observed (data not shown).

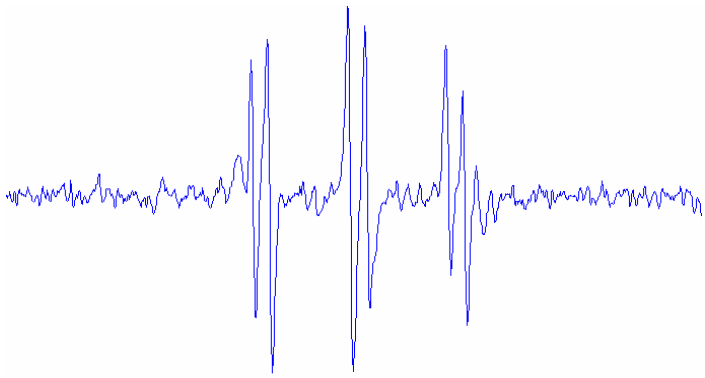

Figure 2. EPR spectrum of PBN adduct registered in liver homogenates of mice treated by alpha amanitin

As is seen on **Figure 4**, twice higher levels of ROS production (calculated as double integrated plots of EPR spectra of the PBN adducts and expressed in arb. units were found in liver homogenates of tested mice comparing to those of the control mice. Typical EPR doublet signal of ascorbate free radical (10) was registered either in liver homogenates of treated mice or in control mice with a g value = 2.0070± 0.0002 and a^H = 1.88±0.02 G (**Figure 3**). The level of the ascorbate radicals in liver homogenates of treated mice was 1.6 times higher than that of the control group of mice (**Figure 5**).

Results from study the level of MDA products in livers of tested and control mice are shown on **Figure 6.** As is seen the level of MDA products in liver supernatants isolated from

mice treated by alpha amanitin was slightly higher compared to that of the controls.

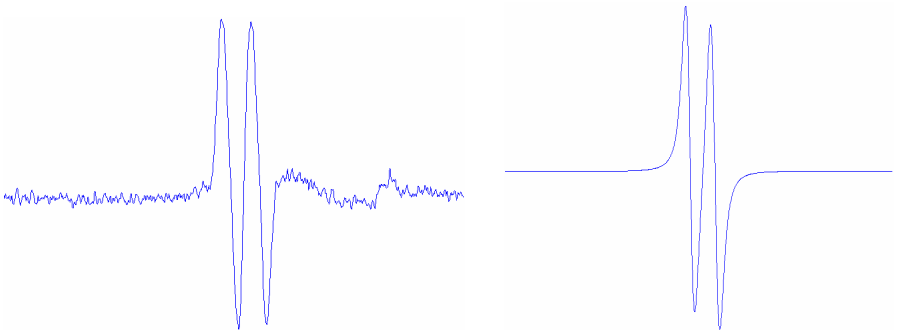

Figure 3. EPR spectrum of ascorbate radical registered in liver homogenates of mice treated by alpha amanitin (A) and simulated EPR spectrum (B) of the same radical

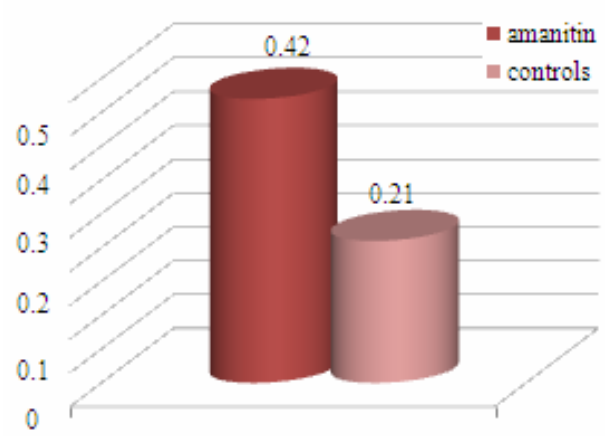

Figure 4. Level of PBN-adduct radicals (arb. units) in liver homogenates of mice treated by alpha amanitin

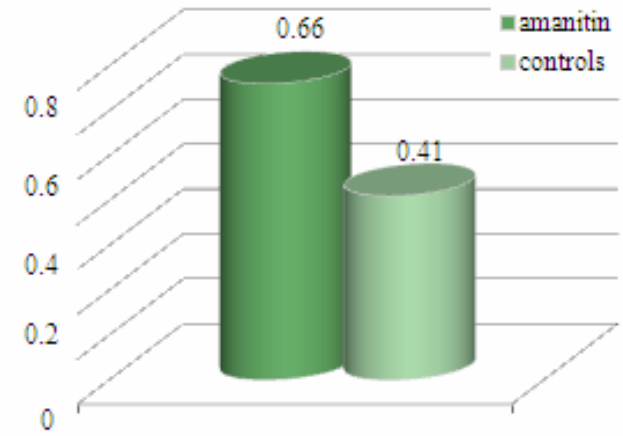

Figure 5. Level of ascorbate radicals (arb. units) in liver homogenates of mice treated by subletal dose alpha amanitin

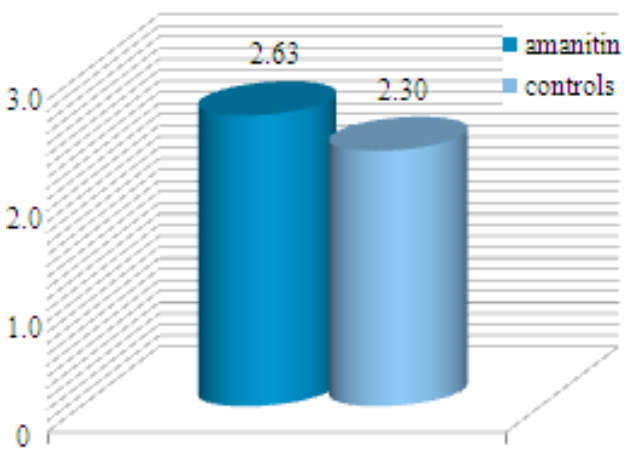

Figure 6. MDA products levels (µM/gPr) in liver homogenates of mice treated by alpha amanitin

DISSCUSSION

It was demonstrated that the reaction of DMSO with hydroxyl free radicals ($^{\cdot}OH$) produced $^{\cdot}CH_3$ radicals, and that oxidation of $^{\cdot}CH_3$ in aerobic conditions produced $^{\cdot}OCH_3$ radicals (9, 11). Since, in the present *ex vivo* EPR spin trapping study the radicals formed in mice liver homogenates (prepared in aerobic conditions) were identified as PBN/$^{\cdot}OCH_3$, we accept that *in vivo* alpha amanitin can cause generation of $^{\cdot}OH$ trapped at our experimental conditions as PBN/$^{\cdot}OCH_3$ adduct (9, 12, 13).

Moreover, Sharma et al., 2005, have studied possibility the level of ascorbate free radicals (AFS) to be used as a real-time marker of free radical generation in briefly and ischemic and reperfushed hearts (14). Having in mind above reported findings and that after one-electron oxidation ascorbic acid can produce relatively stable ascorbate free radicals that can be detected by direct EPR spectroscopy (15, 16) we accept that the reason to be registered increased levels of ascorbate free radicals in the liver tissues of poisonous mice in comparison with those of the controls are $^{\cdot}OH$ radicals generated in mice liver and confirmed by the present EPR spin trapping technique.

The slight elevated levels of MDA products registered in livers of poisonous mice might be explained by the less stability of MAD products and on the other hand by the well known fact that TBAS method is less sensitive enough to detect little variation of LPO process (17).

Based on this preliminary comparative study we might conclude that for *in vivo* characterization of the oxidative process in livers of mice treated by the toxin is more proper to be used not only the TBAS spectrophotometrical method but to be combined by a proper direct and indirect EPR spectroscopy.

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