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Original Contribution

PHENOXYL RADICALS FORMATION MIGHT CONTRIBUTE TO SEVERE TOXICITY OF MUSHROOOM TOXIN ALPHA AMANITIN – AN ELECTRON PARAMAGNETIC RESONANCE STUDY

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ABSTRACT

The aim of the present research was by Electron Paramagnetic Resonance (EPR) spectroscopy using spin trapping technique to study *in vitro* oxidation of severe mushroom toxin alpha amanitin and second to evaluate its *in vivo* effect on oxidative status in mice kidney tissue.

During *in vitro* oxidation of alpha amanitin by PbO_2 in presence of nitrosobenzene as a spin-trapping agent, a triplet EPR signal was registered. Moreover, statistical significant increased levels of ROS products were found in kidney tissue homogenates isolated from the toxin treated mice comparing to those of the non-treated controls. Based on the present EPR study we assume that *in vivo*, alpha amanitin forms unstable phenoxyl radicals that might be a trigger for initiation of oxidative stress in kidney tissues of the toxin treated mice.

Key words*:* alpha amanitin; EPR spin trapping technique; phenoxyl radicals.

INTRODUCTION

Deadly poisonous *Amanita phalloides* mushroom causes dramatic toxic consequences mainly within the liver and kidneys of humans. It was established that *Amanita phalloides* severe toxicity was due to the group of natural toxins named amatoxins (alpha-amanitin, beta-amanitin,… ets.), isolated from this mushroom (1). It has been reported that the molecular mechanism of amatoxin toxicity was due to inhibition of RNA polymerase II of eukaryotic cells (2, 3). To the present does not exist a proper and efficient antidote for the treatment of humans *Amanita phalloides* mushroom intoxications.

By our former *in vivo* studies we reported increased levels of lipid peroxidation products in the livers of mice treated by alpha amnitin **(Figure 1)** in comparison with those of non treated controls (4). Recently by *in vitro* and *ex vivo* EPR spectroscopy studies we have demonstrated that alpha amanitin can form free radicals by itself, which are probably responsible for the elevated levels of ROS products registered in livers of mice treated with subletal dose of alpha amanitin comparing to the controls (5).

In the present EPR spectroscopy study we have reported formation of unstable phenoxyl radicals by alpha amanitin during *in vitro* oxidation with PbO_2 in presence of nitrosobenzene as a spin trapping agent. By *ex vivo* EPR spectroscopy were also found higher levels of ROS production in the kidneys of alpha amanitin treated mice comparing to those of non treated controls.

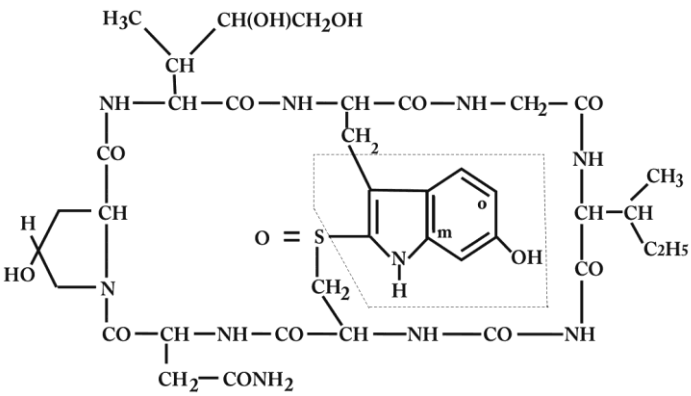

Figure 1. Chemical structure of alpha amanitin

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MATERIALS AND METHODS

Chemicals

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

Animals and treatment

Male albino non-inbred 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 (6 mice in each group) and inoculated i.p. with alpha amanitin in saline. Before treatment every mouse was weighed out and inoculated i.p. 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 anesthesia and the kidneys were immediately collected, washed in cool saline and start to prepare tissues homogenates.

Electron paramagnetic resonance (EPR) studies

EPR measurements were performed at room temperature on an X-band EMX^{micro}, spectrometer Bruker, Germany, equipped with standard resonator. All EPR experiments were performed in triplicate. Quartz sample tubes of 1 mm i.d. were used in all experiments. Spectral processings were performed using Bruker WIN-EPR and Simfonia software.

a) In vitro oxidation of alpha amanitin by PbO_2 in presence of nitrosobenzene as a spin-trapping agent

Alpha amanitin was oxidized in the presence of spin trapping agent nitrosobenzene, according to (6) with some modifications. Briefly, 150 mg of PbO_2 were suspended in a mixture, containing 1.9 ml of 50 mM solution of the spin trap dissolved in benzene plus 0.1 ml of 0.5 mM alpha amanitin and stirred for 2 min under a nitrogen atmosphere. After sedimentation of the heterogeneous phases the upper layer was collected and a quartz tube was filled with the sample and placed in the EPR cavity. Blank sample containing 0.1 ml of water instead of the toxin was also studied. EPR spectra were recorded at the follows EPR spectrometer settings: 3515.730 G center field, 2.032 mW microwave power, 10.00 G modulation amplitude, 200 G sweep width, a receiver gain $2x10^4$, 81.92 ms time constant, 40.96 s sweep time, 5 scans per sample.

b) EPR ex vivo ROS production in mice kidney tissues after alpha amanitin treatment

Tissues homogenates preparation and EPR study on ROS production were performed according to Shi et al., 2005 (7) with some modifications. Briefly, about 0.1 g of kidney 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

c) *EPR ex vivo evaluation the level of ascorbate free radicals in mice kidney tissue after alpha amanitin treatment*.

Kidney homogenates from mice were prepared in DMSO in a ratio of 1:3. After centrifugation the supernatants were collected and immediately transferred into 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.

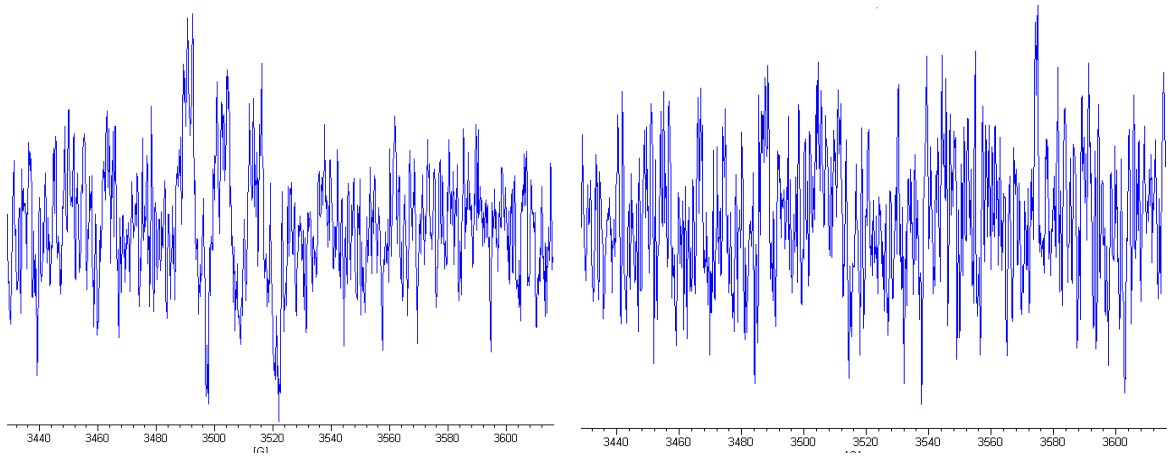
Statistical analysis

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

To check whether alpha amanitin is able to form phenoxyl free radicals, we have studied *in vitro* toxin oxidation by PbO_2 in presence of nitrosobenzene. As shown on **Figure 2 A** an

EPR triplet signal was registered in the studied sample. No EPR signal was registered when the toxin was omitted (**Figure 2 B**). A benzene solution of nitrosobenzene and a mixture containing benzene solution of nitrosobenzene plus PbO_2 were also studied but no EPR signals were registered (results are not shown). The shape of the EPR spectrum and calculated values of hyperfine splitting constants (a^N = 11.45 G, a^H (2, 4, 6) = 3.04 G, a^H (3, 5) = 1.35 G) were similar to those reported by Omelka and Kovacova for different sterically unhindered phenoxyl radicals formed during oxidation by PbO_2 in presence of nitrosobenzene as a spin trap (6).



A **B**

Figure 2. EPR spectrum of spin adduct registered in the sample containing 50 mM solution of spin trapping agent nitrozobenzene dissolved in benzene, 0.5 mM alpha amanitin and PbO_2 (A) and EPR spectrum of the same sample but without alpha amanitin (B).

Results from EPR *ex vivo* study on the levels of ROS production in the kidney tissues of mice treated by the toxin and control mice are presented on **Figure 3 and Figure 4**. Twenty hours after toxin treatment, ROS production marked by EPR spectra of PBN spin – adducts consisting of six spectral lines in the studied mice kidney homogenates could be detected (**Figure 3**). The calculated hyperfine splitting constants of the PBN spin adducts registered were: a^N = 13.90 G and a^H = 2.35 G.

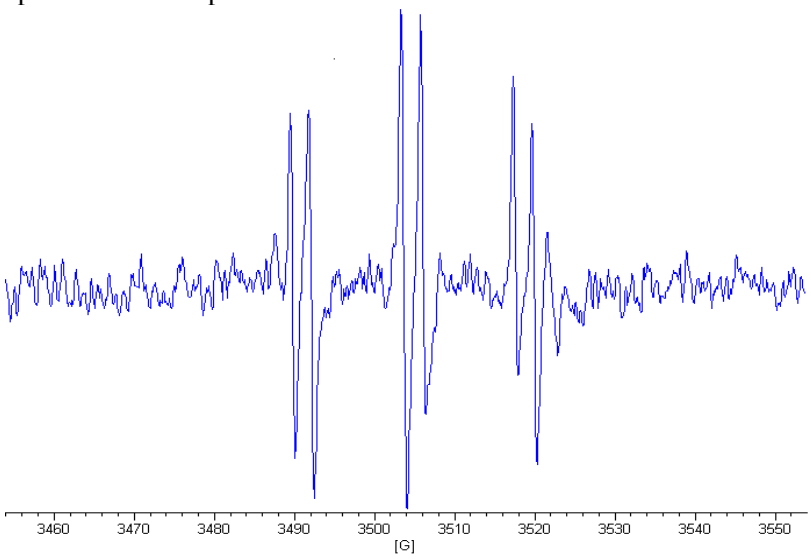


Figure 3. EPR spectrum of the PBN-adduct registered in kidney homogenates of toxin treated mice and control mice.

Based on the values of splitting constants the spin adducts were identified as PBN/$^{\cdot}OCH_3$ radicals (8). To confirm that the radicals trapped by PBN originated, only from the

kidneys 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).

As is seen on **Figure 4**, statistical significant increased levels of ROS production (calculated as double integrated plots of EPR spectra of the PBN adducts) were found in kidney homogenates of tested mice comparing to those of the controls.

A typical duoblete EPR signal of an ascorbate free radical was registered in kidney tissues isolated from toxin treated and control mice (not presented). Moreover, the level of the ascorbate radical registered in the kidneys of alpha amanitin treated mice calculated by the intensities of the EPR siglnals was almost twice higher (183 600 ± 25 000 arb. units) than that of the nontreated controls (96 480 ± 19 400 arb. units).

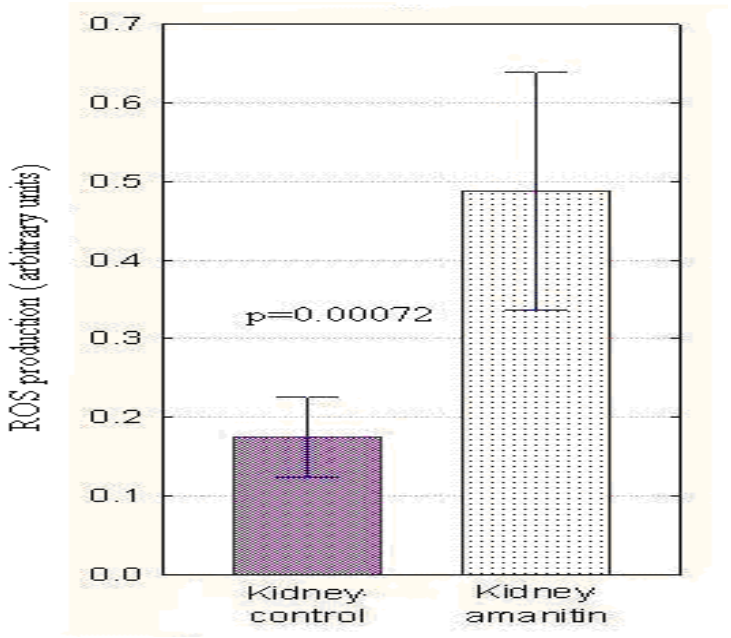


Figure 4. Levels of ROS production in kidney homogenates of alpha amanitin treated mice and control mice (expressed in arbitrary units)

DISCCUSION

Detection of either *in vitro* or *in vivo* generated endogenous free radicals is difficult because they have extremely short half-lives and are in low concentrations. Spin-trapping followed by EPR analysis overcomes the limit of sensitivity of endogenous radicals in biological systems, and has proved to be the least ambiguous method to detect short-lived reactive free radicals generated in low concentrations in biological systems (8, 9, 10). Omelka and Kovacova studied *in vitro* oxidation of variously substituted unhindered phenols using nitrozobenzene as a spin trap and reported that phenoxyl radicals formed in the oxidation with PbO_2 added to the nitroso group of the spin-trap as *ortho*-carbon-centered radicals (primary adducts) and rapidly rearranged to the phenoxazine-10-oxyl radicals as final radical products. They also established that the adducts of some unhindered *meta*–substituted phenoxyls with nitrosobenzene cannot rearranged to phenoxazine-10-oxyl radicals because the steric hindrance of coplanarity required for formation of the phenoxazine skeleton. Because the presence of a phenolic group and a bulky substitute on one of the *meta*-positions of alpha amanitin tryptophan moiety (see **Figure 1**) we assume that during oxidation alpha amanitin forms phenoxyl radicals which were added to the spin trapping agent as *ortho*-carbon-centered radicals (primary adducts) **(Figure 5)** according to the reaction scheme proposed by Omelka and Kovacova (8).

Mason and co-workers have studied hydroxyl radical formation in biological systems by a scavenging reaction in which this radical is converted into methyl radical via its interaction with DMSO (9). In a number of EPR studies methyl radical ($^{\cdot}CH_3$) was detected as its long-lived PBN adduct in presence of PBN as a spin trap. By these studies were demonstrated that the reaction of DMSO with $^{\cdot}OH$ produced $^{\cdot}CH_3$, and that oxidation of $^{\cdot}CH_3$ in aerobic conditions produced $^{\cdot}OCH_3$ (8, 9, 10). Bearing in mind that our *in vivo* EPR spectroscopy study was also carried out at aerobic conditions, registration of PBN/$^{\cdot}OCH_3$ adducts in the mice kidney homogenates might be explained by the generation of $^{\cdot}OH$. Since, the reactivity of phenoxyl radicals is surprisingly high and the rate constants of these radicals for hydrogen atom abstraction are 100-300 times greater than those for peroxyl radicals (11) we suppose that during its *in vivo* accumulation alpha amanitin probably forms phenoxyl free radicals that might be involved in generation of reactive oxygen species, in particular $^{\cdot}OH$ radicals confirmed by higher levels of PBN/$^{\cdot}OCH_3$ adducts in the kidney tissues of alpha amanitin treated mice comparing to those of non-treated control mice.

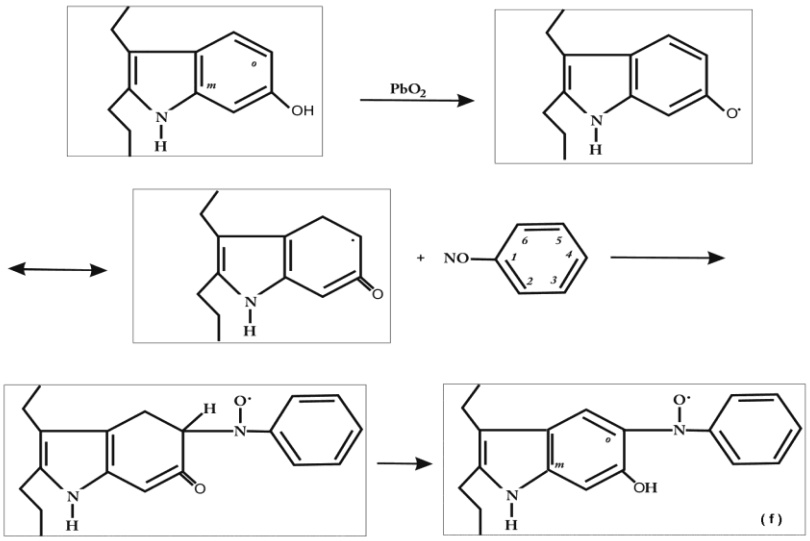


Figure 5. Proposal mechanism for the spin adduct formed between alpha amanitin phenoxyl radical and nitrosobenzene

It is well documented that the intensity of EPR signal of the spectrum of ascorbate free radical is an indicator of oxidative stress *in vitro* and *in vivo* (12) and might be used as a real-time marker for free radical generation in biological systems (13,14). By our *ex vivo* EPR study we established increased levels of ascorbate free radicals in the kidneys tissues of alpha amanitin treated mice comparing to those of the control mice which result correlates with the increased levels of ROS production (PBN/$^{\cdot}OCH_3$ adducts) registered in the kidneys of poisonous mice.

By the present EPR spectroscopy studies we have demonstrated that during *in vitro* oxidation mushroom amatoxin alpha amanitin can form unstable phenoxyl radicals by itself and *in vivo* exhibits pro-oxidant properties. In conclusion we accept that hepatotoxin alpha amanitin during its *in vivo* accumulation could form highly reactive phenoxyl radicals which are probably the reason for generation of ROS not only in livers but in kidney cells of the poisonous mice and this might contribute to severe toxicity of the toxin.

ACKNOWLEDGMENT

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