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Original Contribution

IN VIVO ANTIOXIDANT AND PROOXIDANT PROPERTIES OF *AMANITA PHALLOIDES* MUSHROOM TOXINS

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ABSTRACT

This is an *in vivo* study detailing the effects of catalase (CAT), superoxide dismutase (SOD) and the level of lipid peroxidation (LPO) products in liver homogenates from mice treated with alpha amanitin or phalloidin. Spectrophotometry was the main method used in this study. Our results showed that both toxins increased SOD activity, confirming an earlier *in vitro* study. In addition, our present study showed that alpha amanitin inhibited CAT activity and showed prooxidant properties, while phallodin showed an increase in CAT activity, which confirmed its *in vivo* antioxidant properties.

Keywords: alpha amanitin, phalloidin, antioxidant, prooxidant, lipid peroxidation.

INTRODUCTION

Amatoxins (alpha amanitin and beta amanitin), phallotoxin (phalloidin) are bridged oligopeptides found in a few Amanita mushroom species, mainly *Amanita phalloides* [1, 2] The basic molecular mechanism of amatoxin toxicity is the inhibition of RNA polymerase II in eukaryotic cells [3, 4]. They cause dramatic toxic consequences mainly within the liver [5] and kidney [6]. However, among the different classes of amatoxins (alpha-, beta-, etc), a straight correlation between *in vivo* LD50 and *in vitro* inhibitory constants (Ki) has not been determined [5].

Hepatotoxic effects of the phallotoxins, including phalloidin, have been attributed to their specific binding to F-actin, thus strongly stabilising the structure of the assembled filaments [2]. Subsequently, a depletion of the mitochondrial Ca^{2+} pool would occur as is shown in intact hepatocytes. This finding was related to the primary toxic effects of the phalloidin poisoning [7].

Our earlier *in vitro* studies demonstrated that alpha and beta amanitin could interact in a concentration dependent manner with the stable free radical α,α-diphenyl-β-picrylhydrazyl (DPPH) [8]. It is a well-known fact that this very stable radical is used extensively in electron spin resonance studies as a standard for measurement of free radical concentrations [9] and for scavenging of free radicals as proof of its antioxidant properties [10]. Moreover, Richer and Frei 1988, have discussed Ca^{2+} release from mitochondria induced by prooxidants [11]. Bearing in mind both, the last article discussion and depletion of mitochondrial Ca^{2+} pool after treatment of intact hepatocytes by phalliodin we assumed that *in vivo* this toxin might exhibit prooxidant properties.

Recently, Enjalbert et al, have discussed in detail treatment of amatoxin poisoning. In their 20-years retrospective analysis they concluded that no specific amatoxin antidote is available, but therapeutic agents such as benzylpenicillin or other β-lactam antibiotics, silymarin complex, thioctic acid, antioxidant drugs and other drugs are used for the treatment of amatoxin poisoning [12].

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At the present moment it is obvious that hepatotoxic effect of the amatoxins and phallotoxins is not completely explained and some other additional biochemical processes might be involved in their toxicity. Using *in vitro* studies we have demonstrated that alpha amanitin, beta amanitin and phalliodin could inhibit CAT activity to a considerable degree [13]. Our previous study done *in vitro* demonstrated activating effect of alpha amanitin, beta amanitin and phalliodin on SOD activity. We showed also that this effect was due to superoxide anion scavenging activity (SSA) of the toxins [14]. We concluded in this previous study that alpha amanitin and phalloidin exhibited prooxidant or antioxidant properties *in vitro*. This would suggest its ability to carry out free radical reactions *in vivo*. This therefore informed the decision to study the *in vivo* effect of alpha amanitin and phalloidin on CAT and SOD activity and the level of lipid peroxidation (LPO) products in hepatocytes from mice treated with these toxins.

MATERIALS AND METHODS

1. Chemicals

Alpha amanitin, phalloidin, enzymes SOD and CAT and all other reagents were purchased from Sigma Chemical Co (St. Louis, USA).

2. Animals

White male laboratory mice with weight 20-22 g were used. All animals received food and water *ad libitum* and were in good health.

3. Methods

CAT and SOD activity and the level of LPO products were determined in tissue homogenates from liver isolated from mice treated with the corresponding toxin. Briefly, two groups of mice, 10 in each, were injected i. p. either 200 μl alpha amanitin 1 mg/kg (LD50) or 200 μl alpha amanitin 0.5 mg/kg (sublethal dose). Another group of 10 mice were injected i. p. with 200 μl phalloidin, 2mg/kg (LD50). At 20th h after injection, 5 animals from each group were sacrificed under ether anaesthesia; the livers were removed and kept in cool saline. After several washes with cool saline, 500 mg of livers were homogenised carefully in PBS buffer containing 1 mmol EDTA and centrifuged at $4^{o}C$ for 10 min at 14,000rpm. Supernatants were collected and assessed for CAT and SOD activities, level of LPO products and total protein concentration. Survival of the remaining 5 treated animals in each group was followed for the next days. Since all mice in the group injected with sublethal dose of alpha amanitin survived, they were eventually sacrificed on the 6th day and CAT activity, SOD activity and level of LPO products studied. A control group of untreated mice (5 in the group) was also studied. They were treated i.p. with 200 μl saline.

CAT and SOD activities were determined as previously described [13, 14].

CAT activity was determined following decrease in the initial H_2O_2 concentration (30 mM, used as a substrate) at 240 nm at 25^0C in 60 s according to Johanson and Borg 1988, with some modifications [15]. Briefly, 100 μl of supernatants isolated from mice liver homogenates were placed in a cuvette in PBS buffer (50 mM) to a final volume 2 ml. After adding of 1 ml H_2O_2 the decrease of the absorption at 240 nm for 60 s was followed. One unit of CAT was defined as amount of enzyme that degraded 1 μl H_2O_2 per min.

Determination of SOD activity was based on the generation of superoxide anion radical $^{\bullet}O_2^-$ in a system containing hypoxanthine/xanthine oxidase in which $^{\bullet}O_2^-$ reduced nitroblue tetrazolium to formazan; the absorbance of the latter was measured at 560 nm. SOD activity was determined according to Sun et al., 1988 [16] with slight modifications. Briefly, in glass tubes were added consecutively the following reagents: 200 μl hypoxanthine (0.075mg/ml of PBS), 20 μl EDTA (0.085 mM), 400 μl NBT (0.05mg/ml of PBS), 100 μl of supernatants isolated from mice liver homogenates, 880 μl PBS buffer and 400 μl xanthine oxidase (0.25 mg/ml PBS) were added to a final volume of 2 ml. Tubes were stirred, stoppered and incubated at 37^oC for 20 min. After incubation, samples were put on ice and absorbance at 560 nm was measured. In the control sample the amount of the liver supernatant was replaced by equivalent volume of PBS buffer. One unit SOD activity was defined as the amount of enzyme causing 50 % inhibition of NBT reduction to formazan.

CAT and SOD activity were expressed as enzyme units per mg total protein (U/mg protein).

Total protein concentrations were determined by the Human total protein indicator assay kit (Human, Germany) based on the biuret method, with bovine serum

albumin as a standard.

Total amount of LPO products in the supernatant was estimated using thiobarbituric acid (TBA) method, which measures the malondialdehyde (MDA) reactive products [17]. Briefly, 1 ml of supernatant, 1 ml of saline and 1 ml of 25% trichloroacetic acid (TCA) were mixed and centrifuged for 20 min at 2000 rpm. 1 ml of protein free supernatant was mixed with 0.25 ml of 1% TBA and boiled for 1 h at 95°C. After cooling, the intensity of the pink colour of the resulting fraction product was read at 532 nm. Amount of MDA reactive products was expressed in nmol/mg protein.

4. Apparatus

All visible and UV spectrophotometric studies were performed on a Pharmacia LKB Ultrospec spectrophotometer (Sweden).

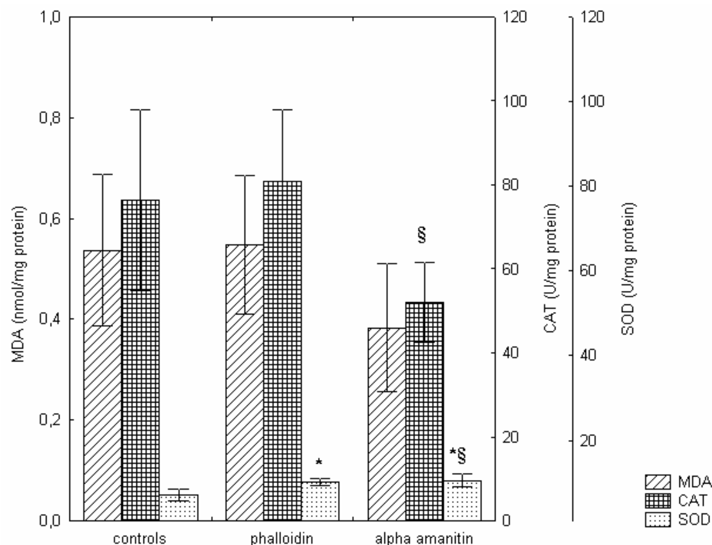
5. Statistical analysis

All data were expressed as means and standard deviations. Student's t-test was used to determine the statistical differences between mean values. Differences were considered significant when the p value was less than 0.05.

RESULTS AND DISCUSSION

In vivo effects of the toxins on CAT and SOD activities and the level of MDA products in supernatant isolated from the liver of mice treated with alpha amanitin (LD50) and phalloidin (LD50) are shown in **Figure 1**. Both groups treated with the toxins expressed increase in SOD activity (mean 9.64 ± 1.63 for alpha amanitin and 9.15 ± 0.85 for phalloidin), when compared to the control group (mean 6.04 ± 1.41). This finding confirms that both toxins exhibit antioxidant properties not only at *in vitro* [14], but at *in vivo* conditions. This increase was attributed to their superoxide anion scavenging activity (SSA) [14]. Increase in SOD activity for both toxins was significant, compared to the controls ($p<0.05$).

***Figure 1**. In vivo CAT and SOD activity and levels of MDA products in supernatant isolated from liver homogenates of mice treated with alpha amanitin 1 mg/kg (LD50) or phalloidin 2 mg/kg (LD50).*



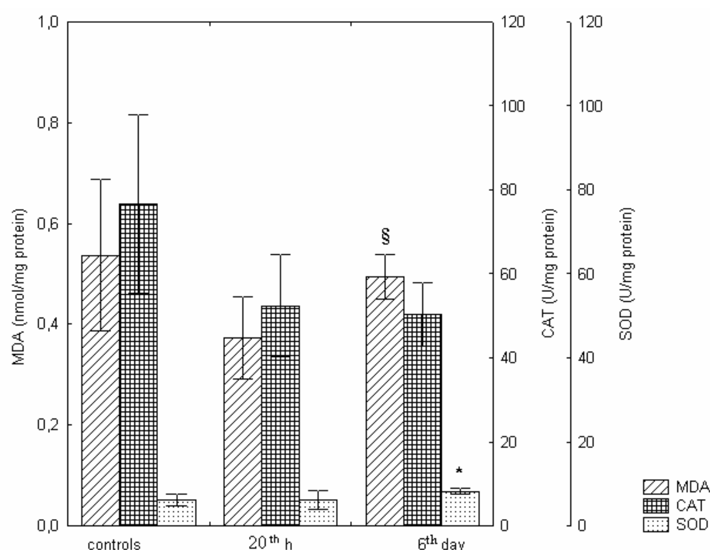
[*] Significant values for both toxins compared to the controls, $p < 0.05$; [§] significant values for alpha amanitin compared to phalloidin, $p < 0.05$.

CAT activity of liver supernatant isolated from mice treated with alpha amanitin was inhibited to a considerable degree (mean 52.58 ± 9.44) in comparison with the controls (mean 76.63 ± 21.38), while CAT activity of liver supernatant isolated from mice treated with phalloidin exhibited slight increase (mean 81.31 ± 16.89, not significant ($p>0.05$) compared to the controls. Our previous *in vitro* studies demonstrated a well - expressed inhibiting effect of alpha amanitin on CAT activity (prooxidant properties of the toxin) [13]. It was obvious that alpha amanitin inhibited CAT activity either at in *vitro* or at *in vivo* conditions (prooxidant properties), while phalloidin exhibited prooxidant properties *in vitro* and slight antioxidant properties *in vivo*.

The level of MDA products in liver supernatant isolated from mice treated with phalloidin was slightly increased (mean 0.55 ± 0.14, not significant, $p >0.05$) when

compared to the controls (mean 0.54 ± 0.15). Based on this result initiation of some peroxidative process in hepatocytes might be assumed. Moreover, at this stage of investigation this last finding cannot be explained because of *in vivo* phalloidin antioxidant properties demonstrated. The level of MDA products in liver supernatant isolated from mice treated with alpha amanitin was lower (mean 0.38 ± 0.13, significant, $p<0.05$) compared to that of the controls (mean 0.54 ± 0.15) which supported the lack of peroxidative process. To explain this finding we propose that antioxidant properties of alpha amanitin in some way prevailed over its prooxidant properties at a dose of LD50 at 20th h before killing the mice. To make clearer this finding we also studied CAT and SOD activities and MDA products of a group of mice treated with sublethal dose of the toxin. Results are presented in **Figure 2**. SOD activity of the supernatant isolated from liver homogenates of mice treated with sublethal dose of alpha amanitin showed slight increase at 20th h (mean 6.11 ± 2.17, not significant, $p>0.05$) and considerable increase at 6th day (mean 8.22 ± 0.68), significant, $p<0.05$) compared to the controls (mean 6.04 ± 1.41). This result confirmed above-mentioned results with the group of mice treated with alpha amanitin at a dose of LD50 and supports the idea for *in vivo* antioxidant properties of the toxin.

Figure 2. *In vivo CAT and SOD activity and levels of MDA products in supernatant isolated from liver homogenates of mice treated with sublethal dose of alpha amanitin (0.5 mg/kg), measured at 20th h and 6st day*



* *Significant values for alpha amanitin compared to the controls, $p < 0.05$; § significant values for 6th day from the start of treatment with sublethal dose of alpha amanitin compared to 20th h of the same treatment, $p < 0.05$.*

CAT activity of the same group of mice (see **Figure 2**) was considerably lower in comparison with the controls (mean 76.63 ± 21.38), for both, 20th h (mean 52.42 ± 12.14, not significant, $p>0.05$) and 6th day (mean 50.34 ± 7.46, not significant, $p>0.05$). This finding confirmed prooxidant properties of alpha amanitin at a dose of LD50 (see **Figure 1**). Interesting result was obtained for the level of MDA products. Considerable decrease for the last was established at 20th h compared to the controls. At the same time a significant increase ($p<0.05$) was registered at the 6th day (mean 0.49 ± 0.04) in comparison with the 20th h (mean 0.37 ±. 0.08). These results make us propose a development of an oxidative process in the hepatocytes of mice treated with sublethal doses of alpha amanitin.

CONCLUSION

Our previous and present results show that alpha amanitin and phalloidin exhibit prooxidant and antioxidant properties not only at *in vitro* but at *in vivo* conditions, as well. These findings support the idea that both toxins penetrating through the liver cell membranes might generate free radicals and / or be involved in free radical reactions that contribute to their severe hepatotoxicity.

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