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Toxicon 48 (2006) 264–271

TOXICON

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Toxic and lethal effects of ostreolysin, a cytolytic protein from edible oyster mushroom (*Pleurotus ostreatus*), in rodents

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Received 20 February 2006; received in revised form 16 May 2006; accepted 30 May 2006
Available online 14 June 2006

Abstract

Ostreolysin (Oly), an acidic, 15 kDa protein from the edible oyster mushroom (*Pleurotus ostreatus*), is a toxic, pore-forming cytolysin. In this paper, its toxic properties have been studied in rodents and the LD_{50} in mice shown to be 1170 μg/kg. Electrocardiogram, arterial blood pressure and respiratory activity were recorded under general anaesthesia, in intact, pharmacologically vagotomised and artificially respirated rats injected with one mouse LD_{50}. A few seconds after intravenous Oly injection, a transient increase in arterial blood pressure was recorded, followed by a progressive fall to mid-circulatory pressure accompanied by bradicardia, myocardial ischaemia and ventricular extrasystoles. Similar changes produced by Oly were observed in vagotomised and artificially respirated animals, indicating that vagotomy and hypoxia play no primary role in toxicity. Oly induced lysis of rat erythrocytes *in vitro*, and probably also *in vivo* as indicated by the increase in serum potassium. Although direct action of the protein on the cardiomyocytes or heart circulation cannot be excluded, the hyperkalaemia resulting from the haemolytic activity probably plays an important role in its toxicity. The lethality and cardiorespiratory toxic action of Oly are thus shown to be candidates for the cause of the recorded adverse effects of oyster mushroom.

Keywords: Acute toxicity; Cardiorespiratory effect; Haemolysis; LD_{50}; Ostreolysin; *Pleurotus ostreatus*; Rats; Mice

1. Introduction

The edible oyster mushroom (*Pleurotus ostreatus*) is a massively cultivated species with important medicinal, biotechnological, and environmental applications (Kües and Liu, 2000; Cohen et al., 2002). However, sporadic local intoxications following human and animal ingestion of large quantities of the fresh mushroom were recorded, and it was suggested that the toxicity is associated with thermolabile proteinaceous molecules (Al-Deen et al., 1987). Recently, water extracts of oyster mushroom were reported to provoke contraction of nonvascular tracheal smooth muscle *in vitro* (Schachter et al., 2005).

Bernheimer and Avigad (1979) reported that *P. ostreatus* produces a 12 kDa protein pleurotolysin, which was haemolytic for mammalian erythrocytes. Recently, purification and isolation of a novel

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0041-0101/$ - see front matter © 2006 Published by Elsevier Ltd.
doi:10.1016/j.toxicon.2006.05.011

haemolytic protein ostreolysin (Oly) has been described (Berne et al., 2002). The protein has a molecular weight of 15 kDa, an isoelectric point of pH 5.0, and belongs to the aegerolysin family of small acidic proteins found in bacteria, fungi and moulds (Berne et al., 2002, 2005). Its haemolytic activity can be abolished by heating (Berne et al., 2002) or by extremes of pH (Berne et al., 2005). The biological role of Oly is not known, however its absence in vegetative mycelium and appearance at the stage of primordial formation suggests its involvement in the process of fructification of the mushroom (Berne et al., 2002; Vidic et al., 2005). Oly lyses bovine, sheep and human erythrocytes in sub-micromolar concentrations (Sepčić et al., 2003), as well as various normal and transformed cell lines (Sepčić et al., 2003, 2004). Experiments on model lipid membranes revealed an exclusive interaction of Oly with cholesterol–sphingomyelin membranes in the liquid-ordered state (Sepčić et al., 2004). Both binding and permeabilisation were promoted by increasing cholesterol concentration. After incubation with Chinese hamster ovary cells, Oly was found in their detergent-resistant membranes, corresponding to cholesterol-enriched membrane microdomains or lipid rafts (Sepčić et al., 2004).

The aim of this work was to determine the lethal and toxic action of Oly, and to study the mechanisms that could be responsible for the toxicity of the oyster mushroom (Al-Deen et al., 1987).

2. Materials and methods

2.1. Oly isolation and preparation

Oly was purified from the fruiting bodies of freshly collected oyster mushrooms (strain Plo 5) as described (Berne et al., 2002). The protein solution was desalted and kept in aliquots at −20 °C. Before *i.v.* injection, sodium chloride was added to a final concentration of 0.9%.

2.2. Animals

Male Balb/C mice, body weight between 20–25 g, were from the animal breeding house (Veterinary faculty, University of Ljubljana, Slovenia). To estimate the LD_{50} of Oly, 24 males were used. For the study of cardiorespiratory effects, 18 male Wistar albino rats, weighing 230–270 g, were obtained from the Medical Faculty, University of Ljubljana. The experiments were approved by the Veterinary administration of the Republic of Slovenia (Permit no. 323–318/2003).

2.3. Determination of haemolytic activity

Rat and bovine erythrocytes were centrifuged from freshly collected citrated blood and washed twice with excess 0.9% saline and once with 140 mM NaCl, 20 mM Tris–HCl buffer, pH 8.0. Haemolytic activity was measured by a turbidimetric method as described (Sepčić et al., 2003). Oly was progressively diluted in 140 mM NaCl, 20 mM Tris–HCl buffer, pH 8.0. 100 µl of the resulting solutions were combined with 100 µl of erythrocyte suspension. The initial absorbance of the lysing mixture at 650 nm was 0.5. The decrease of absorbance, due to haemolysis, was recorded for 20 min at 650 nm using a Kinetic Microplate Reader (Dynex Technologies, USA) in order to determine the time required for 50% haemolysis, t_{50}. All experiments were performed at 25 °C, and each measurement was repeated three times.

2.4. In vivo experiments

2.4.1. Lethality determination of Oly in mice

Oly was dissolved in 0.9% NaCl and 100 µl of a final dilution injected into the right tail vein of male mice as a *bolus*. Oly concentrations of 800, 900, 1000, 1100, 1200, 1300, and 1400 µg/kg were injected into mice, each concentration being applied to three animals. The control group ($n = 3$) received 100 µl of saline. The mice were observed for 24 h for signs of intoxication and lethality. Following the 3R principles the LD_{50} was determined by the Reed and Muench (1938) method.

2.4.2. Spontaneously breathing animals

The anaesthetics Rompun® [5,6-dihydro-2-(2,6-xylidino)-4H-1,3-thiazin] (Bayer AG, Leverkusen), at a dose of 15 mg/kg, and Ketanest® (ketamin hydrochloride, Parke-Davis, Berlin), at a dose 100 mg/kg of body weight, were together injected *i.p.* into rats to induce general anaesthesia. Oly, dissolved in 0.9% saline, was administered *i.v.* through a heparinised (50 I.U./ml saline) cannula inserted into the left *Vena jugularis* at a dose of one mouse LD_{50} (1170 µg/kg) ($n = 6$). Saline was injected similarly into control rats ($n = 3$). The lower number of control experimental animals was chosen according to the 3R principle, since

we already had data from several other control experiments.

The right common carotid artery was cannulated with a polyethylene catheter in order to measure arterial blood pressure. It was determined using a mechanoelectrical transducer previously calibrated with a mercury manometer. The voltage signal was amplified and recorded on an IBM compatible computer using a 12 bit A/D converter NI 6023E (National Instruments, USA) and Biobench 1.2 software (National Instruments, USA) with 1 kHz sampling frequency. Mean arterial pressure (MAP) was calculated according to

$$\mathrm{MAP} = S - D/3 + D,$$

where S is the systolic, and D the diastolic arterial pressure. MAP was expressed as the average of all pulse pressure changes per minute.

The ECG signal was registered using needle electrodes (inserted subcutaneously in limbs, standard lead II of ECG) connected to the A/D converter noted above, and amplified and sampled at 1 kHz. Respiratory activity was recorded using a mechanoelectrical transducer fixed to the sternum and connected to a DC amplifier, and stored via the A/D converter on an IBM compatible PC.

2.4.3. Pharmacologically vagotomised animals

Atropin sulphate (Belupo, Macedonia), which abolishes cholinergic effects on heart, was injected *i.p.* to rats at a dose 0.5 mg/kg 10 min before application of one mouse LD_{50} of Oly. Oly was injected in the same way and at the same dose as described for spontaneously breathing rats ($n = 5$).

2.4.4. Artificially respirated animals

An Anaesthesia EMC WorkStation (Hallowell, USA) was connected to the tracheal tube and rats were artificially respirated by pure oxygen with the following settings: tidal volume 3–4 ml and frequency of ventilation 54, intrapulmonary pressure < 10 mm H_2O. Oly was injected after the artificial respiration had started, and 15 min after the blood pressure and heart rate had stabilised ($n = 4$).

2.5. Blood chemistry

Rat blood (0.7 ml per animal) was collected from the sublingual vein under deep general anaesthesia, 15–20 min after the injection of anaesthetics (control). Five minutes after injecting 1 LD_{50} of Oly (and approximately 70 min after induction of anaesthesia) blood samples were taken from the heart. Electrolyte composition (Na^+, K^+, Ca^{2+}, Cl^-), and haematocrit were measured. Serum calcium level was determined using an RA-XT analyser (Bayer, Germany), and sodium, potassium and chloride using an Analyser Ilyte Na/K/Cl (Instrumentation Laboratory, Lexington, USA). The micro-haematocrit method was used for haematocrit determination.

2.6. Statistical analysis

Data were analysed using Biobench 1.2 (National instruments, USA) and statistical software SPSS 13 (SPSS Inc., USA). The results are presented as average $\pm$ S.D. The Student two-tailed test was used for the statistical analysis. A P value of $\leqslant 0.05$ was considered as statistically significant.

3. Results

3.1. Haemolytic activity of Oly

Sheep, bovine and human erythrocytes have been found to exhibit similar sensitivity to Oly haemolytic activity (Sepčić et al., 2003), whereas rat erythrocytes are less sensitive (Fig. 1). The time courses of both rat and bovine erythrocyte haemolysis showed a typical lag phase, followed by rapid lysis (not shown), which is characteristic of pore-forming toxins with a colloid osmotic mechanism of action.

3.2. LD_{50} determination in mice

The lethality test was performed in order to determine toxic action and to estimate a single LD_{50}, which was used to study lethal effects of Oly in the *in vivo* study on rats.

The *i.v.* LD_{50} of Oly was determined to be 1170 μg/kg mouse body weight. Mice injected with Oly exhibited dose-dependent signs of intoxication. Cyanosis, cessation of movement and hair bristle were observed. In some animals, signs of intoxication started at doses below 0.9 mg/kg, however, most of these recovered in less than 1 h. The injection of 1.1 mg of Oly per kg was lethal and, at a dose of 1.4 mg Oly, all mice died in less than 20 min.

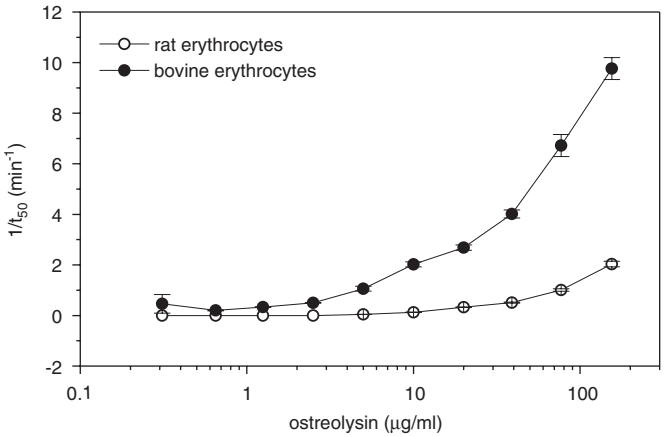

Fig. 1. Haemolytic activity of Oly on rat (○) and bovine (●) erythrocytes *in vitro*. The symbols represent the dependence of the reciprocal half time of haemolysis, ($1/t_{50}$), on the concentration of Oly (0.3–150 μg/ml). t_{50} is the time in which half of the erythrocytes were lysed, e.g., the time at which the apparent absorbance at 650 nm dropped from 0.5 to 0.25. Each point represents the mean of three measurements with corresponding standard errors.

3.3. Effect of Oly on arterial blood pressure, ECG and respiratory activity in spontaneously breathing anaesthetised rats

Fig. 2 shows a representative experiment in which the effects of Oly on electrocardiogram (ECG), arterial blood pressure and breathing were determined.

In all the experiments, Oly caused a rapid and transient increase in arterial blood pressure (aBP) for the first few seconds, followed by a transient return to close to the normal value and then a short, temporary increase over 1–3 min (Fig. 2). Mean aBP increased from the control value of 87 ± 7.6 mmHg to the maximum of 116 ± 9.7 mmHg. Finally, a progressive and irreversible fall in aBP to the mid-circulatory pressure (6–8 mmHg) was observed.

Respiratory arrest was observed approximately 10–20 s after Oly application. Respiratory activity appeared again after 131 ± 10.4 s for a short time (60 ± 26.3 s). Oly produced marked bradycardia immediately after the injection of Oly (Figs. 2 and 3). The heart rate decreased from the control value of 266 ± 14.6 beats/min (BPM) before the toxin injection, to 121 ± 28.9 BPM, 2 min after the toxin injection ($n = 6$). Sinus arrhythmia, peaking and increase of *T* wave amplitude, a prolonged *P–R* interval (Fig. 2A_2, A_3), *S–T* elevation and biphasic wave *P* (A_3) were also observed. Shortly after, ventricular extrasystoles (bigemini) appeared as widened QTS complexes (A_4), followed by decrease of HR and amplitudes of all ECG waves (A_5).

Blood samples collected 5 min after the injection of Oly revealed increased serum potassium levels, probably caused by intravascular haemolysis (Table 1).

3.4. Effect of Oly on arterial blood pressure, ECG and respiratory activity in pharmacologically vagotomised rats

Application of atropine in a dose which prevents cholinergic effects in the heart did not alter the cardiorespiratory effects of Oly described above, although one animal out of four survived. However, the serum potassium level of that animal, following Oly injection was lower (4.98 mmol/l) than in other affected animals (13.18 ± 3.80 mmol/l). In all other cases, the time-course of ECG, aBP and breathing were very similar in amplitude and duration, as described for intact animals injected with one mouse LD_{50} of Oly. In contrast to the intact animals injected only with Oly, less pronounced bradicardia was observed.

3.5. Effect of Oly on arterial blood pressure, ECG and respiratory activity in artificially respirated anaesthetised rats

After respiratory arrest, artificial ventilation was performed to determine the effect of hypoxia on the time course of blood pressure and ECG. In artificially respirated animals ($n = 5$), blood pressure and ECG exhibited similar time courses to

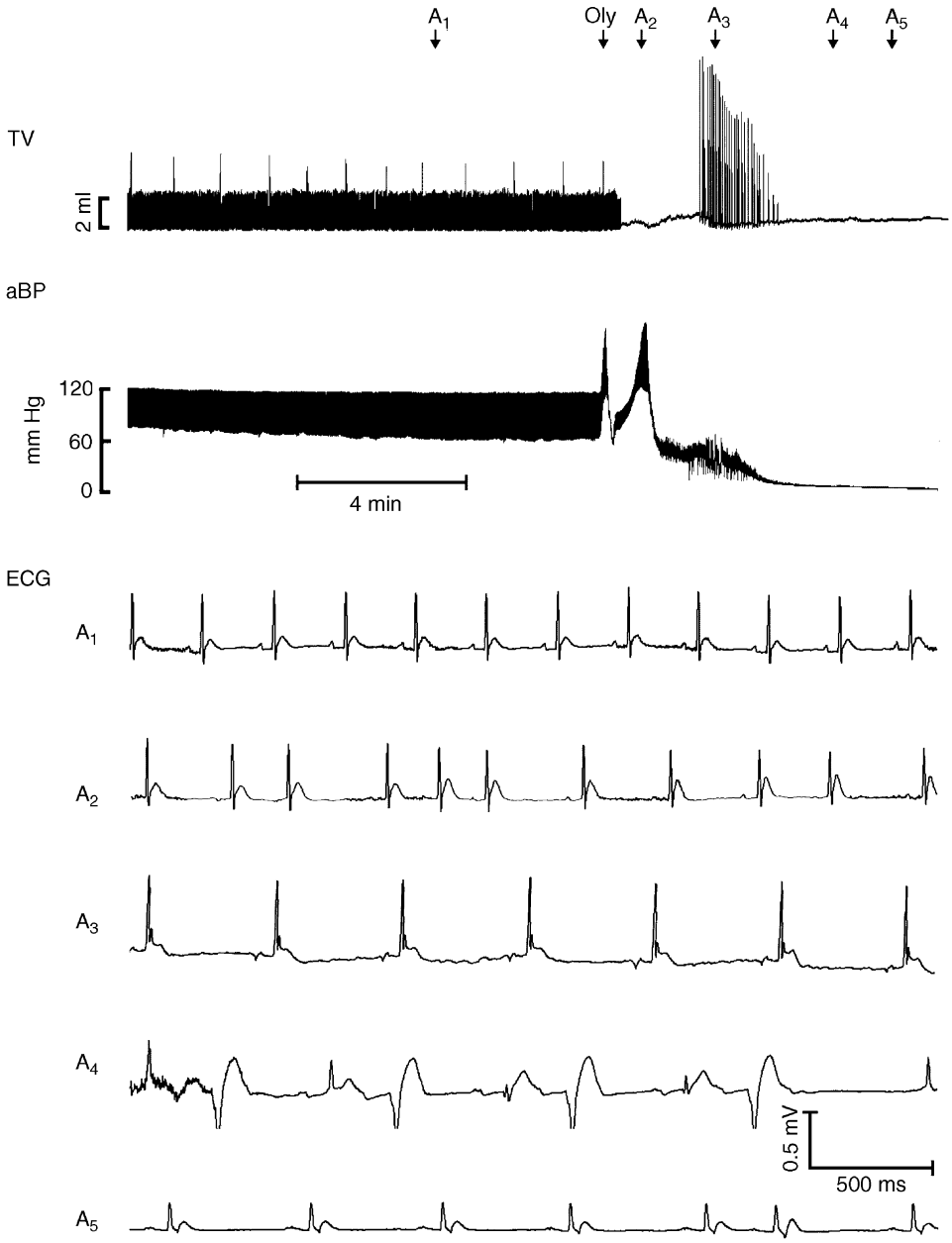

Fig. 2. Effect of an intravenous injection of one mouse LD_{50} of ostreolysin on arterial blood pressure, ECG and respiratory activity in anesthetised, spontaneously breathing rats. Tidal volume (TV) and arterial blood pressure (aBP) recordings represent the time course of respiratory activity and arterial blood pressure during the whole experiment. The ECG recordings A_1–A_5 represent corresponding time intervals marked on a whole experiment. The ECG on outline A_1 represents rhythmic control of ECG activity before the toxin injection. Traces in A_2–A_5 represent changes in the ECG time course after injection of one mouse LD_{50}.

those in spontaneously respirated animals. Progressive fall of aBP, drop of HR, arrhythmia and respiratory arrest were noted after intravenous injection of Oly. Potassium levels in artificially respirated animals were similar to those obtained in other experiments with Oly (8.68 ± 0.49 mmol/l).

4. Discussion

In this work, the toxicity of Oly has been studied in anaesthetised rats, and the LD_{50} determined in mice. Oly was shown to cause cardiorespiratory effects and death when injected intravenously into

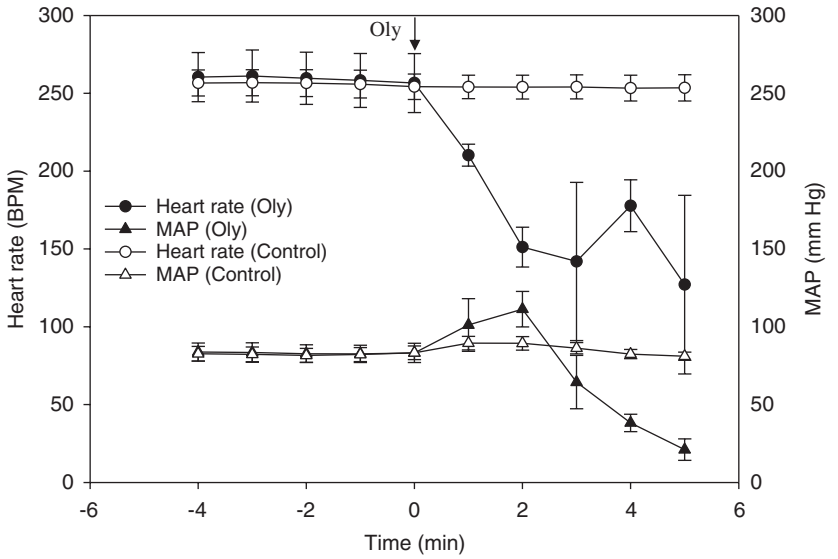

Fig. 3. Effects of the mouse 1 LD_{50} of Oly on heart frequency (beats per minute) and mean arterial blood pressure (MAP). One group of rats was treated with Oly ($n = 6$) and another group with saline ($n = 3$). Note the transient increase followed by progressive drop of mean arterial blood pressure and heart rate following Oly administration. Time 0: injection of Oly or saline.

Table 1
Effects of one mouse LD_{50} of ostreolysin on haematocryte and some blood serum electrolytes (mmol/l) in rats

	Animals treated with Oly		Animals treated only with physiological solution	
	Control (15–20 min.) ($n = 6$)	Oly (70 min) ($n = 6$)	Control (15–20 min.) ($n = 3$)	Control (70 min) ($n = 3$)
Hct (%)	50.4 ± 0.92	**58.00 ± 4.38**	51.00 ± 1.15	**47.33 ± 2.02**
Na^+	141.74 ± 0.69	**136.94 ± 1.89***	143.90 ± 1.24	**143.53 ± 0.54**
Cl^-	98.58 ± 1.15	**95.20 ± 3.86**	95.53 ± 5.41	**102.70 ± 2.38**
K^+	4.33 ± 0.13	**10.28 ± 1.25***	3.93 ± 0.25	**4.36 ± 0.23**
Ca^{2+}	2.91 ± 0.20	**2.83 ± 0.24**	2.59 ± 0.087	**2.73 ± 0.04**

Comparison of mean values of data obtained from Control (last column in bold) and treated group (second column in bold) by the Student *t* test. Control group of animals ($n = 3$) received only 0.9 ml of saline solution *i.v.* Blood samples were collected 15–20 min after the injection of anaesthetics (control) and 5 min after injecting 1 LD_{50} of Oly (approximately 70 min after induction of anaesthesia). Hct = hematocryte

Values are presented as $X \pm SD$.

*Difference significant at 0.05

rodents. Similar effects have been already reported for another member of the aegerolysin protein family, asp-haemolysin from *Aspergillus fumigatus* (Sakaguchi et al., 1975). This pathogenic mould is the main cause of aspergillosis, an invasive systemic disease with high mortality rates. Besides asp-haemolysin, a plethora of other molecules isolated from *A. fumigatus* have been reported to be associated with its toxicity (Rementeria et al., 2005). Purified asp-haemolysin shows 43% sequence identity to Oly (Berne et al., 2005), and is lethal for experimental animals, with LD_{50} values of 750 μg/kg and 350 μg/kg for mice and chicken, respectively (Sakaguchi et al., 1975). When applied intravenously to experimental animals, it binds to arterial walls in the kidneys and in the brain (Ebina et al., 1983).

Some of the signs of intoxication observed in our experiments with Oly are consistent with those observed by Al-Deen et al. (1987), who reported increased rate of respiration, loss of locomotor activities and coordination, and eventual death

following intravenous application of a water extract of oyster mushroom to mice. In their study, both oral and intraperitoneal application of the extract resulted in marked haemorrhages in most organs, with 24-h LD_{50} values being higher than 3 g/kg (Al-Deen et al., 1987). In comparison, *i.v.* LD_{50} of pure Oly was determined to be 1170 μg/kg in our study.

In this study, the intravenous injection of one mouse LD_{50} of Oly led to marked bradicardia and a biphasic effect on arterial blood pressure. The *S–T* elevation observed on the ECG trace within two minutes of Oly injection indicates myocardial hypoxia. Artificial ventilation was performed in order to study changes in arterial blood pressure and ECG connected with the hypoxia caused by respiratory arrest after injection of Oly. However, changes in the time course of blood pressure do not appear to depend on hypoxia, since the same changes in measured parameters were observed in artificially respirated animals.

The cardiotoxic and lethal effects of Oly may be caused by haemolysis and, probably, cellular damage resulting in hyperkalaemia. Previous *in vitro* data obtained on bovine, sheep and human erythrocytes showed that Oly exerts considerable haemolytic activity (Sepčić et al., 2003). In comparison, rat erythrocytes appear to be more resistant to Oly induced haemolysis. Another member of the aegerolysins, asp-haemolysin from *A. fumigatus*, was also reported to possess lower haemolytic activity on rodent erythrocytes than on those from chicken and humans (Sakaguchi et al., 1975). However, a significant increase in serum potassium was observed after Oly administration *in vivo* as well, although haematocrit was even higher after injection of the toxin, suggesting escape of intravascular fluid. Hence, hyperkalaemia is most probably responsible for the arrhythmia. This assumption is supported by the changes in ECG (bradicardia, atrioventricular block of the first or second degree, high-peaked *T* wave, etc.), usually produced by high plasma potassium levels in advanced hyperkalaemia (Kuwahara et al., 1992).

On the other hand, no direct action on heart tissue can be excluded. The observed mechanism of cardiotoxic action of Oly resembles that produced by another pore-forming toxin, sea anemone equinatoxin II (Budihna et al., 1992).

In conclusion, our results suggest that the cardiotoxic effects of Oly observed *in vivo* are caused mainly by the high potassium level that appears after the cytolytic action of the protein on blood and other exposed cells. However, the direct action of the toxin on cardiomyocytes cannot be excluded at present. The observed effects could be the primary cause of the intoxication induced by oyster mushroom.

Acknowledgements

The authors gratefully acknowledge the financial support of the Slovenian Research Agency, by grant P4-0053-0406-04. We thank Prof. Roger Pain for critical reading of the manuscript.

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