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Research Article

# Results of Thirteen Years Prospective Study of Diagnostics and Treatment of Amatoxin Intoxication in Slovakia -

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## ABSTRACT

The paper presents results of the prospective study of diagnosis and treatment of Amatoxin Intoxications (AI) during the period from 2004 to 2016 in the Slovak Republic.

**Methods:** AI diagnosis was suspected by/ suggested by mycological history, clinical course and confirmed by definition of amanitins in serum (ATOs) and in urine (ATOu) ELISA. The therapeutic protocol, designated and used by the authors since 1978, was recommended for hospitals treating confirmed AI patients. The study evaluated the diagnostic validity of ATOs and ATOu, the dynamics of ATOu levels after initiation of treatment and results of selected AI parameters and mortality - in relation to the protocol of treatment chosen for treating hospital.

**Results:** During 13 years of study 520 cases have met the anamnestic and clinical criteria of AI. In 418 of them the ATO levels were below the limit of positivity and AI was excluded. In 102 cases the ATO results confirmed AI and the treatment followed. While ATOu levels reliably reflect the severity of AI in the ranging from 6 to 60 hours since ingestion, ATOs levels were positive only up to six hours since ingestion of mushroom, thus their diagnostic value was limited. After start of the treatment ATOu levels drop below cut-off limit in the first 24 hours. The treatment protocol with mega dose of Penicillin (PNC) Plus Silibinin (PP group) was applied in eighty nine AI patients, and the protocol with the silibinin, without PNC (LP group) was applied in 13 patients. Five patients out of 102 died of confirmed AI, two of them immediately after admission and initiation of treatment as Acute Kidney Injury (AKI). Other three patients were from LP group because of the possible allergy to PNC in their history. Mortality in PP group was 2,25%, and mortality of LP group 23,05% - significantly higher in LP group ( p = 0,00058).

**Conclusions:** The identification of ATOu ELISA within the time ranging from 6 to 60 hours since ingestion of mushroom with 100% certainty confirms and/or excludes AI, while ATOs positivity is up to 6 hours since ingestion and hence its diagnostic value is limited. Early vigorous treatment using the recommended protocol REELDADCOM assures the survival of AI patients without any sequelae. A similar protocol using only silibinin as an antidote is not fully effective in severe form of AI and the liver transplantation is the last lifesaving modality. Early vigorous treatment using the recommended protocol REELDADCOM assures the survival of AI patients without any sequelae. A similar protocol using only silibinin as an antidote was not fully effective in severe forms of AI and the liver transplantation may be the last life-saving modality.


**Keywords:** Amanita phalloides; ELISA amanitin test; Diagnosis and treatment protocol

## INTRODUCTION

Amatoxin Intoxications (AI) are the most frequent fatal intoxications of biological origin [1-4] occurring namely in countries where picking and consumption of wild mushroom is a tradition [2,5,6]. The severity of AI has arisen by the nature of the toxin itself, as well as by the wide range of other circumstances. However, diagnostic practices for AI are still unreliable, treatment procedures variable, noncomplex and leading to liver transplantation [1,3,7-11]. The complex protocol of AI treatment was designed in 70s of 20th century by authors, based on actual pharmacokinetic, experimental and clinical knowledge [12]. This protocol had been used in children till 2003 with more than 90% effectivity and survival without sequelae [13,14]. The weakness of this procedure was the exact diagnostics based on mycological history, clinical picture of AI, and discovery of spores of amatoxin-containing mushrooms. Because the main goal of the treatment protocol had to be introduced in early phase of AI, over 30% of children were over-treated. Since 2003 has been established ELISA - quantitative identification of amanitins in serum (ATOs) and in urine (ATOu) and this have led to the assumptions for the performance of prospective clinical study of AI diagnostics and treatment. The authors report the results of this study.

## MATERIALS AND METHODS

### The criteria for entry into the study

The incriminated mushroom was identified by a picker as genus Russula, Agaricus, Lepiota, respectively an unknown mushroom, respectively their mixture. The interval since ingestion until the first clinical manifestations was minimum 4 hours. Dominant clinical symptom was profuse diarrhoea, commonly with dehydration. Considered were also the experiences of a mushroom picker and other circumstances (method of food processing, suicidal attempt, etc.). Exclusion criteria AI were the cases without diarrhoea (cardinal AI symptom), ingested mushrooms were other than the above mentioned, reliably identified by the picker, and interval since ingestion to the onset of symptoms was shorter than four hours (except the ingestion of mixture of various kinds of mushrooms). Any doubts or uncertainties were an indication for the ATO examination.

The ATOs and ATOu examination was done in urgent mode within 24 hours a day, and results – either negative – were announced to the sending hospital via phone, usually after two hours since the initiation of samples examination (duration of the analysis). The patient was recommended to undertake the first steps of treatment protocol (REELD, respectively REELDAD-M – see the text below) if was met AI anamnestic criteria, even before ATO identification. The ATOs and ATOu over the cut-off limits were considered as definitive confirmation of the diagnosis AI, indicating the initiation of the full treatment protocol (REELDADCOM). Negative ATOu excluded AI diagnosis and treatment was terminated.

ATOs and ATOu test AMANITIN ELISA EK-AMI (90 tests) testing set Bühlmann Lab. AG. Schönenbuch (Switzerland) defines alfa and gamma amanitins concentrations in serum (ATOs) and in urine (ATOu) using the method of competitive inhibition, blocking polyclonal antibody on microplates (ELISA). In the first cultivation amanitins from the examined sample compete for the place on chemical bond with the amanitin-biotin conjugate. After washing, the enzyme streptavidin conjugated is adding to peroxidase – bounding biotin complex as an antibody. An Unbound enzyme is removed by washing and during the third incubation, together with tetramethylbenzidine, produces a color product, which is inversely proportional to the concentration of amanitins in the sample. Enzyme activity of the streptavidin-amanitin complex, biotin-antibody is measured using luminometer LM01A with the wavelength of 450 nm. The calibration curve is set for each analysis.

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Control samples and standard measures were evaluated using LIANA Windows ver. 4x software. Chosen calculation for the proximity of calibration curves is a parabolically weighted regression, with the standards for concentrations 0, 1, 3, 10, 30, 100 ng/ml, ED50 7,6 ng/ml on the y-axis. Control samples (axis x) in concentrations K1 by 3,3 and K2 ranging from 18,3 to 48,4 ng/ml. Analytic specificity of the kit is given by the specificity of rabbit antibody, anti-alfa amanitin and is 100%, beta amanitin 0,1%, gamma amanitins 90%, and epsilon amanitin 0,1%. Analytic sensitivity stated by the producer, is on 0,22 ng/ml, functional sensitivity (FLDD) is 1,5 ng/ml. ATOs positivity declared by the producer, is for a time ranging from 6 to 48 hours since ingestion, ATOu positivity ranges from 6 – 60 hours. ATOs and ATOu cut-off values stated by the producer are on 1,5 ng/ml [15]. Samples in the study were predominantly processed immediately; though they maintained stability for seven days, when kept out the fridge and over seven days, kept in the freezer [15,16].

AI treatment protocol have adhered to the principles and procedures of the original protocol designed in 1977 [12], over the years it has been adjusted to "pure" form by removing drugs with an unproven efficacy [13,14,17]. Basic principles of the treatment were: Initiation of the treatment as soon as possible, ideally within the 48 hours since mushroom ingestion, and every step of the protocol is of equal value in the chain of treatment. Individual steps are expressed in the mnemonic acronym

**REELDADCOM**

**RE:** Vigorous rehydration with the substitution of body fluid loss plus daily requirement, and prevention of hypoglycemia using dextrose and mineral solution. The aim is to supply the body fluid loss, to prevent the prerenal failure, acute kidney injury (AKI) and to enhance the elimination of amanitins by urine [18-20].

**El:** Elimination of the mushroom residue and spores from the gut, as well as amanitins from enterohepatal recirculation by administration of highly-disperse charcoal with lactulose for minimum three days of the treatment.

**D:** Osmotic diuresis by low molecular dextran 40 [20].

**AD:** Antidotes used in the protocol: crystalline Penicillin G Potassium Salt (PNC) in the dosage of one million IU per body weight kg per day, divided to four doses i.v., and Silibinin (Legalon) in dosage 20 mg per body weight kg per day, BID, i.v.

**CO:** Coagulation: To prevent the risk of life threatening haemorrhage and substitution of coagulation factors by administering the fresh frozen plasma.

**M:** Monitoring of body fluid balance, biochemical parameters of the hepatic and renal lesions. Diagnostic and treatment procedures are shown in Algorithm. Detailed treatment protocol was published in the previous papers [12-17].

Algorithm for diagnostic and treatment procedures

Organization of the study: In case of any mushroom intoxication the patient receiving hospital usually consults the National Toxicological Information Centre SK (NTIC). This institution has recommended to contact the consulting "hot line" (Children Faculty Hospital & Newborn Screening Center Banská Bystrica NSCCFH) providing nonstop consulting service and ATOs and ATOu examination of delivered blood and urine samples. ATOu positivity confirmed AI, and vice-versa. The hospital may have decided to implement AI therapy or to transfer the patient to a recommended third-degree center for treatment. When the receiving hospital agreed with application of the treatment protocol and cooperation during the treatment, they stay in permanent consultation. Alternatively, the patient was urgently transported to the center. These differences of the management of individual patients allowed comparison of final results. All the hospitals were asked for the copy of patent´s Report on the Course of Hospitalization, after they recovered. This was filled and evaluated for the purposes of this study. Presented results are from 2004 to 2016, i.e., 13 years.

In processing the results we evaluated the basic characteristics of the whole file of patients, and out of them the groups with confirmed AI. In these groups was compared the spectrum of ingested mushroom, based on their labelling of mushrooms pickers. In the group with negative ATOs and ATOu (below of cut-off limit 1,5 ng/mL,) was checked for the eventual false negativity of AI tests. The values of the ATO over cut-off limit (1,5 ng/mL) were considered to be AI. In these cases the height of ATOs and ATOu levels were evaluated for their dependence on time since the ingestion till examination, as well as the trend of ATOu level decrease within the first 24 hours of treatment. The presence of acute kidney injury (AKI) was based on oliguria/anuria, high serum creatinine, and mineral and acid-base disbalance. Peak values of ALT, AST, bilirubin, prothrombin complex Quick, respectively INR were recorded twice daily. Since ATOu and ALT behave oppositely during the time of mushroom ingestion, we evaluated the so-called Amatoxin Score (ATOS), expressed as the sum of time from ingestion to ATO examination in hours, plus ATOu (ng/ml) plus peak ALT (mmol/ l) [17]. Because the quantity of monitored parameters significantly exceeded the scope of this work, only the main indicators of severity of AI were evaluated. The most important monitored parameter was the survival of the patient without sequelae, or survival after liver transplantation (LTx), or death. Not all hospitals have accepted the use of the recommended antidote combination (PNC plus silibinin). This allowed us to compare the effectiveness of treatment by dividing the AI to the PNC + silibinin (PP) group and silibinin-Legalon, (LP) group.

Statistical analysis was oriented on the file of AI patients. Groups PP and LP were compared by sex and age using Mann-Whitney test in MS Excel 2013 software XLSTAT program. Further were tested statistic significances of the following differences:

- The time since mushroom ingestion till ATOu examination.
- The dynamics of ATOu decrease in time since mushroom ingestion.

Between PP and LP groups in:

- ATOu levels
- Maximal individual ALT values (peak ALT)
- Height of ATOS score (time since ingestion/hours/ + ATOu + peak ALT) and
- Mortality, and its causes

## RESULTS

During the period from 2004 by 2016 were in NTIC consulted 2124 cases of mushroom intoxications. 520 cases of them (24,5%) met AI anamnestic criteria as mentioned above. From them 418 (80,4%) patients had negative ATOs and ATOu tests, thus AI was excluded. In none of them was proven false negativity. In 102 cases (19,6%) were ATOu levels over the cut-off limit of positivity, which confirmed AI

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diagnosis and the patients were treated and were the subject to this study. In the whole file of 102 AI patients were 56 men (54,9%) with the average age of 42,8 years and 46 women (45,1%) with the average age of 39,8 years. The summary data are in table 1.

### ATOs and ATOu levels in the file of AI patients

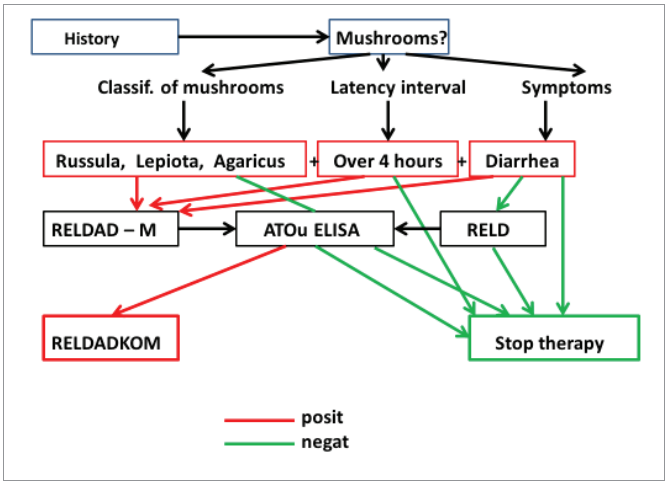
Values of ATOs and ATOu levels in time (hours)-since ingestion of toxic mushroom are presented in table 2 and figure 1.

### Changes in ATO levels in time since mushroom ingestion

Kit producer declares ATOu positivity in time from 6 by 60 hours since mushroom ingestion. Trend of ATOu decrease in AI patients is presented in figure 2. Diagnostic value of ATOu decreases around 60 hours since ingestion, although in individual cases, generally poorly treated, were positive even after 75$^{th}$ hour. The results are in accordance with the producers´ manual. Clinical reliability of the examination is by 60 hours since the mushroom ingestion.

### ATOu levels before and 24 hours after the initiation of treatment protocol

Difference in the first ATOu levels and ATOu levels 24 hours after initiation of treatment in 24 patients with the clinical and laboratory symptoms of severe ATI are presented in table 3. There was a significant decline in ATOu in all patients, in 16 of them (66,7%) even under the cut-off limit of positivity.



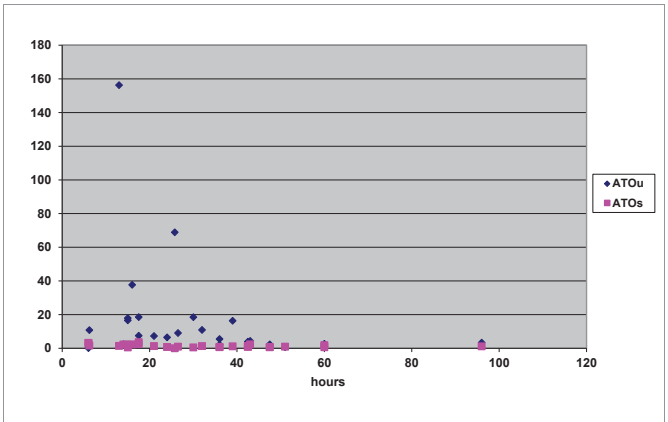




**Figure 1:** ATOs and ATOu Levels (ng/mL) in Intoxicated Patients in Time since Mushroom Ingestion.

**Table 1:** The file of Patients Examined for Suspected AI, Overall Mortality, its Causes.

| Instit | NTIC | SCN | SCN | SCN | SCN | Prot.ch. | Reason | Reason |
|---|---|---|---|---|---|---|---|---|
| Year | Total | Susp. AI Total | ATOu negative | ATOu positive | Exitus | Without PNC | PNC allergy? | AKI |
| 2004 | 97 | 9 | 5 | 4 | 1 | Yes | Yes | |
| 2005 | 155 | 54 | 29 | 25 | 2 | | | 2 Yes |
| 2006 | 136 | 38 | 33 | 5 | 0 | | | |
| 2007 | 155 | 57 | 53 | 4 | 0 | | | |
| 2008 | 128 | 37 | 32 | 5 | 0 | | | |
| 2009 | 114 | 36 | 31 | 5 | 0 | | | |
| 2010 | 297 | 60 | 50 | 18 | 0 | | | |
| 2011 | 73 | 15 | 13 | 2 | 0 | | | |
| 2012 | 171 | 20 | 18 | 2 | 0 | | | |
| 2013 | 208 | 31 | 30 | 1 | 1 | Yes | Yes | |
| 2014 | 260 | 81 | 63 | 18 | 1 | Yes | Yes | |
| 2015 | 160 | 22 | 20 | 2 | 0 | | | |
| 2016 | 170 | 52 | 41 | 11 | 0 | | | |
| Total | 2124 | 520 | 418 | 102 | 5 | 3 | 3 | 2 |

Abb.: NTIC: National Toxicological Information Centre, SCN: Screening Centre of Newborn; AKI: Acute Kidney Injury, ATOs and ATOu levels in the file of AI patients.

### Evaluation of chosen parameters according to the method of treatment

Out of 102 AI patients were 89 (87,2%) patients treated by Protocol in which two antidotes were used: PNC and silibinin (PP group). In 13 (12,8%) patients was only used silibinin as an antidote (LP group). Other steps of the treatment remained alike in both groups. In PP group were 55,06% of men and 44,94% of women. In LP group were 53,85% of men, 46,15% of women. Average age in the PP group was 42,6 years, in LP group 33,38 years (Table 4). Both groups are similar and comparable without significant differences in sex and age.

### The PP and LP groups were compared and tested in the differences of values:

1. Elapsed time in hours since mushroom ingestion till ATOu examination and initiation of the treatment
2. ATOu Levels measured in the time of diagnosis
3. Peak ALT values during treatment
4. Amatoxin score (ATOS), i.e. the sum of parameters 1./+2./+3./ (time + ATOu + ALT).

The results by groups are shown in table 5 and individual parameters in figures 3, 4, and 5.

Average time in hours since ingestion till ATOu examination and the initiation of treatment was in LP 46,4 hours, in PP group 33,5 hours. LP group was diagnosed significantly later than PP one ($p < 0,05$). Average ATOu Levels in LP group were 11,55 ng/ml, in PP group 29,62 ng/ml, significantly higher in PP group ($p < 0,05$). Mean values of peak ALT values in LP group were 30,61 mmol/L, in PP group 36,13 mmol/l, this difference was not statistically significant. Mean ATOS values in LP group were 88,44, in PP group 108,27, the difference was not significant, due to wide variance of STD values. The difference in the number of members in the groups, in

evaluating ALT and ATOS was caused by the fact that 30 final reports on the treatment of individual patients were not complete for their evaluation.

### Mortality and its causes

In the file of 102 patients with confirmed AI diagnosis 5 patients died, thus the overall mortality was 4,9%. Ninety seven patients (95,1%) were discharged with the complete clinical recovery, without any sequelae (Table 1). Two patients died in PP group; the cause of death was Acute Kidney Injury (AKI) in initial early phase of the disease. The mortality in PP group was 2,25%. In LP group 3 patients died – all having possible PNC allergy in their history. It was the reason they did not receive PNC in their therapy and all were succumbed as the hepatorenal failure, typical for AI. Mortality in LP group was 23,08%. Expressed statistically, the difference in mortality of PP and LP groups is highly significant on the level p = 0,00058 in favour of survival in PP group.

## DISCUSSION

The presented prospective study has some weaknesses. At first, the cooperation with some of the hospitals, as well as the acceptance of the recommended treatment protocol was not fully sufficient in the first years of the study. This was the reason of two cases of AKI deaths. Similarly, some patients were treated without PNC. Despite the efforts, we were not able to establish a prompt diagnosis and commence treatment ASAP because of delayed AI suspicion. On the other hand, this allowed us to compare the outcome according to the used treatment protocol.

The strength of the study was the exact and prompt confirmation of AI diagnosis, suspected by mycological and clinical history by ATOu evaluation. The study confirmed 100% specificity and sensitivity of ATOu results. Moreover, ATOu levels in interval since ingestion reflect the severity of intoxication. Negative levels occurred only within intervals less than 6 hours and over 60 hours since mushroom ingestion. This finding is consistent with the data provided by the kit producer. In contrast to that ATOs levels were positive only up to six hours after ingestion and after this time were negative, even in patients with high ATOu positivity. This finding is in contradiction to the data in the information sheet of the laboratory set, stating the ATOs positivity from 6 to 48 hours since ingestion. We considered ATOs as a parameter with the limited diagnostic value. In contrast ATOu testing allowed exact AI diagnosis and enabled us the effective use of the treatment protocol. We consider the ATOu statim examination in patients with suspected AI as an

**Table 2:** ATOs and ATOu Levels (ng/mL) in Intoxicated Patients in Time since Mushroom Ingestion .

| Time (h) | ATOu | ATOs |
|---|---|---|
| 6 | 0,1 | 3,14 |
| 6 | 2,4 | 3,15 |
| 47,5 | 2,32 | 0,61 |
| 47,5 | 0,98 | 0,77 |
| 6,2 | 10,81 | 1,61 |
| 25,75 | 68,87 | 0,01 |
| 96 | 3,37 | 1,11 |
| 42,5 | 3,91 | 1,32 |
| 30 | 18,47 | 0,49 |
| 36 | 5,5 | 0,65 |
| 36 | 2,14 | 0,78 |
| 26,5 | 9,06 | 0,96 |
| 42,5 | 1,54 | 0,87 |
| 42,5 | 1,79 | 0,9 |
| 15 | 17,91 | 0,49 |
| 15 | 16,58 | 1,98 |
| 17,5 | 18,51 | 3,6 |
| 17,5 | 7,47 | 2,44 |
| 43 | 4,37 | 2,32 |
| 60 | 2,68 | 1,82 |
| 60 | 1,27 | 0,6 |
| 16 | 37,7 | 2,27 |
| 13 | 156,3 | 1,4 |
| 15 | 2,25 | 2,12 |
| 14 | 2,4 | 2,24 |
| 51 | 0,61 | 0,95 |
| 39 | 16,32 | 1,14 |
| 24 | 6,46 | 0,72 |
| 21 | 7,26 | 1,26 |
| 32 | 10,88 | 1,3 |

**Table 3:** Decline in ATOu (ng(mL) : ATOu1 before, ATOu2 24 Hours after the Initiation of Treatment (n = 24).

| Patient | ATOu1 | ATOu2 |
|---|---|---|
| AK | 5,25 | 1,62 |
| FZ | 67,4 | 1,87 |
| HB | 17,5 | 1,06 |
| IG | 3,1 | 2,01 |
| JK | 40,67 | 2,09 |
| JO | 27,6 | 1,4 |
| MG | 0,48 | 1,96 |
| MŠ | 70 | 1,07 |
| ŠJ | 11,42 | 1,35 |
| DD | 74,79 | 0,37 |
| ZM | 10,81 | 3,87 |
| CT | 68,87 | 39,22 |
| MT | 26,5 | 9,06 |
| KMm | 17,91 | 0,88 |
| KJ | 16,58 | 0,44 |
| KMd | 18,51 | 0,48 |
| KI | 7,47 | 0,27 |
| FE | 4,37 | 0,35 |
| NO | 2,46 | 0,73 |
| RO | 1,93 | 0,73 |
| KP | 53,65 | 1,48 |
| AO | 1,78 | 0,73 |
| JB | 529 | 13,4 |
| GO | 0,73 | 0,76 |

**Table 4 -** Distribution of Groups by Sex and Age.

| Parameter | PP group | LP group | Statistical signf |
|---|---|---|---|
| Male % | 55,06 | 53,85 | |
| Female % | 44,94 | 46,15 | p = 0,467 /NS/ |
| Age /y/ | 42,6 | 33,4 | |
| SD | 19,1 | 21,6 | p = 0,064 /NS/ |

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essential and crucial step not only for individual diagnostic, but also - and especially - for the comparison of treatment protocols that do not currently have this unifying base. Other aspects contributing to the strengths of the study are its nationwide coverage along with the standardization of diagnostic procedure and the large number of treated patients and close cooperation of all participants. The complex protocol of AI management was designed based on the wide spectrum of experimental and clinical studies from the end of 70s of the 20th century which are still being cited today. They are the basis of the recent knowledge about toxicity, pharmacokinetics and the

**Table 5:** Values in Time since Ingestion till the Diagnosis and Treatment, ATOu Levels, Peak ALT and ATOS in LP and PP Groups.

| Parameter | Number | AM | STD | Median | Mode | Min | Max | Stat. sign. |
|---|---|---|---|---|---|---|---|---|
| Time LP | 13 | 46,38 | 21,41 | 46 | 47 | 15 | 96 | |
| Time PP | 89 | 33,48 | 17,31 | 27 | 26 | 6 | 90 | $p = 0,013$ |
| ATOu LP | 13 | 11,55 | 15,16 | 4,2 | 0,74 | 0,48 | 47,9 | |
| ATOu PP | 89 | 29,62 | 63,74 | 8,53 | 2,4 | 0,1 | 529 | $p = 0,042$ |
| ALT LP | 13 | 30,61 | 50,04 | 7,51 | no | 0,34 | 147 | |
| ALT PP | 59 | 36,13 | 49,66 | 10,38 | 0,44 | 0,2 | 229,3 | $p = 0,281$ |
| ATOS LP | 13 | 88,44 | 59,56 | 68,85 | no | 25,31 | 208,48 | |
| ATOS PP | 59 | 108,27 | 96,23 | 78,8 | no | 8,84 | 647,42 | $p = 0,257$ |

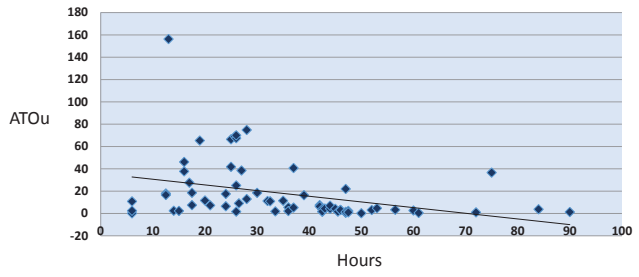



**Figure 2:** ATOu Levels (ng(mL) in Time (hours) since Ingestion of Mushroom in AI Patients (n = 67).

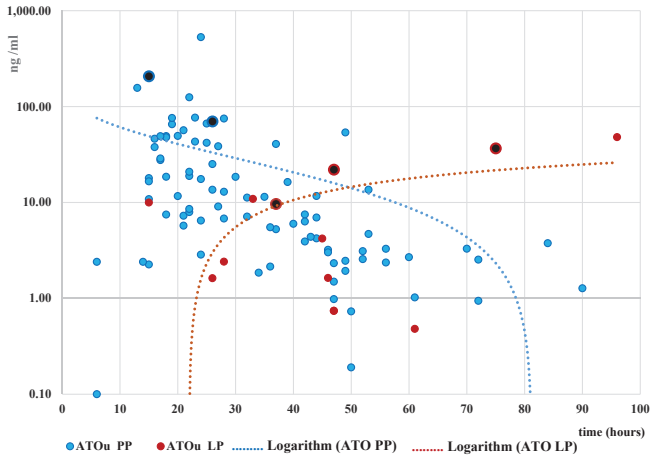



**Figure 3:** Individual values of ATOu (ng/mL) in time (hours) since ingestion of mushrooms in 67 AI patients of PP and LP groups (black dots in died patients).

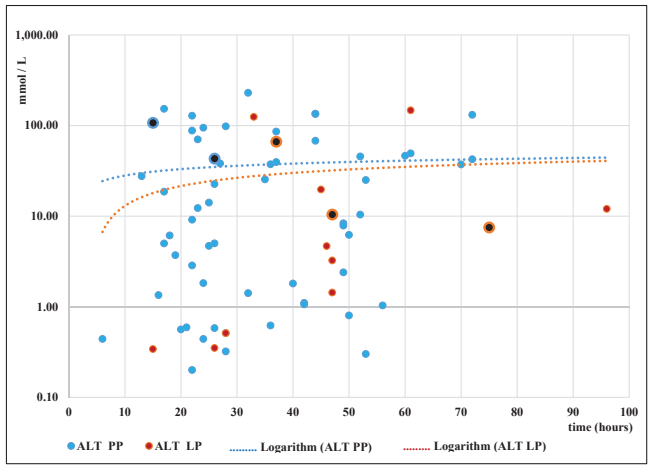



**Figure 4:** Individual peak ALT (mmol/ L) values in 67 AI patients of PP and LP groups (black dots in died patients).

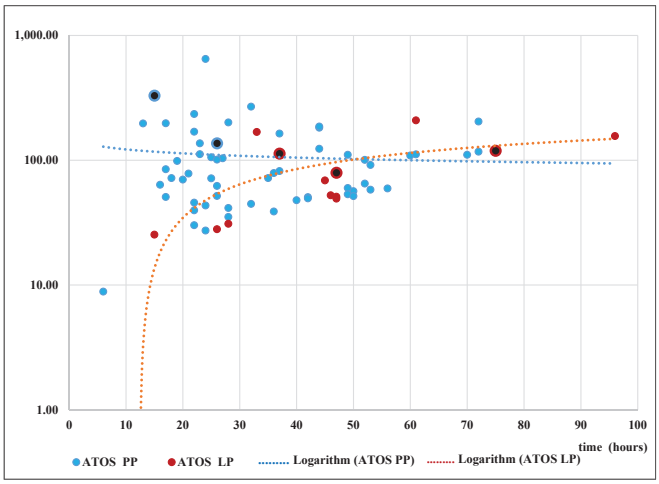
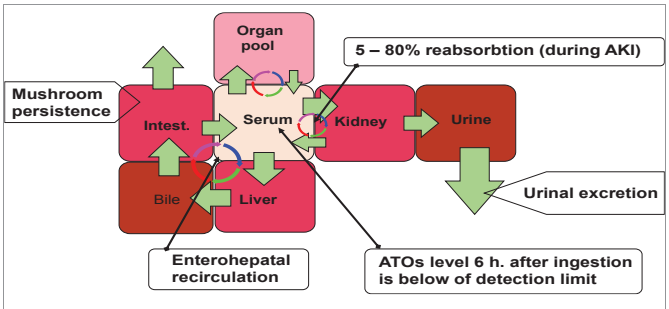



**Figure 5:** Individual ATOS score in 67 AI patients of PP and LP groups (black dots in died patients).




**Scheme 1:** The ATO Distribution and Kinetics in Body Compartments – Concentration ATO Depicted in Colours.

mechanism of amatoxins effects – amanitins and phalloidins [18]. Our study has repeatedly proven that the amatoxin levels in blood are after six hours since ingestion undetectable [14], they do not bind to plasma proteins [4,21] and their persistence in the organism is due to reduced renal excretion in hypovolemia [19], to persistence of mushroom and spores in intestine, and enterohepatal recirculation [22] (scheme 1) modified [18].

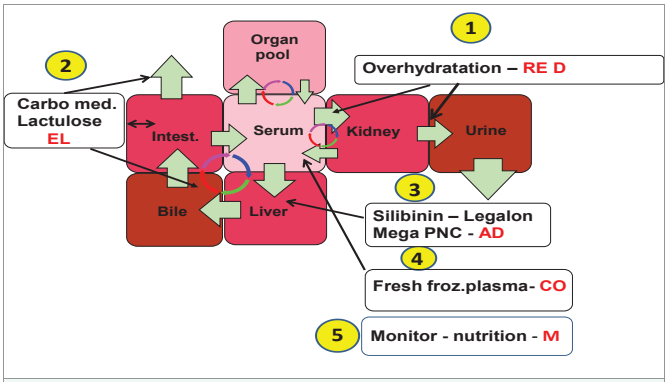
This knowledge forms the principles of treatment protocol (REELDADCOM): Extracorporal elimination procedures, incl. plasmapheresis [23] cannot be effective – ATO is not present in the circulation after six hours since mushroom ingestion. Vigorous rehydration support of ATO elimination through renal way, thorough elimination of spores and ATO from intestine and blockage of enterohepatic recirculation, Combination of antidotes mega doses of PNC and silibinin. Supplementation of coagulation factors by fresh frozen plasma. Fighting hypoglycemia and haemorrhage (Scheme 2).

## SCHEME 2

The REELDADCOM protocol in its target effects

Although this protocol was created at the time of the first experimental work - in the 1970s, the individual steps in the treatment do not differ from those currently used. These are vigorous rehydration with forced diuresis, elimination of mushroom and amanitins from the intestine, blockage of their enterohepatic recirculation. As an antidote, solo silibinin, as well as the combination with PNC in many works, are also used. Early substitution of fresh frozen plasma is a logical prevention of fatal hemorrhage. The benefit of the work is the timeliness and accuracy of AI diagnostics, the timeliness of the use of a complete protocol with an overlapping phase of acute hepatic injury.

This protocol was used in the AI treatment in children by 2003 and over 25 years [13,14] and has undergone only little changes. The survival of treated patients was over 93%, and early liver biopsies found only reversible changes [13,23] and survived patients were without any sequelae. The main problem was, that without exact and prompt diagnosis of AI the treatment protocol was applied to up to 30% of patients who met anamnestic and clinical AI criteria, however the further course of the disease ruled out AI. These patients were subsequently excluded from the study. Exact statim AI diagnostics by the confirmation of amanitins in blood/urine is absent in most published papers [5,7,24-30]. Not even in the papers, where the examination of amanitin (RIA, HPLC) was realised afterwards [10,11,19,20,31]. The introduction of the ELISA individual statim examination meant the key step for effective early treatment of the patients, as well as for the exact verification of the effectivity of the treatment protocol in this prospective study. Our study confirmed the fact that reliably verified AI cases (n = 102) represented only 4,8% out of total mushroom intoxications (Table 1). Without ATOu ELISA examination 520 patients met the anamnestic and initial clinical criteria of AI, presenting the fourth of the total mushroom intoxication in Slovakia (24,5%). We believe that this fact modifies the results in many published studies of AI. In one of the most




**Scheme 2:** The REELDADCOM Protocol in its Target Effects.

frequently cited papers of F. Enjalbert et al., [7] analysing 20 years of AI treatment over the world, the chapter concerning diagnostic methods is absolutely absent. Similarly, there are no data on the timing of the treatment steps, the initiation and duration of individual drugs, the antidote, etc. It's an exact analysis of inaccurate data. An unreliable medical history and atypical AI course in the early stages of poisoning does not allow for an accurate diagnosis. The finding of spores confirms the diagnosis of AI, but their absence does not rule out this diagnosis. These differences do not allow an exact comparison of treatment results published works, including our study. We found only one clinical study of estimation the amanitin ELISA, but it was a study evaluating the validity of this test [16].

Our presented study has brought some new knowledge about pharmacokinetics of amanitin during AI

ATOs negativity (i.e. level under 1,5 ng/ml) is in the serum after 6 hours since ingestion, confirming the absence of amanitin in blood. It is fully consistent with Faulstich et al. finding [18]. In addition, this observation indicates that ATOs estimation after 6 hours since mushroom ingestion has no diagnostic value [13,17]. Zero levels of ATOs exclude the possibility of binding amanitins to serum albumin [19,21].

Amanitins are immediately eliminated by urine and by bile. Their body hemodynamic pool is maintained by resorption from mushroom and spore residues in gut, plus enterohepatic recirculation, respectively by renal tubular reabsorption, mainly during dehydration in the early phase of AI.

Binding of amanitin to DNA-dependent RNA polymerase II [19,31] is not irreversible, but is probably the result of persistent reabsorption of amanitin from the intestine, enterohepatal recirculation and re-absorption of amanitin in renal tubules [18]. We can speak of "endogenous re-intoxication". This thesis is supported that ATOu levels after introduction of the treatment protocol are mostly negative within 24 hours (Table 4), Severely elevated aminotransferase, bilirubin, impaired synthesis of coagulation factors are corrected after approximately five days of protocol treatment, Early liver biopsy (14 days since ingestion) shows reversible signs of damage with the absence of necrosis [13]. The initial steps of Protocol – REELD – early Rehydration, intestinal Elimination, and osmotic Diuresis appear to be crucial for the elimination of amanitin from the body. This is the way to avoid initial acute kidney failure. Twenty two of our patients (21,6%) met criteria for Acute Kidney Injury (AKI) of 1th and 2nd grade32 immediately after admission and two of them died in this early phase of AI.

In discussions about treatment is the greatest emphasis devoted to the AI antidotes [7,22]. First results in 70s of the 20th century have found a number of seemingly incongruous substances that had a protective effect in AI in in vitro and animal experiments [33,34]. Paradoxically, all of these substances exhibited some degree of hepatotoxicity, for example chlorophorm, phenylbutazone, cinchocaine, streptomycin, ethanol, cyclosporine A, etc [33]. Most of these substances directly block the target enzyme of amanitin, DNA dependent RNA polymerase II, and they also block Organic Anionic Transport Proteins (OATP) in membrane of hepatocytes, which trasport amanitin into cell [31]. Protective effect of these substances, including ethanol could be attributed to the direct and competitive inhibition of the enzyme [33,34]. This temporary knock-out of transcriptional activity of the eukaryotic cell by antidotes protects it from binding amanitin. In AI therapy routine has been used silibinin

[2,35,36] and mega dosed penicillin G [5,12,13,17]. The first antidote of AI, penicillin, usable in clinical practice, was described in 1971 by Floersheim / 33 / has become the basis of our protocol /12/. Silibinin was used in the treatment of AI later (Hruby 1979 /35/, Thaler 1983 /36/), initially as a hepatoprotective agent. It was included to our protocol together with mega PNC. Despite the majority of clinical studies [7, 22, 36] prefer silibinin (Legalon) as the antidote, but the combination silibinin plus PNC is used in others /1, 5, 17, 21/. The exact comparison of the effectiveness of these different procedures is limited by variable diagnostic procedures. For example, the frequently cited work by Ganzert, et al. [40] analyses 604 suspected cases retrospectively were only 367 cases were diagnosed without the definition of amanitine levels. Many of these publications calculate with Liver Transplantation (LTx) as the standard step of AI therapy [1,6-9,37,38,39], we consider it as a failure of conservative treatment. In our study the silibinin treated group (LP) has significantly higher mortality than the silibinin + PNC group (PP). Only two patients died in our PP group as severe AKI immediately after hospital admission, before introducing of REELD steps of treatment. All other PP patients have survived without sequelae and without LTx. The PNC was given to several patients only during the first two days of treatment and overall treatment protocol lasted up to 7 days. Three patients from LP group were not administered PNC because of possible PNC allergy in their medical history. All of these patients died of hepatic failure, typical of AI. In LP group altogether 10 patients survived. In the comparison of chosen parameters (Table 5), is evident that it was milder intoxication. We consider PNC and silibinin applied ASAP along with prompt AI diagnosis by quantitative (ELISA) identification of amanitins in urine. Uncertainty about PNC allergy can be easily verified by prick – test, respectively in vitro one. In case of uncertainty, it is better to administer a high steroid dose before the PNC because the risk of death is higher for AI than for an allergic reaction.

## CONCLUSION

Our 25 years of experiences with AI treatment protocol in children and adults, have proven its high efficiency. Thirteen years of prospective nationwide study based on prompt and accurate diagnostics of AI through ELISA identification of amanitins in urine have confirmed these results in adults, as well as the fact, that the effective antidote is a combination of high doses of PNC with silibinin. The group treated only by silibinin in our file had statistically significantly worse prognosis. In 2017 were diagnosed and successfully treated other seven patients.

## AUTHORS CONTRIBUTION

S. Dluholucky - the author of diagnostic and treatment protocol, a consultant for diagnostics and treatment for external workplaces, the main author of the study design and data interpretation, final approval of the version.

Maria Knapkova - responsible for introduction and implementation of diagnostics by identification of amanitins using ELISA test, organization of services and continuous evaluation of results of examinations for individual patients, responsible for data collection and analysis.

K. Kralinsky - co-author of the amatoxin intoxications protocol, the head of workplace realizing the treatment in children patients, responsible for data collection, co-author of the manuscript.

L. Skladany - the head of workplace realizing treatment of adult patients with amatoxin intoxications, responsible for data collection, co-author of the manuscript.

D. Kapusta - the head physician realizing the therapy in adult patients responsible for data collection, cooperating in data processing for the manuscript.

M. Snitkova - co-author of manuscript, language editor and translator.

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Data collection and their further analysis were conducted with patients´ approval.

Trial design was based on the current knowledge and existing treating protocol in force.

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