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Enzyme N-acetyl-β-D-glucosaminidase (NAG) as an early marker of intoxications by the Cortinarius species (nephrotoxic syndrom)

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The enzyme N-acetyl-β-D-glucosaminidase (EC 3.2.1.30; NAG) was evaluated as a marker of intoxications by the Cortinarius mushroom (nephrotoxic syndrom). Enzyme activity was measured in the urine after i.p. applications of Cortinarius orellanus (Fr.) Fr. and C. rubellus Cooke species, respectively, by fluorimetric and/or colorimetric methods. Considerably higher level of the enzyme (up to 50 times) was observed already on the first day after intoxication, when others markers of renal damage (e.g. hematuria) were without changes. The high level of enzyme activity was detected up to 4th day after intoxication. The picture of intoxication was completed by determination of the urea level in serum and by histological examinations.

Key words: *Cortinarius* species, nephrotoxic syndrom, enzyme N-acetyl-β-glucosaminidase

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Enzym N-acetyl-β-D-glucosaminidasa (EC 3.2.1.30) byl posuzován jako časný marker intoxikace nefrotoxickými druhy rodu Cortinarius. Aktivita enzymu byla měřena v moči laboratorních potkanů po intraperitoneální aplikaci druhů Cortinarius orellanus (Fr.) Fr. a C. rubellus Cooke fluorimetricky a kolorimetricky. K významnému zvýšení aktivity (až padesátinásobnému), došlo již první den po intoxikaci, kdy další markery renálního poškození (např. hematurie) ještě změněny nebyly. Zvýšená hladina aktivity enzymu byla u neuhynulých zvířat zaznamenána až do 4. dne po intoxikaci. Výsledky jsou doplněny stanovením močoviny v séru a histologickým vyšetřením ledvin.

INTRODUCTION

A severe poisoning resulting in acute renal failure and several cases of fatal intoxications can be caused by certain *Cortinarius* species (Lincoff and Mitchel 1977, Rumack and Salzman 1978). It have been firstly reported by Grzymala (1957). Post mortem microscopic examination revealed tubular necrosis and interstitial nephritis. A slightly acidic crude substance was isolated from *Cortinarius orellanus* and demonstrated to be toxic in animal experiments (Grzymala 1962). Antkowiak and Gessner (1979) reported the structure of orellanine as 3,3',4,4',-tetrahydroxy-2,2'-bipyridine-N,N',-dioxide which was later confirmed by other authors (Holmdahl et al. 1987, Prast et al. 1988, Rapior et al. 1989, Richard et al. 1988). Kürnsteiner and Moser (1981) express some doubts with respect to some physical and chemical

properties of drug, but they isolated orellanine as Na-salt. In addition to orellanine Prast et al. (1988) found a nonfluorescent compound of minor toxicity. Quite different conclusions were published by Testa (1970) and Tebett and Caddy (1983) who assumed that the toxic compounds are polypeptides – cortinarins. Matthies and Laatsch (1991) and Matthies et al. (1991) were not able to reproduce the isolation of fluorescent or any other cyclic peptides related to cortinarins.

Nephrotoxic syndrom belongs to the most serious mushroom poisoning. The toxicity was proved in two species – *Cortinarius orellanus* (Fr.) Fr. and *C. rubellus* Cooke (Syn.: *C. speciosissimus* Kühn. et Romagn., *C. orellanoides* Henry) and it is also probable in *C. gentilis* (Fr.) Fr. and *C. splendens* Henry. Poisonings caused by nephrotoxic *Cortinarius spp.* were noted in our country (Bouška et al. 1980, Středová et al. 1978).

The selective damage of kidney was studied by a number of authors using histological methods. Two days after intoxication Nieminen et al. (1976) observed first symptoms of kidney damage intersticial infiltrates occuring mainly in the outer medullary zone and necrotic changes mainly in tubuli of the corticial zone. Richard et al. (1988) administered pure orellanine isolated from *Cortinarius orellanus* fruit bodies to mice. Histological examinations revealed tubular necrosis in the cortex corticis, frequent in distal convulated tubules. Glomeruli and proximal tubules were undamaged. Holmdahl et al. (1987) who also used pure orellanine isolated from *Cortinarius speciosissimus* described the histological picture of kidneys of experimental animals as intersticial nephritis and tubular necrosis. The proximal tubules were dilated and flattened. Pigment casts were found in the collecting tubules and in the proximal tubules of the cortex. Bouška and Klán (1987) described histological changes in rat kidney after i.p. administration of powdered *Cortinarius speciosissimus* as tubulointerstitial nephritis. Epitelium of the proximal tubule was first damaged and inflammatory changes in the intersticium occured later. Human intoxications by *Cortinarius* species has a very long latency period (more than 48 h). In experimental intoxications in rats by *Cortinarius orellanoides* histological changes in kidney could be observed already on the first day after intoxication (Bouška and Klán 1987, Prast and Pfaller 1988). Therefore, a biochemical marker reflecting the above mentioned changes and which could be used as an early indication of intoxication is looked for.

Urinary enzymes are particularly useful for the detection of acute renal damage, as e.g. in acute renal tubular necrosis (Price 1982, Bernard et al. 1984). For instance, decreasing of alkaline phosphatase level in urine on the first day after intoxication by *Cortinarius orellanus* is described (Prast and Pfaller 1988, Moser 1981).

Since the proximal tubule seems to be primary target site N-acetyl-β-D-glucosaminidase (NAG) was chosen as a marker of tubular damage. In the kidney it accumulates in the proximal tubule cells and also in the papilla and glomeruli; its increase level in urine could indicate cell damage. Although the enzyme is

ubiquitous, it is not filtered through the glomerulus due to its high molecular weigh (300–400 kD) and its presence in urine has to stem from the cells of tubules. The enzyme exists in the form of two isoenzymes: isoenzyme A, which amounts to 95 % of the total and occurs in the soluble contents of lysosomes, whereas isoenzyme B (5 %) is bound to lysosomal membranes and its excretion indicates a severe renal damage. We examined the total amount of the enzyme in urine after an intraperitoneal application of suspensions of homogenized fruit bodies *Cortinarius orellanus* (Fr.) Fr. and *C. rubellus* Cooke (Syn. *C. orellanoides* Henry, *C. speciosissimus* Kühn. et Romang.).

MATHERIAL AND METHODS

Suspensions of dried homogenized fruit bodies of *Cortinarius orellanus* Fr.: Fr. and *C. rubellus* Cooke in 0.2 % agar were applied intraperitoneally at doses of 177 ± 9.5 mg/kg and 124 ± 13 mg/kg body wt to male Wistar rats ($n = 22$, preclinical data: weight (g) $\bar{x} = 166.45$, S_D 24.35; NAG in urine (nkat/1) 2.115, S_D 1.85; urea in serum (mmol/1) 3.52, S_D 0.328; pH of urine 6.5–7.5; proteinuria less than 0.1 g/1; hematuria less that 5.10^6 ery/1. Controls received the same amount of nontoxic Cortinarius armillatus (Fr.) Fr. ($n = 6$) or the only 0.2 % agar suspension (2 ml) ($n = 6$). The animals were housed individually in metabolic cages. They were allowed water ad libidum. Urine was collected twice a day in intervals 12 hrs and frozen to −25° C.

Total NAG activity was assayed in intervals 24 hrs fluorimetrically using 4-methyl-umbelliferyl-N-acetyl-β-D-glucosaminidase as substrate (excitation 360 nm, emission 450 nm), incubation was at pH 5.0 (Na-citrate buffer) and 37° C (Leaback and Walker 1961), modification by Haragsim et al. (1990), and colorimetrically using p-nitrophenyl-N-acetyl-β-D-glucosaminidase as substrate ($A_{max} = 420$ nm). Urea in serum was assayed using the o-phtaldialdehyde method (Statim test Urea 13F), hematuria was measured in intervals 24 hrs using diagnostic test stirps "Hexaphan". In the end of experiment kidneys were examined histologically, paraffine slices stained with hematoxyline-eosine according to Bouška and Klán (1987).

Statistical evaluation was performed in a Vectra computer using methods of the program block Statgraphics, version 2.6 (Two sample analysis, standard deviation, t-test, HO hypothesis).

RESULTS AND DISCUSSION

Activities of NAG after intoxication by *Cortinarius orellanus* and *C. rubellus* species are summarized in Tables 2 and 3. It follows from the tables that the remarkable increase of activity of N-acetyl-β-D-glucosaminidase was proved after intoxication by both species studied. The activity increased 6–7 times as compared with controls (day 0) and control groups with nontoxic Cortinarius armillatus application (tab. 1) or without mushroom application already on the first day after introxication (see Fig. 1). Absolute activity values were up to 40–50 nkat/1. In all experimental animals the absolute activity increased at least 4 times (as compared with values before intoxication), in more that 50 % at least 10 times, in 80 % animals this increase could be observed 24 h after intoxication.

Hematuria occured in most animals in about 48–60 h after intoxication. The increase (as compared to controls) was not proved on the first day (see Tab. 4).

Determination of urea in serum on the 4th day after intoxication served as biochemical control of renal damage. A 7-fold increase $\bar{x} = 26.80$ mmol/1, S_D 8.35, $t = 9.63$, $P < 0.001$) with respect of values before intoxication could be observed.

Six rats of 22 experimental animals (i.e. 27 %) died 3–4 day after intoxication.

Dissections at the end of experiments showed hypertrophy of kidney (Fig. 2) in majority of animals, histological examination proved dystrophic changes of proximal tubules (Fig. 3) in all animals in agreement with Holmdahl et al. (1987), while control animals had no changes in their proximal tubules.

Any significant correlation between the absolute value of activity of the excrete enzyme and intensity of renal damage (histological findings, urea level in the serum, death) was not observed.

Renal of damage after application of Cortinarius rubellus appeared to be more serious, however, the amount of toxins in fruit bodies was not determined quantitatively.

When investigating the enzyme kinetics three possibilities were found:

1. In 32 % of animals ($n = 8$) majority of the enzyme was excreted in 24 h after intoxication ($\bar{x} = 21.69$ nkat/1, S_D 13.86, $P < 0.001$) and the excretion decreased continuously later on. Increasing of NAG activity was not proved on the 5th day after intoxication as compared with controls ($P>0.05$). In such cases, in spite of the fact, that absolute activity values of the excreted enzyme were higher (71 % of animals more than 20 nkat/1), percentage of dead animals was low (only 14 %) (see Fig. 4., curve A).

2. In 41 % of animals ($n = 9$) the enzyme excreted in two waves after 1 d ($\bar{x} =$ 10.4 nkat/1, $S_D = 7.27$, $P < 0.001$) and after 3 d ($\bar{x} = 11.11$ nkat/1, $S_D = 7.27$, $P < 0.001$) after intoxication. Such cases were the most frequent, but the activity peaks were low (more that 20 nkat/1 in only 44 % of animals). Lethality was 33 %. In these cases a gradual damage of the tubule probably occured (Fig. 4, curve B).

3. In 37 % ($n = 6$) of animals a "delayed" enzyme excretion occured 3 d after intoxication ($\bar{x} = 21.64$ nkat/1, $S_D = 16.25$, $P < 0.005$). Such cases were observed least frequently, however, lethality was the same as in case B (33 %). In 66 % of animals maximum NAG activity values reached more than 20 nkat/1. (see Fig. 4, curve C).

When evaluating results it should be kept in mind that a model is involved which only resembles the state in human pathology. However, it is clear already now that NAG activity cannot be used in diabetes, high blood pressure and in renal damage of other fungal origin. Theoretically even Retinol binding protein (RPB) in urine is more reliable, stable and probably more specific index of proximal tubular dysfunction. However this assumption would have to be demonstrated experimentally.

Generally, it can be concluded, that intoxications by nephrotoxic *Cortinarius* species are associated with decreasing of the level of alkaline phosphatase (measured by Prast and Pfaller 1988, Pfaller et al. 1991) and increasing of NAG activity in urine already on the first day after administration. In this time, all others markers of renal damage (e.g. hematuria) are without changes.

Tab. 1
NAG activity after application of nontoxic *Cortinarius armillatus* (control group) ($n = 6$)

Day	$\bar{x}$ (nkat/1)	S_D	t-test	P-values
0	2.48	2.51		
1	3.00	1.18	0.460	more than 0.05
2	2.74	2.69	0.165	more than 0.05
3	3.10	3.63	0.564	more than 0.05
4	2.99	2.51	0.224	more than 0.05

Tab. 2
NAG activity after intoxication by *Cortinarius orellanus* ($n = 16$)

Day	$\bar{x}$ (nkat/1)	S_D	t-test	P-values
0	2.48	1.89		
1	14.87	12.49	3.983	less than 0.001
2	6.93	4.19	3.848	less than 0.01
3	7.87	6.80	3.047	less than 0.01
4	5.74	4.20	2.733	less than 0.05
5	5.74	4.24	2.723	more than 0.05
6	7.29	4.53	3.715	less than 0.01

Tab. 3
NAG activity after intoxication by *Cortinarius rubellus* ($n = 6$)

Day	$\bar{x}$ (nkat/1)	S_D	t-test	P-values
0	1.40	2.04		
1	13.67	11.15	2.406	less than 0.05
2	11.36	7.13	2.996	less than 0.05
3	22.16	12.89	3.534	less than 0.01
4	8.50	2.12	4.121	less than 0.01

Tab. 4
Hematuria after intoxications by the *Cortinarius orellanus* and *C. rubellus species* (day 1–4 $n = 22$, day 5–6 $n = 16$)

Day	$\bar{x}$ (ery10^6/1)	S_D	t-test	P-values
1	0.26	0.73	1.54	more than 0.05
2	5.78	9.47	2.66	less than 0.05
3	10.22	11.52	3.76	less than 0.01
4	6.46	11.08	2.25	less than 0.05
5	6.36	12.06	1.75	more than 0.05
6	1.11	3.33	1	more than 0.05

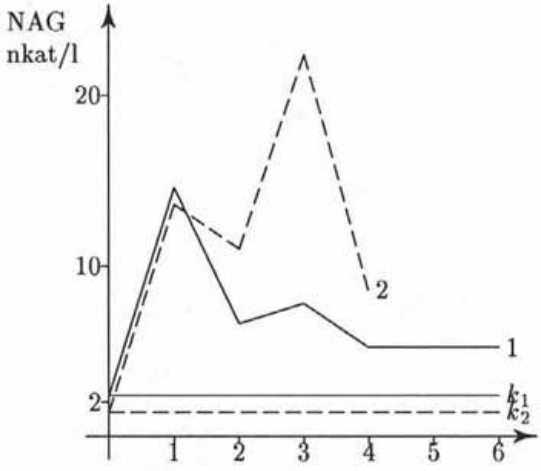

Fig. 1. The activity of NAG in rat urine after intoxications by *Cortinarius orellanus* (1) and *C. rubellus* (2) (k_1 and k_2 are values before intoxication).

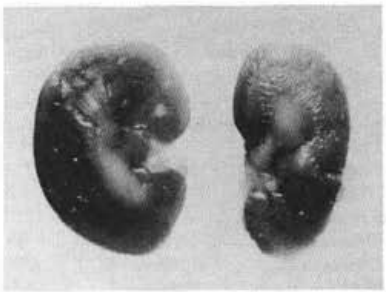

Fig. 2. Hypertrophy of kidney on the 5th day after intoxication by *Cortinarius rubellus* species (to the left of the control kidney).

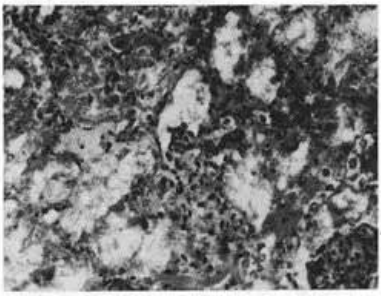

Fig. 3. Dystrophic changes of proximal tubule on the 5th day after intoxication by *Cortinarius rubellus* species.

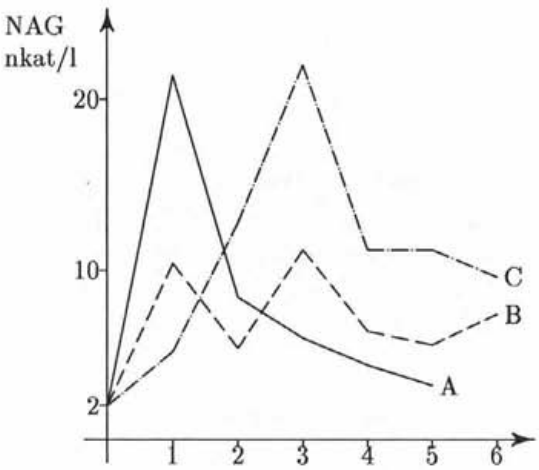

Fig. 4. Kinetics of NAG excretion after *Cortinarius* intoxications (curves A, B, C show different way of excretions the enzyme in rats).

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