



The influence of poisoning with patulin on activity of acid phosphatase, cathepsin B and D in mice kidneys and livers

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Borzęcki A, Nieradko-Iwanicka B, Mikocka J. The influence of poisoning with patulin on activity of acid phosphatase, cathepsin B and D in mice kidneys and livers. *J Pre Clin Clin Res.* 2020; 14(3): 94–97. 10.26444/jpccr/126833

Abstract

Introduction. Patulin is a mycotoxin produced by a variety of moulds, for instance, *Aspergillus*, *Penicillium* and *Byssoschlamys*, and found most often in rotten apples. Previous studies showed toxic effects of patulin in the gastro-intestinal tract, impairment of kidney function, as well as neurotoxicity.

Objective. The aim of the study was to investigate whether intoxication with patulin affects the activity of acid phosphatase, cathepsin B and D in mice kidneys and livers.

Materials and method. Experiments were conducted on 36 female mice. Animals were divided into 6 groups of 6: 1 – control, 2 – received 0.1 LD50 patulin i.p. 28 days, 3 – saline i.p. once, 4 – patulin i.p. 0.1 LD50 once, 5 patulin i.p. 0.2 LD50 once, 6 – patulin i.p. 0.5 LD50 once. 6 hours after patulin or saline administration, animals from groups 3, 4, 5, 6 were sacrificed. Kidneys and livers were obtained. Animals from groups 1 and 2 were sacrificed on day 29. Acid phosphatase activity was measured in the tissue supernatants with colorimetric method. Cathepsin B and D activities were determined with an ELISA-kit.

Results. The activities of acid phosphatase, cathepsin B and D in the kidneys and livers of mice exposed to patulin for 28 days was higher than in controls. A proportionate increase in acid phosphatase and cathepsin B activity in the kidneys and livers was observed for a single dose of the xenobiotic.

Conclusions. Acute and subacute poisoning with patulin negatively affects the functioning of lysosomes and induces an increase in the activity of lysosomal enzymes in mice livers and kidneys. Activities of acid phosphatase and selected cathepsins in the livers and kidneys are markers of cell damage due to patulin's toxicity.

Key words

hepatotoxicity, nephrotoxicity, Patulin

INTRODUCTION

Patulin is a mycotoxin produced by a variety of moulds, for instance, *Aspergillus*, *Penicillium* and *Byssoschlamys* [1, 2], and found most often in rotten apples [3]. Patulin has been detected not only in apple juices, jam and ciders, but also in cherries, strawberries, plums, bananas, blueberries and grapes [4, 5, 6]. The World Health Organization recommends a maximum concentration of 50 µg/L of patulin in apple juice [7]. There are reports that patulin has also been detected in grains: barley, corn, wheat, and their products, as well as in shellfish [8]. Pasteurization or thermal denaturation do not destroy patulin, as the compound is heat-stable [9].

Patulin was previously used as an antibiotic against Gram-positive and Gram-negative bacteria. It was recognized for use as anticancer compound and a drug to treat the common cold [9]. However after several reports about its' toxicity in 1950–1960, it is no longer used as a medicine [10].

Patulin exerts its toxicity through covalent binding to the sulfhydryl group of amino-acids [9].

Studies summarized by Pal et al. showed that patulin *in*

vivo damaged liver, kidneys, intestinal tissues and immune system in mammals. Several studies revealed mutagenic, teratogenic, DNA damaging and leading to the micronuclei formation effect of patulin in mammalian cells. Patulin reduces the expression of interleukin-23, interleukin-10, TGF-β, decreases interferon γ production [9]. The mycotoxin at the dose of 0.1 mg/kg b.w. administered to male rats for 60 or 90 days caused haemorrhage, plasma cell hyperplasia, dilatation and fibrosis in the cortex, enlargement of the interstitial tissue between thymic lobules, thinning of the cortex, and blurring of the cortico-medullary demarcation in the thymus [9].

Acid phosphatase is an enzyme that hydrolyses orthophosphate monoesters in pH 4–6 during digestion. It can be further classified as a phosphomonoesterase. Acid phosphatase is stored in lysosomes and functions when these fuse with endosomes, which are acidified while they function; therefore, it has an acid pH optimum [11, 12, 13]. Acid phosphatase is present in the liver, kidneys, pancreas, placenta, bone marrow, bones, erythrocytes, granulocytes, platelets, serum, bile and urine. It regulates immune response, enables iron absorption, use of calcium and phosphorus by osteoblasts, helps in phagocytosis and mitosis [13].

Cathepsins are a group of endolysosomal proteases that regulate the mechanisms of innate and acquired

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Received: 01.07.2020; accepted: 25.08.2020; first published: 07.09.2020

immunity, including cell adhesion and migration, antigen processing and presentation, and resistance to numerous viral infections. Their abnormal activity can lead to many disease states, including the development of cancer. They regulate the mechanisms of immunity: cell adhesion and migration, resistance to viral infections, antigen processing and presentation. The name cathepsin comes from Greek *kathapsein*, meaning 'to digest' [14]. The cathepsins are: A-H, L, K, O, S, V, W, X [15]. Some enzymes of this group are required for Toll-like receptor cleavage and for the formation of functional receptors which play a role in sensing viruses (especially nucleic acids). Cathepsins are able to stimulate or inhibit the secretion of cytokines that are involved in the regulation of antiviral immune response. Cathepsins are very important in the entry of filoviruses, retroviruses and reoviruses into the host cell. Cathepsins are necessary for some enveloped viruses for fusion with membranes of infected cells. The inhibition of cathepsins may result in a reduction of virus replication. Moreover, viruses utilize endocytosis or low pH within the endosome for their penetration into the cell, to disassembly of the viral capsid, and for productive viral replication [15].

Cathepsins B, C, F, H, L, K, O, S, V, W and X are cysteine proteases from the papain family and make up the largest and best known class of cathepsins. On the other hand, cathepsins A and G belong to the serine proteases, and cathepsins D and E to the aspartate proteases.

Humans can be exposed to patulin *via* oral route chronically. Therefore, the question arises whether exposure to food contaminated with this mycotoxin could affect activity of lysosomal enzyme acid phosphatase and selected cathepsins in organs most important for metabolism and elimination of xenobiotics: liver and kidney.

OBJECTIVE

The aim of the study was to investigate whether acute and subacute intoxication of mice with patulin at the dose of 0.1–0.5 LD₅₀ intraperitoneally (*i.p.*) affects the activity of acid phosphatase, cathepsin B and D in mice kidneys and livers.

MATERIALS AND METHOD

The study was approved by the Ethical Committee in Lublin (Decision No. 22/2013) and conducted according to Polish and European law regulations. The authors had been trained in the planning and performance of experiments on animals. The experiment was conducted in the Centre for Experimental Medicine at the Medical University in Lublin. The experiment was supported by Grant No. DS227/13 from the Medical University in Lublin.

Patulin was purchased from Sigma Aldrich Merck (Germany). Saline was purchased from Glenmark Pharmaceuticals (India) in 5ml ampules. According to Pubchem [16], LD₅₀ for patulin administered intraperitoneally to mice (without data about their gender) is 5mg/kg b.w. and 10 mg/kg if administered *via* subcutaneous injection. According to our previous experience with female mice we considered patulin's intraperitoneal LD₅₀ for female mice to be 10 mg/kg.

Experiments were conducted on 36 female Albino Swiss mice. Female mice were used because our experience

indicated, that they were more sensitive to xenobiotics when compared to males. They were housed in standard laboratory conditions. Animals were randomly selected and divided into 6 groups of 6 mice each:

- Group 1 – control group received saline *i.p.* for 28 subsequent days;
- Group 2 – received patulin *i.p.* at the dose of 0.1 LD₅₀ (1mg/kg b.w.) for 28 subsequent days;
- Group 3 – received saline *i.p.* once;
- Group 4 – received patulin *i.p.* at the dose of 0.1 LD₅₀ once (1mg/kg b.w.);
- Group 5 – received patulin *i.p.* at the dose of 0.2 LD₅₀ once (2mg/kg b.w.);
- Group 6 – received patulin *i.p.* at the dose of 0.5 LD₅₀ once (5mg/kg b.w.).

Our previous experience showed that 6 hours after *i.p.* patulin administration, signs of its toxicity are best visible. Therefore, 6 hours after patulin or saline administration, animals from groups 3, 4, 5, 6, were sacrificed, their kidneys and livers immediately retained. Animals from groups 1 and 2 were sacrificed on day 29, and their kidneys and livers were also retained. The tissues were homogenized in phosphate buffered saline 0.5mg tissue/2mL of buffer purchased from Gibco (Scotland) and then centrifuged (14,000 × g, 4°C, 15 minutes) to yield the supernatant. Acid phosphatase activity was measured with a colorimetric method (wavelength 415nm) [17]. COBAS INEGRA 400 Plus analyzer manufactured by Roche (France) was used.

In the study, cathepsin B and D were chosen as the former belongs to the cysteine proteases from the papain family, and the latter is an example of aspartate proteases.

Cathepsin B and D activities were determined with an ELISA-kit purchased from EIAab Science Co., Ltd. (China) and used according to manufacturer's instructions. The kit allows for precise and specific determination of cathepsin B activity within the range 0.78–50ng/mL, and cathepsin D within the range 78.0–5,000 pg/mL. The measurement uses the sandwich ELISA method and was performed at wavelength 450 ± 10 nm. The minimum concentrations detected with this method were: cathepsin B – 0.78ng/mL and for cathepsin D – 78.0pg/mL. (Tab. 1), calculated per volume (0.5g tissue/2mL) of supernatant used for enzyme determination.

The results were statistically analyzed. Shapiro – Wilk, U Mann – Whitney, ANOVA rang Kruskal – Wallis and Dunn's *post hoc* tests were used. $P < 0.05$ was considered statistically significant.

RESULTS

The activity of acid phosphatase in the kidneys and livers of mice exposed to patulin for 28 days was significantly higher than in controls (Tab. 1). The single dose proportionate increase in acid phosphatase activity in the kidneys and livers was observed after exposure to single dose of the xenobiotic. However, comparison of acid phosphatase activity in mice kidneys in groups 4,5,6 *versus* group 3 was considered not significant ($p > 0.05$). Comparison of the acid phosphatase activity in mice livers in group 6 *vs* group 3 was statistically significant ($p < 0.05$). Cathepsin B concentration was higher in the kidneys of mice after 28 days of intoxication with patulin, compared to controls, but the difference was not

Table 1. Influence of poisoning with patulin on activity of acid phosphatase, cathepsin B and D in mice kidneys and livers

Enzyme activity	Group 1 – Control group receiving saline for 28 days	Group 2 – receiving patulin 0.1 LD50 for 28 days	Group 3 – receiving saline <i>i.p.</i> once	Group 4 – receiving patulin 0.1LD50 once	Group 5 –receiving patulin 0.2LD50 once	Group 6 – receiving patulin 0.5LD50 once
Acid phosphatase activity in mice kidneys (mean±SD) [U/L]	14.37±10.483	118.37±33.03*	76.22±37.523	85.08±48.58	96.65±15.33	112.75±32.71
Acid phosphatase activity in mice livers (mean±SD) [U/L]	56.43±36.47	125.13±31.28*	49.12±24.19	59.67±40.79	77.38±39.54	99.57±45.97#
Cathepsin B in mice kidneys (mean±SD) [ng/mL]	1.60±0.55	5.73±0.75	3.24±0.81	4.41±1.46	4.84±1.59	5.54±0.52#
Cathepsin B in mice livers (mean±SD)[ng/mL]	2.27±1.79	2.79±1.01	1.03±0.43	1.84±0.57#	2.62±1.45#	3.52±1.29#
Cathepsin D in mice kidneys (mean±SD) [pg/mL]	1292.5±500.5	4657.5±997.5	13330±975	3332.5±1310	5280±1020	5280±1040#
Cathepsin D in mice livers (mean±SD)[pg/mL]	5330±1080	1056±1190	5550±480	5850±860	6880±1970	7670±1860#

*p<0.05 vs group 1 # p<0.05 vs group 3

statistically significant. Cathepsin B and D concentrations in acute poisoning with patulin were single dose proportionate and there was a statistically significant increase after exposure to the highest dose of the mycotoxin *vs* control. Cathepsin B concentration in mice livers was significantly increased ($p < 0.05$) *vs* control after exposure to each dose administered in an acute poisoning model. In the case of chronic poisoning, the increase was not significant ($p > 0.05$). Cathepsin D concentration in mice livers was elevated after 28 days of intoxication with patulin, but the increase was not statistically significant. In the case of exposure to a single dose of the mycotoxin, there was a dose proportionate increase in the level of cathepsin D in the livers, but only after the highest dose (0.5LD50) the increase was statistically significant compared to the control group (Tab. 1).

DISCUSSION

Patulin is produced by various fungi which can infect fruits and vegetables. It can accumulate in apples where patulin producing fungi cause blue rot [18]. Intoxication with this mycotoxin causes neurological, gastrointestinal, immunological adverse effects, hepatotoxicity and kidney damage in mammals [7].

The presented study was intended to be a model of possible human exposure to patulin. Humans usually absorb patulin *via* the oral route, but in experiments on animals different routes of administration were tested. Administration *via* the oral route is usually associated with the first pass of the portal blood through the liver effect. Intravenous or *i.p.* administration requires lower doses of the xenobiotics and produces stronger toxic effects than by the oral route; therefore, in the current experiment, the intraperitoneal route of administration was chosen. In the study by Fuks-Holmber, patulin at the dose of 3mg/kg b.w. per day in Tris-acetate buffer (pH 5) was administered to rats orally. During the first 7 days, 3 of 7 rats treated with patulin died [19]. In order to avoid animal loss during the experiment, an alternative route of dosing was sought. Three different doses of patulin were administered, looking for the dose-effect correlation. As the oral dose of LD50 for patulin in rats is 27.79 mg/kg and in the cited publication a much lower dose was used, perhaps there was another reason for such a high mortality (possibly

gastroenteritis, dehydration, hypersensitivity to *vehiculum*). In the current study, it was considered that *i.p.* administration guaranteed that the full dose of the xenobiotic was given to the animal, and had the opportunity to be absorbed into the bloodstream. The large surface of the peritoneum with its rich network of blood vessels allow the fast absorption of substances administered in this way. Group 2 in this experiment was a model of subacute poisoning with patulin, while groups 4, 5 and 6 – acute poisoning. Acute, subacute and chronic exposures of humans to patulin are possible and depends on how often a person consumes apple juice and other contaminated foods and drinks.

In subchronic studies, patulin led to hyperaemia of the duodenal epithelium and kidney function impairment, agitation, convulsions, dyspnea, pulmonary congestion, oedema, ulceration and distention of the gastrointestinal tract [9]. The major retention sites of patulin are erythrocytes and the spleen, kidney, liver and lungs [9]. At cellular level, patulin produces plasma membrane disruption, inhibits protein synthesis, and Na^+ coupled amino acid transport, disrupts transcription and translation, inhibits DNA synthesis, inhibits interferon γ producing T-helper type 1 cells [9]. This mycotoxin induces apoptosis either through the mitochondrial pathway or without the involvement of cytochrome p52. The interaction of patulin with sulfhydryl groups of macromolecules and subsequent generation of reactive oxygen species play major role in the apoptotic process after intoxication with the mycotoxin [20]. Glutathione is considered as the scavenger of patulin-induced toxicity as the mycotoxin exerts its toxicity by reacting with thiol groups [9]. Jayashree et al. showed that green tea leaves produced remarkable antioxidant and hepatoprotective effects in mice after poisoning with patulin [21].

Patulin interacts with cysteine-containing proteins. Many enzymes containing sulfhydryl group in their active site (Na^+ - K^+ dependent ATPase, RNA polymerase, aminoacyl-tRNA synthetase, muscle aldolase) are inhibited by this mycotoxin [9]. Vinken et al. established deleterious effects of patulin on hepatic gap junctions [22].

Little information is available on the oral toxicity of patulin to primates. Garza et al., gave daily oral doses of patulin (5, 50, 500 $\mu\text{g}/\text{kg}$ b.w.) to pig-tail monkeys for 4 weeks, after which the monkeys exposed to the highest dose were given 5mg daily of the mycotoxin/kg b.w. for another 2 weeks. The

parameters determined after the experiment ended were: blood morphology, blood urea nitrogen, glucose, sodium and potassium, serum protein electrophoresis, serum glutamate oxalacetate transaminase, alkaline phosphatase, cholesterol and protein. No statistically significant changes were observed in laboratory blood tests between the experimental groups of animals and controls, except for alkaline phosphatase, which decreased in the blood sera of animals exposed to the highest doses of patulin [23].

The liver transforms xenobiotics to metabolites. Exposure of mice to patulin causes an increase in the activity of serum alanine transaminase (ALT), aspartate transaminase (AST) and increased lipid peroxidation [9]. Primary cultures of adult human hepatocytes exposed to this mycotoxin showed reduced cell viability [9]. Since the liver, like other blood rich organs, is a possible toxic target of patulin, Lu et al. tested whether exposure to patulin can cause enhanced hepatotoxicity by using both cell culture (wild-type p53 cell lines and knockout p53 cell lines) and animal models. Results obtained in their study confirmed that the xenobiotic boosted reactive oxygen species generation both *in vitro* and *in vivo*. Patulin also inhibited catalase activity [24]. Results published by Jin et al. are in agreement with the above mentioned studies: patulin-induced hepatotoxicity develops in the mechanism of oxidative stress [25].

Kidneys are also sensitive to xenobiotics due to the high renal flow. Male albino mice receiving patulin orally at the dose of 152 ppb/ml for 6 weeks had increased urea, creatinine and uric acid in their blood sera [9]. The increase in the activity of acid phosphatase, catalase B and D in the liver and kidney of experimental mice after acute or subacute poisoning with patulin in the current experiment probably resulted from oxidative stress in the livers and kidneys, and accelerated the degradation processes. Damaged cells in the liver and kidney undergo cell death. Unfortunately, due to lack of means, no histopathological examinations of the livers or kidneys were carried out. However, it is suspected that accumulation of undigested molecules in the lysosomes increased the synthesis of acid phosphatase, which resulted in a high activity of the enzyme. Increased acid phosphatase activity is a marker of cell damage.

CONCLUSIONS

Acute and subacute poisoning with patulin negatively affects the functioning of lysosomes and induces an increase in the activity of lysosomal enzymes in mice livers and kidneys. Activities of acid phosphatase and selected cathepsins in the livers and kidneys are markers of cell damage due to patulin's toxicity.

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