

EVALUATION OF PATULIN TOXICITY IN THE THYMUS OF GROWING MALE RATS

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Patulin is a mycotoxin produced by several *Penicillium*, *Aspergillus*, and *Byssachlamys* species growing on food products. In this study, we investigated the effects of patulin on the thymus of growing male rats aged five to six weeks. The rats were receiving it orally at a dose of 0.1 mg kg⁻¹ bw a day for either 60 or 90 days. At the end of the experiment, the thymus was examined for histopathology by light microscopy and for epidermal growth factor (EGF) and its receptor (EGFR) by immunolocalisation. For morphometry we used the Bs200prop program to analyse images obtained with the Olympus BX51 light microscope. Cell ultrastructure was studied by electron microscopy. In rats treated with patulin, the thymus showed haemorrhage, plasma cell hyperplasia, a dilation and fibrosis in the cortex, enlarged interstitial tissue between the thymic lobules, enlarged fat tissue, thinning of the cortex, and blurring of the cortico-medullary demarcation. Electron microscopy showed signs of cell destruction, abnormalities of the nucleus and organelles, and loss of mitochondrial cristae. However, no differences were observed in thymus EGF and EGFR immunoreactivity between treated and control rats.

KEY WORDS: *histopathology, immunotoxicity, light microscopy, morphometry, mycotoxin, cell ultrastructure*

Patulin is a mycotoxin produced by several *Penicillium*, *Aspergillus*, and *Byssachlamys* species. It is almost exclusively found in apples and apple-derived products and sometimes in peaches, pears, apricots, grapes, cheese, and grain, or their products (1-3). The World Health Organization (WHO) has recommended a limit on patulin concentration in apple juice to 50 µg L⁻¹ (4). International Agency for Research on Cancer (IARC) has classified patulin as a group 3 carcinogen, since there is not enough evidence of its carcinogenicity in experimental animals (5).

The thymus plays a major role in the development and maintenance of the immune system. It is the most developed right after birth and starts to involute after puberty (6). Involution is considered the major cause of age-related decline in the immune function (7). It may be accelerated by a wide variety of stimuli such as disease, stress, dietary deficiencies, ionising radiation,

colloidal substances, bacterial endotoxins, and some hormones (8).

Thymic tissue produces epidermal growth factor (EGF), which is one of the critical regulatory molecules for the production of cytokines (including interleukin-6), and may serve as a modulator of human T cell development (9, 10). Thymic epithelial cells contain epidermal growth factor receptor (EGFR) and can be stimulated by EGF for proliferation (11). EGFR is a transmembrane glycoprotein with intrinsic tyrosine kinase activity. Through several pathways it also affects angiogenesis, apoptosis, cell cycle control, cell mobility, and tumour invasiveness (12). Le et al. (10) reported that thymic epithelial cells expressed EGFR both *in vitro* and *in vivo*. EGFR is expressed in many thymic epithelial tumours (13).

Inconsistent reports on patulin toxicity in various species have created an unclear picture of potential

harmful effects of patulin on the immune system (14, 15). The aim of our study was to determine the distribution of EGF and EGFR in thymic cells that might explain malfunctions in the thymus of growing patulin-treated rats. These effects were investigated using morphological, histopathological, morphometric, ultrastructural and immunohistochemical methods.

MATERIALS AND METHODS

Chemicals

Patulin (4-hydroxy-4H-furo[3, 2-c]pyran-2 (6H)-one) was obtained from Sigma Chemical Company (St. Louis, Missouri, USA). Specific antibodies to mouse EGF and mouse EGFR were obtained from SIGMA, Ankara, Turkey.

Experimental design and animals

Wistar albino male rats aged 5 to 6 weeks were obtained from the Production Centre of Experimental Animals of the Hacettepe University, Ankara, Turkey. The rats were randomly divided into four groups, each consisting of 10 animals. Two of the four groups were treated with patulin for either sixty or ninety days (P-60 and P-90, respectively), and the other two were corresponding control groups (C-60 and C-90). Groups P-60 and P-90 were receiving 0.1 mg kg^{-1} bw of patulin a day by gavage. Patulin was prepared afresh every week; it was dissolved in sterile water and stored in dark at $+4^\circ\text{C}$. The dose was extrapolated from estimated exposure levels for humans (16) and is 1000 times above the estimated 90th percentile of human intake (17) to provide an adequate margin of safety. Control rats received equal volumes of sterile water by gavage.

During the experiment, the rats were housed in Plexiglas cages at $(22\pm 2)^\circ\text{C}$, $(68\pm 4)\%$ humidity, and 12 h light and 12 h dark cycle. They were given standard rat diet and had free access to tap water. Food and water consumption were recorded daily, and the animals were weighed weekly. At the end of the study, the rats were killed by cervical dislocation. The thymus was removed and weighed. The ratio of thymus weight to body weight was calculated as relative thymus weight.

Light microscopy

Dissected thymus specimens were fixed in Bouin's solution, processed in a series of graded ethanol, and

embedded in paraffin. Paraffin specimens were cut to $5 \mu\text{m}$ thick serial slices and stained with hematoxylin and eosin for light microscopy. The incidence of histopathological changes is expressed as number of affected animals per number of animals in a group.

For immunohistochemical analysis $5 \mu\text{m}$ deparaffinised tissue slices were prepared using the unlabelled peroxidase-antiperoxidase method according to Elcuman and Akay (18). All the following steps were performed at room temperature. The slices were treated separately in PBS for 60 min with rabbit anti-mouse EGF (1:500 dilution) and anti-mouse EGFR (1:50 dilution). For positive control we used tissue known to stain positively for either respective antibody. Negative control slices were not treated with primary antibodies. After immunohistochemical staining, the sections were counterstained with haematoxylin and mounted on glass slides coated with poly-l-lysine solution (Sigma, Ankara, Turkey). Two observers evaluated labelling intensity using a light microscope. For morphometric analysis, 300 test areas were randomly chosen for each experimental group and the thickness of the thymic cortex from the selected thymic lobule in serial slices was measured under light microscope (Olympus BX51) using the Bs200prop program.

Electron microscopy

Excised thymus glands were immersed in 2.5 % phosphate-buffered glutaraldehyde, pH 7.2, and kept in cold for 2 h. After repeated rinsing in cold phosphate buffer, the tissue was postfixed in 1 % osmium tetroxide solution for another 2 h. Fixed tissue was dehydrated in a series of graded ethanol, placed in propylene oxide, and embedded in araldite (19). Thirty-five ultrathin sections for each experimental group were stained with uranyl acetate and lead citrate, and examined with transmission electron microscopy (TEM) (JEOL 100 CX II) at 80 kV.

Statistical analysis

For statistical analysis we used SPSS 9.0 for Windows. Data were expressed as mean \pm SD. Thymus weight and body weight data were analysed using one-way ANOVA, Games-Howell multiple comparison, and Hochberg's GT 2 test. Histopathology results were compared using Fisher's exact test. The statistical significance was set at the 0.05 level. We also compared the thickness of the thymic cortex between the groups using Student's *t*-test ($P < 0.001$).

RESULTS

Morphology

Table 1 shows body weight, thymus weight, and relative thymus weight of control and patulin-treated rats. Thymus weight was lower in patulin-treated rats, but not significantly. No significant difference was found between the P-60 and P-90 groups either.

Light microscopy

Histopathological changes including haemorrhage, plasma cell hyperplasia, dilations and fibrosis in the cortex were mainly observed in the thymus of patulin-treated rats (Table 2). We also observed enlarged interstitial tissue between the thymic lobules, enlarged fat tissue, and blurring of the cortico-medullary demarcation in the thymic lobules. Patulin-treated groups, P-90 in particular, showed thymic atrophy, cell shrinking, and loss of cells in the cortex.

Immunohistochemical staining showed a weak positive or positive immunoreactivity to EGF and EGFR in epithelial cells of the medulla and cortex

both in control and patulin-treated rats. However, the differences in EGF and EGFR immunoreactivity between the groups were not significant. The thinning of the thymic cortex was significantly greater in group P-90 than in control groups. In contrast, group P-60 showed thickening of the thymic cortex which significantly differed from control groups (Table 3).

Electron microscopy

Ultrastructural analysis showed that the thymus of control and patulin-treated rats generally contained both large and small T cells. The thymus of control rats consisted of mature T cells and cells in various stages of differentiation, maturation, and division (Figure 1A). The mature cells had a large nucleus and a few organelles (mitochondria and ribosomes) in their cytoplasm. Thymic cells of patulin-treated rats showed several changes. Nearly all had lost the mitochondrial cristae and it is remarkable that the cytoplasm of some of cells was rich in free ribosomes (Figure 1B). Although cell destruction was observed in both patulin-treated groups, it was more severe in the P-90. Some thymi in the P-90 group had completely

Table 1 Rat body and absolute and relative thymus weight

Parameters	Groups			
	C-60	P-60	C-90	P-90
Initial body weight / g	114.1±7.20	109.8±4.5	113.6±4.2	106.5±5.2
Final body weight / g	251.2±18.0	248.2±12.9	273.8±7.90	277.8±11.4
Increase / %	120.10	126.00	141.00	160.8
Absolute thymus weight / g	0.54±0.42	0.38±0.12	0.40±0.11	0.36±0.06
Relative thymus weight x10 ⁻³	1.51±0.41	1.46±0.68	1.47±0.33	1.28±0.25

C - control rats; P - patulin-treated rats; 60 and 90 denote days of treatment. Each group counted 10 rats. Results are expressed as mean values ± SD.

Table 2 Histopathological findings in rat thymus

Histopathological findings	Groups			
	C-60	P-60	C-90	P-90
Haemorrhage	0	4 ^a	0	7 ^b
Plasma cell hyperplasia	0	5 ^a	0	6 ^b
Enlargement of interstitial tissue between the thymic lobules	0	5 ^a	0	7 ^b
Enlarged fat tissue	0	4 ^a	0	7 ^b
Dilation in the cortex	0	5 ^a	1	6 ^b
Fibrosis in the cortex	0	4 ^a	0	4 ^b

C - control rats; P - patulin-treated rats; 60 and 90 denote days of treatment. Each group counted 10 rats. Data are expressed as number of affected animals.

^a Significantly different from C-60 group, P≤0.05 (Fisher's exact test).

^b Significantly different from C-90 group, P≤0.05 (Fisher's exact test).

Table 3 Thickness of rat thymic cortex

Parameter	Groups			
	C-60	P-60	C-90	P-90
Thickness of thymic cortex / μm	239.76 \pm 131.7	307.05 \pm 153.0*	226.48 \pm 120.5	191.03 \pm 107.2*

C - control rats; P - patulin-treated rats. 60 and 90 denote days of treatment. Each group counted 10 rats.

* $p < 0.001$ (Student's *t*-test); Values are expressed as mean \pm SD.

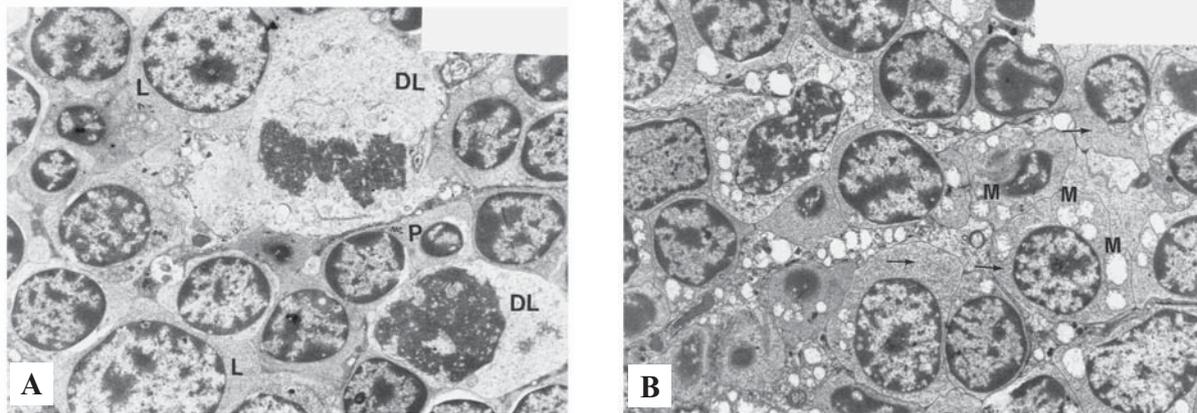


Figure 1 A- Ultrastructure of thymus cells in control rats: lymphocytes (L), precursor cell (P), and dividing lymphocytes (DL). B- Ultrastructure of thymus cells in patulin-treated rats (P-90 group): loss of mitochondrial cristae in several lymphocytes and increase in free ribosomes; Mitochondria (M), Free ribosomes (\rightarrow) (magnification 4350x).

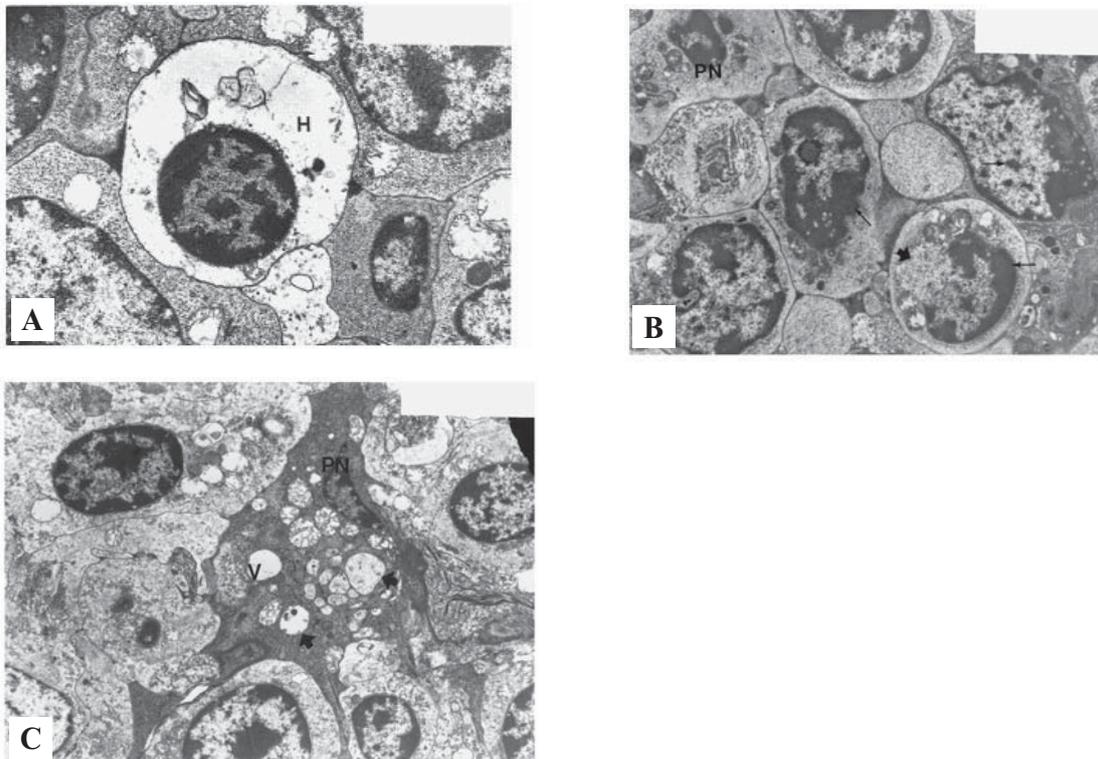


Figure 2 A- Ultrastructure of thymus cells in patulin-treated rats (P-90 group): hyaline cytoplasm; loss of mitochondrial cristae, free ribosomes (\rightarrow) (magnification 10800x); B-Ultrastructural changes of the nucleus: chromatin clumping (\rightarrow) and lysis and nucleus deformation (\rightarrow); pyknotic nucleus (PN) (magnification 7200x); C-Epithelial-reticular cell; Pyknotic nucleus (PN), Vacuole (V) (magnification 5400x); loss of mitochondrial cristae (\rightarrow)

lost their cytoplasmic material and their cytoplasm was hyaline (Figure 2A). Nucleus abnormalities were observed in large lymphocytes of the P-90 group. They included chromatin clumping, pyknosis nucleus, and deformations (Figure 2B). Thymic epithelial-reticular cells of patulin-treated rats also showed pyknotic nucleus (Figure 2C).

DISCUSSION

It is generally accepted that the thymus of young animals is more vulnerable to immunotoxic chemicals than the thymus of adult animals (20). In this study, we wanted to see whether patulin treatment of different duration affected thymus weight, involution, and cell structure in growing male rats. Hormonal and histopathological effects of patulin on the growth and development of growing rats have already been reported (21), but no study investigated the effects of patulin on the rat thymus in detail.

In our study, absolute and relative thymus weights were not significantly lower in patulin-treated rats than in controls. Llewellyn et al. (16) reported similar findings with female B6C3F₁ mice exposed to different doses of patulin for 28 days.

However, unlike control animals, patulin-exposed rats in our study showed enlarged fat tissue, enlarged interstitial tissue between the thymic lobules, and enlarged medulla. These changes closely resemble to the results obtained by Elcüman and Akay (18), Kuper et al. (22), and Utsuyama and Hirokawa (23), who studied age-related changes in the thymus. Histological changes observed in our study may be attributed to the patulin treatment. At the same time, we also observed thinning of the thymic cortex in rats treated with patulin for 90 days. They suggest that prolonged exposure to patulin during growth might cause the accidental thymic involution and decline in the immune function.

EGF is responsible for the production of thymic epithelial cell-derived cytokines. Epithelial cells of the thymic stroma play a critical role in T cell development (24, 25). In addition, EGF enhances Ca²⁺ release from intracellular stores. Ichikawa and Kiyohara (26) reported that capacitative Ca²⁺ entry correlated with cell proliferation in mouse mammary epithelial cells. This is why we decided to determine the distribution of EGF and EGFR, hoping that it could help to explain thymus functional disorders in patulin-treated rats. With ultrastructure findings we hoped to

verify our assumptions. However, immunostaining for EGF and EGFR showed no difference between control and patulin-treated groups. This suggests that patulin did not change EGF and EGFR expression in the thymus. However, an advanced molecular investigation might shed more light on the distribution EGF and EGFR molecules.

Patulin binds to the sulfhydryl groups, amino acids, or proteins in the plasma membrane (27). This mechanism of action depletes glutathione and increases the risk of oxidative damage (28), which in turn causes DNA damage (29). Some reports have also suggest that patulin inhibits other cellular processes, such as nucleic acid and protein synthesis (30, 31). Burghardt et al. (32) reported that patulin could directly affect cellular and mitochondrial glutathione levels and plasma membrane. In our study, thymic ultrastructure clearly differed between patulin-treated and control rats. Rats in the P-90 group suffered more structural damage than in the P-60 group. The most prominent damages were the loss of mitochondrial cristae, abundance of free ribosomes, loss of cytoplasmic material in some cells, and nuclear anomalies, which corroborates other investigations (29-31).

Our findings of pyknotic nuclei in the patulin-treated rats indicate that the cells were degenerating. Although apoptosis is a normal physiological event, some external factors such as radiation can trigger pathological apoptosis and various cell dysfunctions (33). It is still unclear what role cellular organelles play in this process, but investigation of thymus cell ultrastructure may help. Earlier ultrastructural studies in patulin-treated rats showed degeneration of thymus capillary walls (34), apoptotic body formation, and cell apoptosis in interdigitating dendritic cells (35). Our study, too, suggests that T cell anomalies in patulin-treated rats lead to apoptosis. These data are consistent with our previous observations of thymic involution in rats, which was accompanied with thinning of the thymic cortex. Elmore (36) has suggested that thymus is a sensitive target organ to immunotoxicants. Earlier studies on mice showed a number of effects of patulin on the immune system, including increased number of splenic T cells, lower serum Ig concentrations (15), depressed or delayed hypersensitivity response, higher neutrophil count, and resistance to *Candida albicans* infection (14). On the other hand, patulin administration in rabbits resulted in lower serum immunoglobulin, reduced lymphocyte blastogenesis, and reduced leukocyte chemiluminescence (37). Patulin also inhibited DNA

synthesis in peripheral lymphocytes (38). Lymphocyte degeneration induced by patulin in our study may have been mediated by mechanisms similar to those reported in earlier studies (14,15,37,38).

CONCLUSION

Mycotoxins suppress the immune system and increase susceptibility to certain diseases (39). This immunosuppressive effect can be much stronger in combined exposure to mycotoxins even at low concentrations (40). Our morphological, histopathological, morphometric, and ultrastructural findings suggest that exposure to patulin leads to early thymic involution in growing male rats. Since the thymus is fundamental for good health, consumption of food containing patulin and other mycotoxins, should be avoided, especially in childhood.

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Sažetak

TOKSIČNI UČINCI PATULINA NA TIMUS MUŽJAKA ŠTAKORA U RAZVOJU

Patulin je mikotoksin koji proizvode plijesni sojeva *Penicillium*, *Aspergillus* i *Byssachlamys* na različitim prehrambenim proizvodima kao podlozi. Učinke patulina istražili smo na timusu mužjaka štakora u razvoju (dobi 5 do 6 tjedana). Mikotoksin je životinjama davan *per os* u dnevnoj dozi 0,1 mg kg⁻¹ tj. t. 60 odnosno 90 dana. Na kraju pokusa štakori su žrtvovani, timus je podvrgnut histološkim analizama s pomoću svjetlosne mikroskopije, a imunocitokemijskim je metodama istražena stanična lokalizacija epidermalnog faktora rasta (EGF) i njegova receptora (EGFR). Morfometrijska analiza provedena je s pomoću računalnog programa Bs200prop povezanog u sustav sa svjetlosnim mikroskopom Olympus BX51. Elektronskomikroskopski je istražena ultrastruktura stanica timusa. Utvrđeno je da patulin izaziva krvarenja u timusu, hiperplaziju plazma-stanica, dilataciju i fibrozu u kortikalnoj regiji timusa, širenje intersticijskog tkiva između režnjeva timusa, povećanje masnih stanica, smanjenje debljine kore timusa te nestanak kortiko-medularne demarkacije. Elektronskomikroskopski u tkivu timusa štakora tretiranih patulinom uočeni su znakovi raspadanja stanica, abnormalnosti jezgre i organela te gubitak mitohondrijskih krista. Unatoč navedenom, na presjecima tkiva kontrolnih štakora i štakora tretiranih patulinom nismo utvrdili razlike u imunoreaktivnosti EGF i EGFR, što bi trebalo dodatno istražiti osjetljivijim molekularnim metodama.

KLJUČNE RIJEČI: *histopatologija, imunotoksičnost, mikotoksin, morfometrijska analiza, svjetlosna mikroskopija, ultrastruktura*

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