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HISTOCHEMICAL EVALUATION OF T-2 TOXIN-INDUCED CARDIOTOXICITY IN RATS: SEMIQUANTITATIVE ANALYSIS

ABSTRACT: In this study female Wistar rats were treated with T-2 toxin (1 LD_{50} 0.23 mg/kg *sc*) and sacrificed on days 1, 3, 5, 7, 14, 21, 28 and 60 after the treatment. Control groups of rats were treated by saline (1 ml/kg 0.9% NaCl). At each time-schedule, control groups of animals were sacrificed, too. Pathohistological alterations of the heart were evaluated in whole visual fields stained by haematoxylin and eosin (HE), periodic acid-Schiff's (PAS), Masson-Trichrom's (MT) and Giemsa (GIM) methods. The changes observed were scored by using semiquantitative grading scale. The heart alterations detected in T-2 toxin-treated animals ranged from focal parenchymal or hyaline degeneration (HE = 2.5 — 4.0; $p < 0.05$ vs. control) to diffuse necrosis of muscle cells (HE = 5.0; $p < 0.05$ vs. control and 1st day after T-2 treatment). The myofibrils were slightly PAS-positive during the first week of the study (PAS = 2.0 — 3.2; $p < 0.05$ vs. control and 1st day after T-2 treatment), while a diffuse distribution of glycogen granules in endo- and perimisium were observed from day 21 to 60 in the whole heart' tissue (PAS = 4.0; $p < 0.05$ vs. control and 1st day after T-2 treatment). Massive hemorrhagic foci associated with diffuse accumulation and degranulation of MCs were the most intensive from day 28 to 60 of the study (MT = 5.0; $p < 0.05$ vs. control and 1st day after T-2 treatment). During the whole study period, irregular distribution of glycogen granules, intensity and total number of haemorrhages were in correlation with the degree of heart structural lesions, which showed the higest coefficient of correlation (r = 0.8750; $p < 0.001$). Our results indicate that basic histohemical methods can be a useful tool for evaluation of T-2 toxin-induced cardiac damage, which is

probably a result of complex inflammatory mechanisms, eventually leading to vascular lesions and myocardial necrosis, as well as for some potential cardioprotectors in the future.
KEY WORDS: T-2 toxin, Rats, Cardiotoxicity, Pathohistology

INTRODUCTION

Administration of T-2 toxin to various animals produced the signs of a shock-like syndrome characterised by massive haemorrhages, immunological failure, cardiomyopathy and death (Anonymous, 2003). The exact causal mechanism of T-2 toxin-induced cardiomyopathy remains unclear. Many investigators consider its cardiotoxic effects just as results of particular myocardial structural alterations, capillary damages, haemorrhages and a focal accumulation of inflammatory cells (Borison et al., 1991; Jaćević, 2005). Its toxic effects on the plasma membrane caused an increase of membrane permeability, which eventually led to irreversible cell injury (Sherman et al., 1987). T-2 toxin also has profound effects on ribosome, sarcoplasmatic reticulum functions and mitochondrial respiration (Feurstein et al., 1985; Ueno, 1984; Pestka et al., 2004; Speijers and Speijers, 2004).

Some authors showed that pro-inflammatory action of T-2 toxin is probably the most important mechanism of its acute cardiotoxicity (Jaćević, 2005; Newton et al., 1997; Bondy and Pestka, 2000). Regarding all these facts, it seems that T-2 toxin-induced blood vessel and myocyte damages as a result of activation of a large number of mast cells (Jaćević et al., 2003).

The aim of this study was to evaluate basic histohemical methods as a useful tool for analysis of T-2 toxin-induced cardiotoxicity in rats.

MATERIALS AND METHODS

The experiment was performed on adult female Wistar rats, weighing 180—220 g (Animal House, Military Medical Academy, Belgrade). The animals were housed in plastic cages, under standard laboratory conditions (21°C, 12/12h light/dark cycle, commercial food and tap water *ad libitum*). In this study rats were treated with T-2 toxin (1 LD_{50}, 0.23 mg/kg *sc*) and sacrificed on days 1, 3, 5, 7, 14, 21, 28 and 60 after the treatment. Control groups of rats were treated with saline (1 ml/kg 0.9% NaCl). At each time-schedule, control groups of animals were sacrificed, too. Each group consisted of 8 animals. The study protocol was based on the Guidelines for Animal Study no. 282-12/2002 (Ethics Committee of the Military Medical Academy, Belgrade, Republic of Serbia). T-2 toxin used in these experiments was produced under laboratory conditions from *Fusarium sporotrichoides* fungi, cultivated on synthetic GPY (glucose 5%, peptone 0.1%, yeast extract 0.1%, pH 5.4) medium. Extraction and crude purification of the toxin were performed by filtration, while definite purification and determination of T-2 toxin content were performed by gas chromatography with electron capture detection (GC-ECD) (Romer et al., 1987). T-2 toxin was preliminarily tested on animals in order to obtain its

LD_{50} value (Litchfield and Wilcoxon, 1949). The hearts were excised after sacrification, and the samples were fixed in 10% neutral formalin for 5 days. Tissue samples were dehydrated in graded alcohol, xylol and embedded in paraffin blocks. Finally, 1-mm thick paraffin sections were stained by haematoxylin and eosin (HE), periodic acid-Schiff's (PAS), Masson-Trichrom's (MT) and Giemsa (GIM) methods. Pathohistological alterations of the heart were analysed in whole visual fields. The changes observed were scored by using semiquantitative grading scale (scoring scale of various myocardial lesions by using HE, PAS and MT staining) (Table 1). The mast cells (MCs) were counted with standard microscope.

Tab. 1 — Pathohistological grades of myocardial lesions in rats treated with T-2 toxin (scoring scale for myocardial degenerations — HE, glycogen distribution — PAS and vascular alterations — MT

Grade	Definition
0	Normal histological structure of myofibrills
1	Mild damage — Small groups of cells with early myofibrillar loss and normal nuclei (HE). Glicogen fragmentation (PAS). Oedema and hyperemia (MT).
2	Moderate damage — Groups of cells (more than 50%) with marked myofibrillar loss and cytoplasmatic vacuolisation (HE). Glicogen deposit in the cytoplasm (PAS). Small focal heamorrhages in the endomisium (MT).
3	Severe focal damage — Majority of cells with homogenisation and hyalinisation of cytoplasm with karyopicnosis and focal accumulation of inflammatory cells (HE). Large glicogen deposit on the pole of myocites (PAS). Thickening of blood vessels with vacuolisation of endothelial cells (MT).
4	Severe diffuse damage — Diffuse discoidal fragmentation of citoplasm with karyorrhexis or karyolysis and diffuse accumulation of inflammatory cells (HE). Irregular distribution of glicogen granules in the sarcoplasm (PAS). Diffuse and massive haemorrhages in the myocard (MT).
5	Tissue necrosis (HE). Glicogen accumulation in the endo- and perimisium (PAS). Heamorrhagic infiltartion of the heart tissue (MT).

Statistical evaluation was performed using commercial statistical software (Stat for Windows, R.4.5, Stat Soft, Inc., USA, 1993). In table, all results are shown as the mean (χ) ± the standard deviation (SD). Comparison of data was done by one-way ANOVA + post-hock analysis (Tuckey's test) and Pearson's test. The differences with values of $p < 0.05$, $p < 0.01$ and $p < 0.001$ were considered significant.

RESULTS

Myocardial alterations detected in T-2 toxin-treated animals ranged from focal parenchymal degeneration (HE = 2.5; $p < 0.05$ vs. control) or hyaline degeneration (HE = 4.0; $p < 0.05$ vs. control and 1st day after T-2 toxin treatment) to diffuse necrosis of muscle cells (Table 2).

Tab. 2 — Severity of myocardial lesions in T-2 toxin treated rats

	Treatment ($\chi \pm SD$)					
	Control (1 ml/kg *sc* 0.9% NaCl)			T-2 toxin (0.23 mg/kg *sc* T-2 toxin, $1LD_{50}$)		
Days	HE	PAS	MT	HE	PAS	MT
1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.5 ± 0.5 a	2.0 ± 0.5 a	3.0 ± 0.5 a
3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.7 ± 0.4 a	2.5 ± 0.5 a	3.5 ± 0.5 a
5	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	3.0 ± 0.5 a	2.8 ± 0.4 a	3.5 ± 0.5 a b
7	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	3.2 ± 0.5 a	3.2 ± 0.5 a b	4.0 ± 0.5 a b
14	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	4.0 ± 0.5 a b	3.5 ± 0.5 a b	4.5 ± 0.5 a b
21	0.4 ± 0.8	0.4 ± 0.8	0.4 ± 0.8	4.5 ± 0.5 a b	4.0 ± 0.5 a b	4.5 ± 0.5 a b
28	0.4 ± 0.8	0.4 ± 0.8	0.4 ± 0.8	5.0 ± 0.5 a b	4.0 ± 0.5 a b	5.0 ± 0.5 a b
60	0.4 ± 0.8	0.4 ± 0.8	0.4 ± 0.8	5.0 ± 0.5 a b	4.0 ± 0.5 a b	5.0 ± 0.5 a b

Statistical evaluation was performed using Tuckey's test.
a — $p < 0.05$ vs control; b — $p < 0.05$ vs 1st day after treatment.
* According to scoring scale (Table 1).

Necrotic areas were most prominent in the inner part of the myocardium and in all layers of endocardium on 28th and 60th day of the experiment (HE = 5.0; $p < 0.05$ vs. control and 1st day after T-2 toxin treatment). These myofibrils were slightly PAS-positive during the first week (PAS = 2.0—3.2; $p < 0.05$ vs control), while a diffuse distribution of glycogen granules in endo- and perimisium were observed from day 21 to 60 in the whole heart' tissue (PAS = 4.0; $p < 0.05$ vs. control and 1st day after T-2 toxin treatment) (Table 2). From day 3, interstitial haemorrhages appeared uniformly in each of the sections examined, and were located in the middle myocardial or subendocardial areas. Massive hemorrhagic foci associated with a diffuse accumulation of inflammatory cell infiltrates were the most intensive from day 28 to 60 of study (MT = 5.0; $p < 0.05$ vs. control and 1st day after T-2 treatment) (Table 2). During the whole study period, irregular distribution of glycogen granules, intensity and total number of haemorrhages were in correlation with the degree of heart structural lesions, which showed the higest coefficient of correlation (r = 0.8750; $p < 0.001$) (Table 3).

Tab. 3 — Time dependent frequency of myocardial lesions — HE, distribution of glycogen granules — PAS and intensity of haemorrhages — MT in T-2 toxin-treated rats

Parameters	X variable	Y variable	Pearson (r)	Probability (p <)
T-2 (HE)	Days after treatment 0—60	Myocardial degenerations — HE	0.8750	0.001
T-2 (PAS)	Days after treatment 0—60	Glycogen distribution — PAS	0.5218	0.001
T-2 (MT)	Days after treatment 0—60	Vascular alterations — MT	0.2258	0.05

Statistical evaluation was performed using Pearson's test.

The total number of MCs had only a negative coeficient of correlation (r = — 0.329; $p < 0.01$) (Table 4). The total number of all type of MCs were the highest on the 20th day of the study (results not shown).

Tab. 4 — Time dependent frequency of myocardial lesions (HE) and total number of mast cells — GIM in T-2 toxin-treated rats

Parameters	X variable	Y variable	Pearson (r)	Probability (p <)
T-2 (HE)	Days after treatment 0—60	Myocardial degenerations — HE	0.8750	0.001
T-2 (GIM)	Days after treatment 0—60	Total number of mast cells — MCs	–0.3293	0.05

Statistical evaluation was performed using Pearson's test.

DISCUSSION

The present study demonstrates that the subcutaneous administration of a single dose of 0.23 mg/kg (1 LD_{50}) of T-2 toxin to Wistar rats results in myocardial damage, especially in massive hemorrhagic lesions and intensive degranulation of MCs, and provides new insights into the pathogenesis of these alterations. Moreover, our results point to the marked complexity of the pathogenesis of these lesions. Signs of inflammation, degeneration and rapid loss of normal cell architecture, could be found in the heart samples of T-2 toxin treated rats stained by HE method. Similar pathohistological alterations, vacuolisation and hyaline degeneration of myocytes with karyopicnosis, have been reported in the heart of humans and animals during the acute T-2 toxin poisoning (Sudakin, 2003; Jaćević et al., 2006). If the examination lasted long enough, as our experiment did, for 60 days, pathohistological changes in the heart of T-2 toxin-poisoned rats ranged from hyaline degeneration to myocardial necrosis with focal accumulation of mononuclear cells. In this period, diffuse distribution of glycogen granules were observed in PAS-positive endo- and perimisium of the whole heart' tissue. These results suggested that T-2 mycotoxin has a direct toxic effect on capillaries and increases their permeability, as well as on the plasma membrane which led to irreversible cell injury (Borison et al., 1991; Jaćević, 2005; Sherman et al., 1987). In contrast, a total number of MCs was in the correlation with cardiac damages until to day 21 of the study, and could probably contribute to the vascular damages and myofibrilar necrosis. Induction of necrosis by mast cells was suggested by the findings of increased numbers of these cells and extrusion of their granules in areas of vascular injury as early as 24 hours after the treatment with T-2 toxin (Jaćević, 2005; Jaćević et al., 2006). Tissue mast cells possess granules containing substances that can have profound effects on vascular integrity. The release of inflammatory mediators (e.g., histamine) and pro-inflammatory cytokines (IL-1a, IL-1b, IL-6, IL-8, and TNF-a) by mast cells is thought to be involved in vascular permeability changes, induction of leukocyte-endothelium interactions, and acute vascular inflammation (Galli, 1993; Gavrisheva and Tkachenko, 2003). Thus, it seems likely that all of the factors cited here could contribute to the myocardial necrosis and vascular lesions induced by T-2 toxin.

CONCLUSION

Our results indicate that basic histohemical methods can be a useful tool for evaluation of T-2 toxin-induced cardiac damage, which is probably a result of complex inflammatory mechanisms, eventually leading to vascular lesions and myocardial necrosis, as well as for some potential cardioprotectors in the future. Further morphological investigations are needed in order to elucidate further these processess.

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ХИСТОХЕМИЈСКО ИСПИТИВАЊЕ КАРДИОТОКСИЧНИХ ЕФЕКТА КОД ПАЦОВА ТРЕТИРАНИХ Т—2 ТОКСИНОМ: СЕМИКВАНТИТАТИВНА АНАЛИЗА

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Резиме

У овом раду су испитани токсични ефекти на срцу Wistar пацова акутно трованих Т-2 токсином. Животиње, једнократно третиране Т-2 токсином у дози од 0,23 mg/kg *sc* (1 LD_{50}), жртноване су 1, 3, 5, 7, 14, 21, 28. и 60. дана после апликације отрова. Контролне групе животиња третиране су физиолошким раствором (1 ml/kg 0,9% NaCl) и жртноване у истим временским интервалима. Процена патохистолошких промена извршена је на узорцима ткива срца, бојених стандардним хистохемијских методама: хематоксилин и еозин (HE), Гимза (GIM), перјодна киселина Schiff-ов реагенс (PAS) и Masson trichrom (MT), применом семиквантитативне анализе. У срцу пацова третираних Т-2 токсином уочене су промене од фокалне паренхиматозне и хијалине дегенерације миофибрила (HE = 2,5—4,0; $p < 0,05$ у поређењу са контролом) до фокалне или дифузне некрозе мишићних ћелија (HE = 5,0; $p < 0,05$ у поређењу са контролом и 1. даном после апликације Т-2 токсина). Током прве недеље испитивања миофибриле су биле благо PAS-позитивне (PAS = 2,0—3,2; $p < 0,05$ у поређењу са контролом и 1. даном после апликације Т-2 токсина), док је дифузна дистрибуција гранула гликогена у ендо- и перимизијуму запажена од 21. до 60. дана (PAS = 4,0; $p < 0,05$ у поређењу са контролом и 1. даном после апликације Т-2 токсина). Масивна хеморагична поља, окружена многобројним инфламаторним ћелијама, нарочито су изражена у периоду од 28. до 60. дана испитивања (MT = 5,0; $p < 0,05$ у поређењу са контролом и 1. даном после апликације Т-2 токсина). Током целог периода испитивања, неправилна дистрибуција гранула гликогена, интензитет крварења и укупан број мастоцита су били у корелацији са степеном оштећења ткива срца ($r = 0,8750$; $p < 0,001$). Добијени резултати су потврдили раније изнету тезу да су кардиотоксични ефекти Т-2 токсина вероватно резултат комплексних инфламаторних механизама.