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MORPHOMETRIC CHANGES OF CARDIAC MAST CELLS IN RATS ACUTELY POISONED BY T-2 TOXIN

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Wistar rats were treated with T-2 toxin (1 LD_{50}; 0.23 mg/kg sc) and the surviving animals were sacrificed on days 1, 3, 5, 7, 14, 21 and 28 after treatment. At each time, control animals were sacrificed, too. Cardiac mast cells, previously stained by Giemsa method, were analyzed in whole visual fields, magnification x40. In the present study the following quantitative morphometric parameters of cardiac mast cells were evaluated: perimeter (P), area (A) and roundness (R). In the control groups of rats the majority of mast cells were small (P = 6.86 - 7.99 μm), hypogranular (A = 11.60 - 14.30 μm^2) and ovoid (R = 0.60 - 0.65 μm). Mast cells, with discrete granules, hypergranular, had significantly different quantitative parameters (P = 12.80 - 14.90 μm; A = 16.70 - 20.00 μm^2; R = 0.35 - 0.38 μm). The minority of mast cells, classified as degranulated, had a large (P = 20.70 - 23.30 μm), irregular shape (A = 24.40 - 30.90 μm^2) and showed degranulation (R = 0.15 - 0.21 μm). In the heart of T-2 toxin-treated rats the quantitative parametar values of hypogranular mast cells and hypergranular mast cells were similar to the control group during the whole study. However, degranulated mast cells showed a significant increase in perimeter and area values ($p<0.05$), while their roundness was decreased ($p<0.05$) in comparison to the control groups of animals. It could be concluded that the chosen quantitative morphometric parameters of cardiac degranular mast cells are useful for the evaluation of the functional status of the rats' heart during acute T-2 poisoning.

Key words: heart, mast cells, morphometry, T-2 toxin, rat

INTRODUCTION

The trichothecene mycotoxin, T-2 toxin, is one of the most effective cytotoxic metabolites of *Fusarium* fungi (Ciegler, 1975), which produce after inhalation or

consumption of contaminated food and water a toxic reaction called mycotoxicosis (Ciegler, 1980).

Administration of T-2 toxin to various animals produced signs of a shock-like syndrome characterized by massive haemorrhagies, 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 a result of particular myocardial structural alterations, capillary damages, haemorrhagies and focal accumulations of inflammatory cells (Yarom *et al*., 1983; Pang et al., 1987; Borison *et al*., 1991; Jaćević et al., 2006). Its toxic effects on the plasma membrane caused increased membrane permeability, which eventually leads to irreversible cell injury (Sherman *et el*., 1987). T-2 toxin also has profound effects on ribosomes, sarcoplasmatic reticulum functions and mitochondrial respiration (Feurstein *et el*., 1985; Ueno, 1991; Pestka *et al*., 2004; Spijers and Spijers, 2004). However, available data favours the hypothesis that not all these effects are specific. They resemble lesions caused by a number of cardiotoxic drugs especially those used in antidepressant and anticancer therapy. The pathogenic mechanisms of these drugs are varied and often multifactorial (Dragojević-Simić *et al*., 2004). Some authors showed that pro-inflamatory action of the T-2 toxin probably is the most important mechanism of its acute cardiotoxicity (Newton *et al*., 1997a; Newton *et al*., 1997b; Bondy and Pestka, 2000; Jaćević, 2005). Regarding all these facts, it seems that T-2-induced blood vessels and myocyte damages due to the activation of a large number of mast cells (Jaćević *et al*., 2003).

Different activators, one of them probably T-2 toxin, stimulate mast cells to synthesize arachidonic acid metabolites such as prostaglandins (PG), leukotrienes (LT), platelet-activating factor (PAF) and adenosine (Katz *et al*., 1992; Smith *et al*., 2000; Moller *et al*., 2003). These *de novo* sinthesized mediators play an important role during hypersensitivity and inflammation, and especially in non-specific reactions of the heart tissue (Engels *et al*., 1995; Shiota *et al*., 2003). Several authors have suggested that mast cell (MCs) mediators may be involved in the aetiology of some forms of human and animal cardiomyopathy (Masini *et al*., 1985; Masini *et al*., 1988; Sperr *et al*., 1994). They consider that cardiac ischemia and reperfusion induce degranulation of mast cells, which is accompanied by oedema, arrhythmias, histamine and serotonin release, and release of sarcoplasmic enzymes (Dai and Ogle, 1990). However, there is accumulating evidence that indicates that cardiac mast cells are critically involved in cell toxicity, vascular endothelial cell proliferation and integrity, angiogenesis, fibrosis, polimorphonuclear cell activation and differentiation, immunoregulation and immunomodulation (Befus *et al*., 1988; Stevens and Austen, 1989). Synthesis and degranulation of pro-inflammatory mediators involves the migration and activation of macrophages and production of IL-1, IL-2, IL-6, IL-8 and TNF-alpha (Castells *et al*., 1991), which play an important role in the pathogenesis of T-2 mycotoxicosis (Bondy and Pestka, 2000).

The aim of this study was to evaluate the changes of three different types of myocardial mast cells (hypogranular, H0-MCs; hypergranular, H1-MCs; degranular, D-MCs) according to their perimetar, area and roundness in the heart

of acutely T-2 toxin-poisoned rats. The rationale for this experimental study was our previous study and finding that a single administration of T-2 toxin significantly increased the total number of rats' cardiac mast cells (CMCs), which showed degranulation (Jaćević *et al*., 2003; Jaćević, 2005).

MATERIAL AND METHODS

Experimental animals and treatment

The experiment was performed on adult Wistar rats, of both sexes, 6-8 weeks old, weighting 180-200 g (Animal House, Military Medical Academy, Belgrade). The animals were housed in plastic cages, under standard laboratory conditions (21°C, 12/24h light/dark cycle, commercial food and tap water *ad libitum*) before being randomized into corresponding experimental groups. One day before the experiment, the animals were fasted. Rats were treated by T-2 toxin (1 LD_{50}; 0.23 mg/kg *sc*) and the surviving animals were sacrificed on days 1, 3, 5, 7, 14, 21 and 28 after treatment, respectively. At each time, control groups of animals were sacrificed, too. Each experimental group consisted of at least 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, Serbia).

T-2 toxin

The T-2 toxin used in this experiment was produced under laboratory conditions by *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 was performed by filtration, while definite purification and determination of T-2 toxin content was performed by gas chromatography with electron capture detection (GC-ECD) (Romer *et al*., 1991). T-2 toxin was preliminarily tested on animals in order to obtain its LD_{50} value, (Litchfield and Wilcoxon, 1949). It was thereafter used in the current experiment at a single dose of 0.23 mg/kg s.c. (1 LD_{50}).

Histopathological procedure

Animals were sacrificed and their hearts were excised and tissue samples were fixed in 10% neutral formalin for 5 days. Transmural tissue samples from the left and right ventricular wall were dehydrated in graded alcohol, xylol and embedded in paraffin blocks. Finally, 2-μm thick paraffin sections were stained by Giemsa (GIM) method and studied by microscopy (40x; Olympus-2 microscope).

Morphometric analysis

Ten cardiac sections of each tissue sample were examined with a standard microscope connected to a computerized video system and analyzed with image-analysis software (Camia, 2005) to estimate various quantitative mast cell features. From each specimen, 5-10 accidentally selected visual fields, magnified by 40x were analyzed and a minimum of 100 mast cells per animal were measured

automatically and the perimeter calculated. The perimetar, area and roundness of mast cells were measured by calculating the pixels. Data on these parameters were converted by the factor 0.4761 into μm. According to their visible quantitative features (hypo-, hyper- and degranulated cells) all examined MCs classified on quantitative bases and perimetar, area and roudness range during the experiment (28 days) were as follows (Table 1).

Table 1. Morphometric classification of cardiac mast cells of untreated rats (MCs)

Visual characteristics of MCs	Types of cardiac MCs	Perimetar range (P)(μm)	Area range (A) (μm^2)	Roundness range (R) (μm)
MCs without visible granules	Hypogranular H0-MCs	5.0 – 10.0	10.0 – 15.0	>0.6
MCs with visible granules	Hypergranular H1-MCs	10.1 – 15.0	15.1 – 20.0	0.3 – 0.6
MCs which show degranulation	Degranular D-MCs	>15.0	>20.0	<0.3

Statistical analysis

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

RESULTS

General condition of experimental animals

In the surviving rats, during the 28-day period of observation, no significant changes of general health status were seen. All the rats were in good shape. The hair, skin, visible mucoses and muscle tonicity were without any visible changes. Movements and co-ordination were preserved and comparable to the control animals.

Morphometric evaluation of cardiac mast cells perimeter

In the control group of rats, sacrificed after day 1, the perimeter of H0-MCs was 7.57 ± 1.48 μm. It could be seen that the perimeters of H1-MC, and D-MCs were 14.61 ± 2.03 μm, and 20.95 ± 1.64 μm, respectively. These values were not significantly different from the other control groups of rats, during the whole experimental period (Figure 1). Single administration of T-2 toxin induced a significant increase of the perimetar of D-MCs ($p<0.05$). The first significant increase was noticed on the first day after administration of T-2 toxin. This trend could be seen until day 14 of treatment. During the rest of the experiment

perimeters of D-MCs were not significantly different from the control ones. The highest perimeter of D-MCs was seen in the poisoned group sacrificed on the fifth day of the study (33.60 ± 3.83 μm). On the other hand, the perimeter of H0-MCs and H1-MCs was similar to control groups of rats during the whole study.

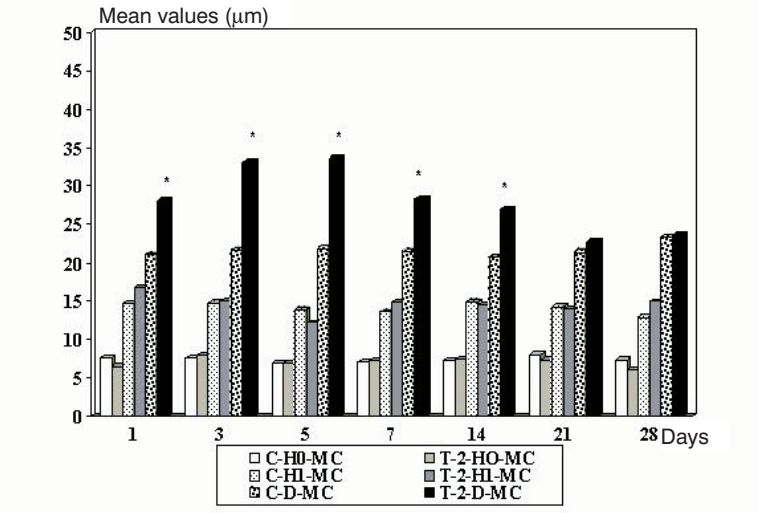

Figure 1. Time-dependent perimeter changes of cardiac MCs in control and T-2 toxin-treated rats
* = $p<0.05$ vs. control group

Morphometric evaluation of cardiac mast cell area

In all control groups the areas of H0-MCs were in the range of 11.62 ± 2.41 to 13.1 ± 2.41 μm^2; H1-MCs (16.70 ± 5.81 to 19.90 ± 3.25 μm^2); values of D-MCs were no higher than 30.60 ± 5.11 μm^2. The median lethal dose of T-2 toxin did not induce significant changes of the areas of H0-MCs and H1-MCs troughout the experimental study, compared to the control groups. As it is shown in Figure 2, T-2 toxin only induced a significant increase of the area of D-MCs from days 1-14 of the experiment compared to control rats. The greatest difference between T-2 toxin treated and the control group was noticed on the fifth day (39.70 ± 5.81 versus 27.00 ± 6.01 μm^2 ; $p<0.05$).

Morphometric evaluation of cardiac mast cell roundness

In the first day in the control group of animals, the roundness of H0-MCs was 0.616 ± 0.071 μm; H1-MCs (0.380 ± 0.06 μm); and D-MCs (0.150 ± 0.04 μm). It was found that the roundness values of this type of cardiac MCs were not significantly different during the overall experimental period. The results presented in Figure 3 also cleary show that the roundness of cardiac H1-MCs and D-MCs in rats treated by T-2 toxin were similar to the ones in the control groups. However, T-2 itself, significantly changed only the roundness of cardiac H0-MCs in rats sacrificed after day 7 and 14 in comparison to the control ones (0.71 ± 0.18 vs. 0.62 ± 0.07 μm; 0.72 ± 0.08 vs. 0.62 ± 0.10 μm; $p<0.05$).

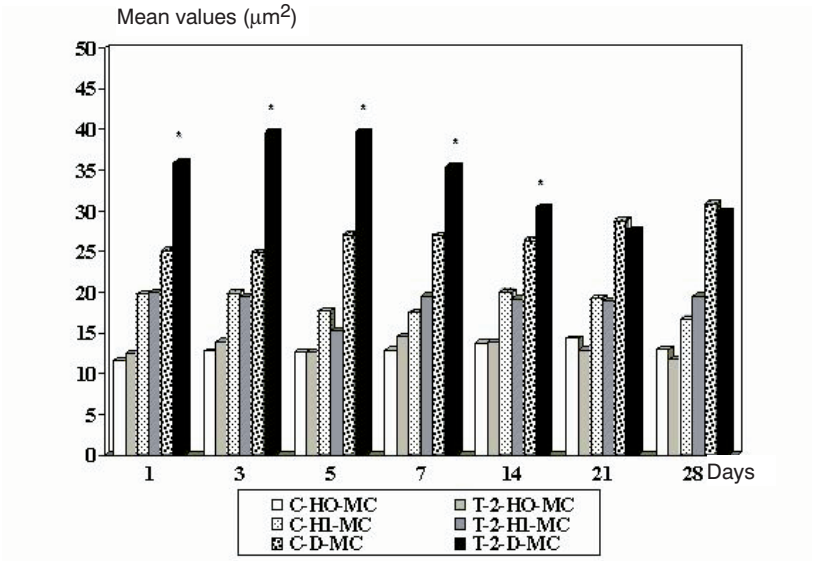

Figure 2. Time-dependent area changes of cardiac MCs in control and T-2 toxin-treated rats
* = p<0.05 vs. control group

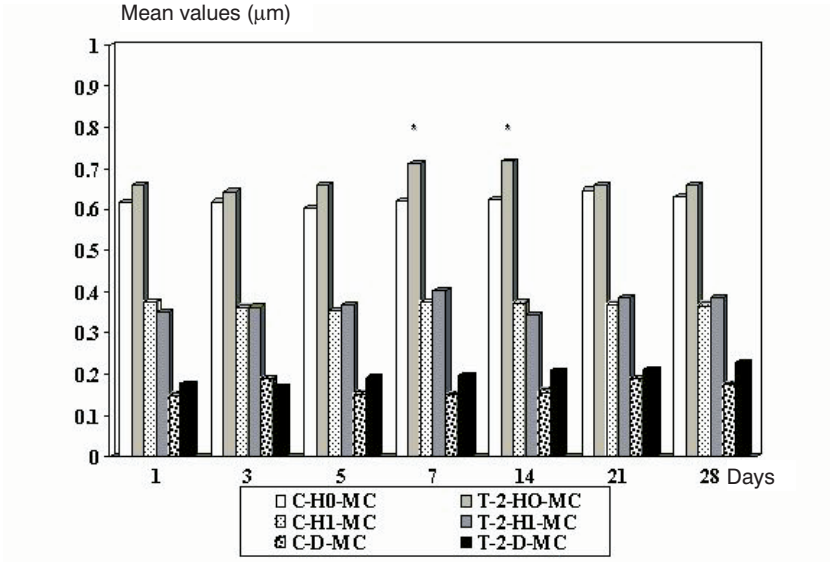

Figure 3. Time-dependent roundness changes of cardiac MCs in control and T-2 toxin-treated
* = p<0.05 vs. control group

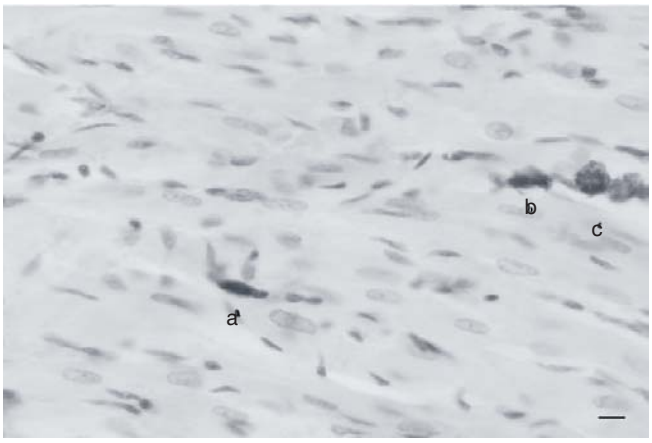

Figure 4. Perivascular localization of different types of cardiac MCs in control rat
a = H0-MCs, b = H1-MCs, c = D-MCs, scale bar = 10 μm

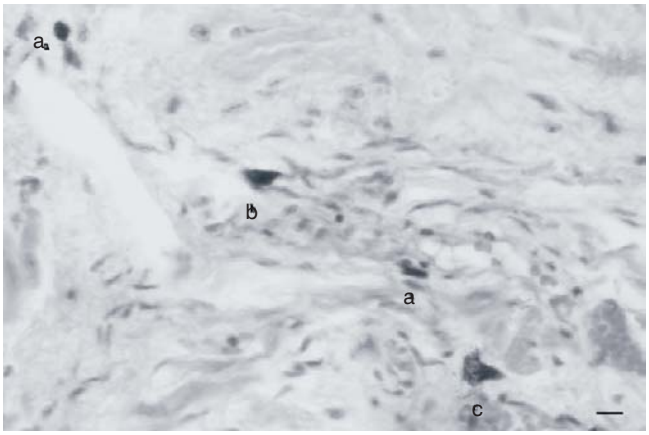

Figure 5. Diffuse tissue spreading of different types of cardiac MCs in T-2 toxin-treated rat
a = H0-MCs, b = H1-MCs, c = D-MCs, scale bar = 10 μm

DISCUSSION

In previous studies different types of cardiac MCs were monitored in the control and T-2 toxin poisoned groups of rats (Jaćević *et al*., 2003; Jaćević, 2005). In the present study, in the control group of rats, cardiac H0-MCs were small (P = 7.57 ± 1.48 μm), tiny (A = 11.62 ± 2.41 μm^2), ovoid (R = 0.616 ± 0.071 μm) and, similarly to results reported in literature (Castells *et al*., 1992), they were situated on the external wall of blood vessels in the subepicardial and subendocardial regions. These findings, together with perivascular localization of H0-MCs, suggest that the basic function of the resident MCs population in the heart served for adaptive vascular changes during physiologic responses (Galli, 1993;

Gavrisheva and Tkachenko, 2003; Boerma *et al*., 2005). In the control group of animals, we found that registrated parameters of H0-MCs were highest on day 21 (P = 7.99 ± 1.35 μm; A = 14.33 ± 2.18 μm^2; R = 0.646 ± 0.095 μm). The highest values of H1-MC were seen after the end of day 14 (P = 14.85 ± 3.10 μm; A = 19.95 ± 4.53 μm^2; R = 0.373 ± 0.05 μm), while these data for D-MCs were the highest on day 28 (P = 23.28 ± 0.27 μm; A = 30.85 ± 2.28 μm^2; R = 0.174 ± 0.029 μm).

Our results have shown that 0.23 mg/kg single dose of T-2 toxin produced a diffuse perivascular and tissue accumulation, as well as a massive degranulation of cardiac mast cells. Later on, a single injection of T-2 toxin changed the perimetar and the area of D-MCs and their values were significantly higher than in the control groups during the study ($p<0.05$). On the other hand, the reduction of roundness was probably associated with multiple, irregular shapes after activation and degranulation of MCs. Significantly the inrease of perimetar and area, as well a significan decrease of roundness of D-MCs in T-2 toxin-induced rats, are considered multifactorial. These are the results of direct lymphocyte-derived and other cytokines effects on mast cell proliferation or maturation/differentiation which are quite different than the normal levels of these products expressed at the same anatomical location under normal circumstances (Matsumori, 2005). The low doses of T-2 toxin have stumulatory effects on immune and inflammation associated genes, Th1 and Th2 cytokines as well as chemokines, COX-2 and inducible nitric oxide synthase. Some authors confirm that T-2 toxin has a great ability to induce interleukin-2 (IL-2) production (Feurstein, 1985). IL-2 is a potent T lymphocyte growth factor, which causes proliferation of natural killer (NK) cells and cytotoxic T lymphocytes. It also interacts with macrophages and enhances the activity of cytotoxic T cells (Robb, 1984). The targeting of such cells onto the heart may be due to their previous sensitization by damaged cardiac tissue in the T-2 toxin-treated rats. The sites of cardiac alterations, with a large numbers of eosinophils, may have a high concentration of molecules (e.g. IgE and specific antigens) which alter mast cell phenotype as a consequence of inducing its degranulation (Fox and Lakshamanan, 1994; Newton *et al*., 1997a; Newton *et al*., 1997b). Similar to these facts, our morphometric evaluation has shown that excessive degranulation of cardiac mast cells was during the first 2 weeks of the experiment. The most prominent perametar of D-MCs was on day 5 (P = 33.6 ± 3.83 μm; A = 39.66 ± 5.68 μm^2; $p<0.05$). The pathophysiological function of the cardiac mast cell population in T-2 toxin-treated rats is still unclear. On the other hand, the activated mast cells, in the present study named as H1-MCs, around the newly formed blood vessels may cause the fragile microvessels to fracture with resulting local hemorrages (Kaartinen *et al*., 1996; Akgul *et* al., 2005). These observations point to a possible role of cardiac mast cells in the local microenvironmental and vasculaar events in the haert, especially during the development of ishaemia caused by T-2 toxin.

Morphological changes of D-MCs in our experiments were verified by functional alterations in these cells during degranulation. It should be mentioned that the mechanism of mast cell degranulation includes the extrusion of granules to the exterior wall of the cell, as well as intracytoplasmic solubilization of granules

and fusion of its membranes, and formation of degranulation channels (Dvorak, 1991). This activation may be induced by macrophages (Ueno, 1991) and T lymphocytes (Lui *et al*., 1986; Sedgewick *et al*., 1981), two pro-inflammatory cell types that are present in perivascular infiltrations in the region of T-2 toxin-induced vascular and cardiac damages are confirmed in our recent studies (*Jaćević et al*., 2001; Jaćević *et al*., 2005).

It could be concluded that the chosen quantitative morphometric parameters of D-MCs are useful only for the evaluation of the functional status of the heart during acute T-2 toxicosis. Therefore, further investigations are necessary to establish the correlation between the MCs degranulation and miocardial alterations in rats acutely poisoned by T-2 toxin.

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PROMENE MORFOMETRIJSKIH PARAMETARA MASTOCITA U SRCU PACOVA AKUTNO TROVANIH T-2 TOKSINOM

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SADRŽAJ

Preživeli Wistar pacovi, tretirani T-2 toksinom (1 LD_{50}; 0,23 mg/kg *sc*), žrtvovani su 1, 3, 5, 7, 14, 21. i 28. dana posle tretmana. U istim vremenskim intervalima žrtvovane su životinje iz kontrolnih grupa. Mastociti srca, prethodno obojeni primenom Giemsa metode bojenja, analizirani su u celom vidnom polju, pod uveličanjem 40. U ovom radu ispitivani su sledeći kvantitativni morfometrijski parametri: perimetar (P), površina (A) i kružnost (R). U srcu kontrolne grupe pacova mastocititi su većinom sitni (P = 6,86 - 7,99 μm), hipogranularni (A = 11,60 - 14,30 μm^2) i ovalnog oblika (R = 0,60 - 0,65 μm). Mastociti blago ispunjeni granulama, hipergranularni mastociti, imali su statistički značajno različite vrednosti kvantitativnih parametera (P = 12,80 - 14,90 μm; A = 16,70 - 20,00 μm^2; R = 0,35 - 0,38 μm). Mali broj mastocita označeni kao deganulirani mastociti su veliki (P = 20,70 - 23,30 μm), nepravilnih oblika (A = 24,40 - 30,90 μm^2) sa granulama ispražnjenim u okolno tkivo (R = 0,15 - 0,21 μm). U srcu pacova tretiranih T-2 toksinom kvantitativni parametari hipogranuliranih i hipergarnuliranih mastocita imali su vrednosti slične kontrolnim grupama životinja tokom celog perioda ispitivanja. Međutim, degranulirani mastociti pokazali su statistički značajno povećanje vrednosti prečnika i površine ($p<0,05$), dok je njihova kružnost bila manja ($p<0,05$) u poređenju sa kontrolnim grupama pacova. Moglo bi se zaključiti da su ispitivani kvantitativni morfometrijski parametri degranuliranih mastocita korisni za ispitivanje funkcionalnog statusa srca pacova akutno trovanih T-2 toksinom.