Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2020.024

RESEARCH ARTICLE

Morphofunctional Assessment of the Glycyrrhizinic Acid Effect on Myocardium of Rats under Adrenaline Loading

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ARTICLE HISTORY (19-478)

Received: October 17, 2019
Revised: February 14, 2020
Accepted: February 15, 2020
Published online: March 05, 2020

Key words:
Cardioprotector
Glycyrrhizinic acid
Histology
Immunohistochemistry
Myocardium

A B S T R A C T

The paper describes the glycyrrhizinic acid effect on rats having an experimental myocardosis using histological and immunohistochemical methods. The studies were performed on 30 sexually mature white Wistar rats of both sexes weighing 280-350 g. Dystrophic and necrotic processes were induced using adrenaline in rats' myocardium. The first experimental group received glycyrrhizinic acid at a dose of 50 mg/kg, together with drinking water for 14 days. The second experimental did not receive glycyrrhizinic acid. The third experimental group was a control group. 14 days after prophylactic use of glycyrrhizinic acid, the first and the second experimental groups were exposed to a single administration of epinephrine at a dose of 4 mg/kg. In the second experimental group, rats received glycyrrhizinic acid orally together with water once a day within 14 days, and then 0.1% adrenaline hydrochloride solution was injected subcutaneously. Morphological changes were studied in the responsive areas of the myocardium. There were found areas of extensive focal cardiosclerosis formed at the site of dead cardiomyoscytes, perivascular sclerosis, and thicker vascular wall due to cells' smooth muscle hyperplasia 30 days after a single injection of adrenaline in the rat myocardium. Glycyrrhizinic acid in rat myocardium stimulates increased cell expression of a tissue inhibitor of metalloproteinase-2 which has an antiapoptotic effect. This promotes the growth and survival of cardiomyocytes, prevents gross myocardial scarring in the affected areas, which ensures the cardioprotective effect of glycyrrhizinic acid in the adrenaline model of myocardial damage.

To Cite This Article: Bazekin G, Gatiyatullin I, Skovorodin E, Sharipov A and Dolinin I, 2020. Morphofunctional assessment of the glycyrrhizinic acid effect on myocardium of rats under adrenaline loading. Pak Vet J, 40(3): 289-294. http://dx.doi.org/10.29261/pakvetj/2020.024

INTRODUCTION

Diseases of a cardiovascular system are widespread in animals and make up about 4 to 22%. They are most often, heart hypertrophy, myocardium dystrophy, and disturbances of the heart muscle trophicity. As a result, the pathology of other organs and systems of the body develops (Janahmadi *et al*., 2015; Moneim *et al*., 2015; Krasnikova *et al*., 2019).

Currently, there is an increasing interest in the treatment of heart diseases with plant-based medicines that are associated both with the high efficiency of herbal medicinal products and their relative safety compared to synthetic drugs (Skrivanova *et al*., 2009; An *et al*., 2017; You *et al*., 2019). In this regard, triterpene glycoside from licorice root - glycyrrhizinic acid (GA) - deserves special attention. It is harmless and of low toxicity compared to chemical analogues.

GA derivatives are attractive for their high and diverse biological activities, low toxicity. Glycyrrhizinic acid affects the metabolic processes in the myocardium, interacts with the sulfhydryl groups of the Na+, K+-AT transport phase of the cardiomyocyte membrane. It reduces the activity of the enzyme, lowers the intracellular content of potassium ions and increases the concentration of sodium ions in myofibrils, which increases the content of free calcium ions in the myocardium. This leads to the formation of a contractile protein-actomyosin. Besides, glycyrrhizinic acid normalizes metabolic processes and energy metabolism in the cardiac muscle and increases oxidative phosphorylation contingency that results in the significant systole increase. GA prevents a decrease in

glycogen in the myocardium, and an increase in the content of total lipids, activation of their peroxidation. It averts lower antioxidant activity of blood serum, improves electrocardiographic parameters. It is prospective in dealing with pathological processes of heart accompanied by inflammatory and necrotic changes of the myocardium, in particular, during myocarditis and myocardial infarction.

While being administered, it enhances the humoral immune response in animals primed with a thymus-dependent antigen. GA has the apparent anti-inflammatory, ulcerogenic, hepatoprotective, and antioxidant properties (Tolstikova *et al*., 2009; Bazekin and Ismagilova, 2015). It exhibits antiarrhythmic, anti-allergic, antiviral, and anti-inflammatory activity (Baltina *et al*., 2010; Stolyarova *et al*., 2014; Jitesh and Geetha, 2017), as well as ability to suppress an increase in fasting blood glucose and insulin and reduces glucose tolerance. The number of adverse reactions observed while using glycyrrhizinic acid is small. The possible mechanisms of antioxidant activity of GA have been studied (Baltina *et al*., 2010; Ming *et al*., 2013).

Glycidipin studies proved its high hypotensive and antiarrhythmic activity as well as its safe use (Tolstikova *et al*., 2009). Besides, GA can directly influence cardiac performance. It is vital in the protection of myocardium and coronal vessels in cardiovascular diseases. Therefore, glycyrrhizinic acid is the remedy deserving attention when considering the strategy of cardiac disease treatment (Di Paola *et al*., 2009; Gao *et al*., 2015; Cai *et al*., 2017).

MATERIALS AND METHODS

Ethical approval: All experiments were performed in conformity with the Law (The European Convention for the protection of vertebrate animals, European convention for the protection of vertebrate animals used for experimental and other scientific purposes; Guide for the care and use of laboratory animals and the Animal Ethics Committee report (No. 6 dated 02.03.2017).

Animal feeding and management: An experimental part of researches is conducted in the conditions of a vivarium of veterinary clinic and pharmacology laboratory of biotechnologies and veterinary medicine faculty of the Bashkir State Agrarian University. The provisions and rules for conducting work using animals were guided by when implementing the experimental part of the experiments and forming the experimental groups of animals, arranging normative feeding, and care of the laboratory animals. The experimental conditions were identical for the control and experimental groups; they met the requirements of SanPIN No. 1045-73 "Sanitary rules for designing, equipping, and keeping up experimental biological clinics" following international GLP standards. The ration of laboratory animals consisted of the granulated meal "ProKorm" balanced in nutritional value. The temperature regime in the vivarium was maintained continuously in the range from 18 to 20 degrees Celsius. Rats were kept on beddings in the solid-bottom cages. Woodcutter shavings were used as bedding material.

Animals, experimental design and treatments: The studies were carried out on 30 sexually mature white Wistar rats of both sexes weighing 280-350 g. The task of our research was not to find out the features of pathology in animals of different sexes. Therefore, each group consisted of 5 males and 5 female rats, being at the diestrum stage of the sex cycle. All the animals were of the same age. During ECD, morphological, and morphometric studies, we did not find significant differences between 4-month-old females and males. Animals were selected on the analogue basis taking into account live weight and sex. The preparations were not administered in the control group rats. Dystrophic and necrotic processes in the myocardium were induced in rats of the first experimental group using adrenaline. A single subcutaneous injection of epinephrine with a dose of 4 mg/kg was made. 24 hours after epinephrine injection rats were decapitated. Rats of the second experimental group were fed with the glycyrrhizinic acid once a day during 14 days, and then a 0.1% solution of adrenaline hydrochloride with dose of 4 mg/kg was injected subcutaneously.

Morphological methods: After a thorough pathomorphological study of the organs, the hearts of rats of all the studied groups were cut across into 3 plates of the same thickness: upper, middle, and lower. Tissue samples were fixed in a 10% solution of neutral formalin dehydrated in ascended alcohol series and embedded in paraffin according to the generally accepted technique. Sections were cut with the LEICA RM 2145 microtome. They were stained with hematoxylin and eosin, according to Mallory, Van Gieson's procedures, and generally accepted techniques.

The paraffin sections were of 4 μm thickness. Staining was performed using the automated stainer for immunohistochemistry and hybridization Leica Microsystems Bond™. There were following primary polyclonal antibodies: MMP 9+ at a dilution of 1: 300; TIMP 2+ at a dilution of 1: 300; Gata 4+ at a dilution of 1: 300 (Santa Cruz Biotechnology, USA). Unmasking was done with the polyclonal indirect streptavidin-biotin detection system Leica BOND (Novocastra™, Germany). Evaluation of the reaction specificity was carried on staining sections without the first antibodies. After the immunohistochemical reaction, the sections were mounted in a Bio Mount synthetic medium (Bio Optica, Italy).

Morphometry and statistical analysis: Calculation of cells was performed in 20 fields of view of each sample (n=6) at magnification ×400. The positively stained cells against antibodies to MMP 9+, TIMP 2+, Gata 4+ in responsive areas of myocardium were calculated. The statistical reliability of the indicator was determined with the Student's t-test. The rank analysis was performed using the non-parametric Kruskell-Wallis test with the licensed software package STATISTICA x (Stat Soft Inc., USA.) due to the relatively small number of animal groups and deviations from the normal distribution of variables.

RESULTS

The adrenaline model of myocardial damage was used in the experimental part of studies. The introduction of large doses of adrenaline causes damage to the b-adrenergic receptors of the heart and development of necrotic changes in the myocardium. It leads to a decrease in physical performance (during the research rats demonstrated apathy, loss of appetite, severe inhibition, inspiratory shortness of breath, convulsions, and cyanosis of the mucous membranes).

Degenerative changes, damage to arteries, myocardium, decrease in hypoxia resistance, and increase in heart rate. These damages are so overt that necrotic foci appear in the hearts that are not distinguishable from infarcted ones. This fact has determined the choice of a methodological approach to the study of the cardioprotective properties of glycyrrhizinic acid. When choosing the adrenaline model, we believe that if glycyrrhizinic acid exhibits protective properties under such harsh conditions, then it will undoubtedly have a cardioprotective effect under the other, milder conditions (Perfilova *et al*., 2006; Jiang *et al*., 2012).

The bundles of muscle fibers in the myocardium of intact rats of the control group are located tightly. The blood vessels are slightly congested, and they contain the formed elements of the blood of a typical structure in an optimal ratio. Myocyte nuclei are basophilic, oval, elongated along the long axis of the cell, with a clear karyolemma. Sarcomeres were detected with periodic transverse striation. Reticular and collagen fibers, when stained according to Mallory, were defined as a thin reticulum in the endomysium and the perivascular space (Fig. 1). Thus, the histological picture of the myocardium corresponds to the morphological norm.

Myocardium of rats of experimental groups 24 hours after administration of adrenaline: Extensive inflammatory cell infiltrates (Fig. 1) were found in the first experimental group rats' left ventricular myocardium 24 hours after a single adrenaline injection. Most of the myocytes showed signs of granular dystrophy. Some of them manifested changes in the tinctorial properties of the cytoplasm, karyopyknosis, and karyolysiscardiomyocyte as collision necrosis of cardiomyocytes.

There was perivascular sclerosis against the background of venous hyperemia in the areas of myocardial damage. Twenty-four hours after a single adrenaline injection, muscle fibers in the myocardium of the left ventricle of the second experimental group were of high cellular density. The animals had been previously fed with glycyrrhizinic acid and didn't show any signs of cardiomyocyte necrosis (Fig. 2).

Myocardial infiltration by macrophages and Anichkov cells predominated. Anichkov cells were of a characteristic arrangement of nuclear chromatin in the form of a dentate band, showing phagocytic activity. These cells were often found in the foci of myocardial damage. Some scholars claim that it can be a result of their participation in the regeneration as "myocytes". There was hypertrophy of cardiomyocytes and their polyploidy in the damaged area. The nuclei of cardiomyocytes are basophilic, large, oval, enlightened with a distinct, not blurred karyolemma. The blood vessels are dilated, lumens are free. There were no signs of stasis or thrombosis.

A loose, thin, cobweblike reticulum of collagen fibers was detected in the area of inflammatory cell infiltrates. There were no pathologically expanded spaces associated with hydropic dystrophy.

Myocardium in rats of experimental groups 30 days after the administration of adrenaline: Thirty days after a single administration of adrenaline in rats of the first experimental group, there were numerous areas of myocardial sclerosis in the left ventricular myocardium manifested as the proliferation of perivascular dense fibrous connective tissue.

Foci of extensive focal cardiosclerosis with fatty degeneration of myocytes were detected against this background. That means that fibroplastic processes associated with myocardial remodeling did not fade out in the myocardium upon the expiration of 30 days period.

Areas with dense fibrous connective tissue formed at the site of dead cardiomyocytes were found quite often. Moreover, cardiomyocytes were located in thick bundles of collagen fibers. There were signs of apparent perivascular sclerosis, thickening of the vascular wall due to hyperplasia of smooth muscle cells. Such the state can result in stenosis and reduction of blood vessels.

Thirty days after a single administration of adrenaline to rats of the second experimental group, having been previously fed with the glycyrrhizinic acid, there was loose fibrous connective tissue in the responsive areas of the left ventricular myocardium. Thin streaks of the tissue penetrated endomysium and perimysium that didn't change the functional syncytium structure and vector orientation of the heart fibers. There were no foci of inflammatory cell infiltrate in the myocardium. Fibroplastic processes in the myocardium are currently not observed. There is no reaction from the blood vessels; there were no signs of spasm or dilatation. The formed blood elements were found in the vessel lumen; thrombosis or erythrocyte sludge were not detected (Fig. 3).

We conducted a quantitative assessment of fibroplastic processes in the myocardium in 22 fields of view of each block of each sample (before the formation of a stable dispersion) at a magnification of × 200. The area occupied by collagen fibers in the left ventricle, i.e., in the area most susceptible to pathomorphological changes, was calculated based on the paraffin sections stained according to Van Gieson. Collagen fibers were found to occupy an area of 67,218 ± 19,804 μm2 in the first experimental group when quantifying the degree of fibrosis in the experimental animals' hearts. The collagen fibers' growth area in the second experimental group is 8,019±3,883 μm2 that is 8.4 times less than in the first experimental group.

Results of an immunohistochemical research: Gata 4+ antigen in rat myocardium in the control group was found in perivascular spaces as a part of intramural cells. Antigen to MMP 9+ was determined mainly in the subepicardial space (Fig. 4). TIMP 2+ was detected in myocardial interstitium in endomysium, in the cytoplasm of histiocytes or fibroblasts.

Table 1: Quantity of cells with antigens in responsive areas of the rats' myocardium 30 days after beginning of the experiment

Positively stained cells for antigens	Gata 4+	MMP 9 +	TIMP 2+
Control group	1.35±0.7	0.35±0.4	0.35±0.4
The first experimental group (adrenaline)	19.5±2.9*	1.3±1.5	0.65±0.47
The second experimental group (HA, then adrenaline)	18.05±3.05*	1.05±0.8	9.65±1.1*

Note: the difference is significant when: * - P<0,05.

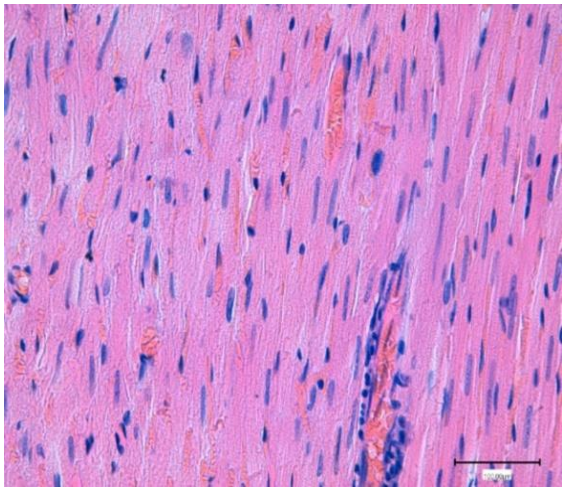

Fig. 1: Myocardium of the left ventricle in the control group rats. The optimal structure of muscle tissue. H&E stain, x200.

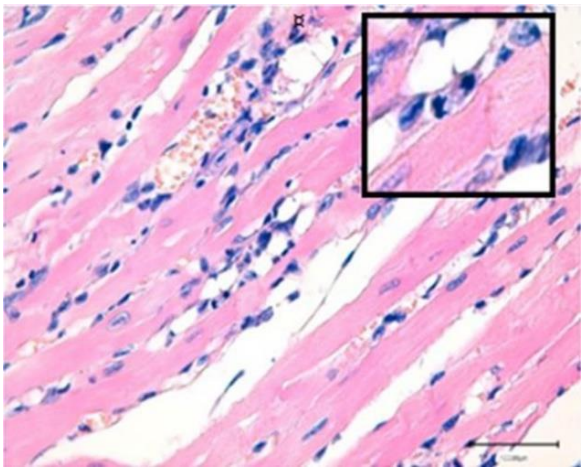

Fig. 2: Myocardium of the left ventricle in the second experimental group rats: day one after the administration of adrenaline. Myocardial infiltration by macrophages, Anitschkow cells. Mitosis of poorly differentiated cells (↑). H&E stain, x200. Insert: x400.

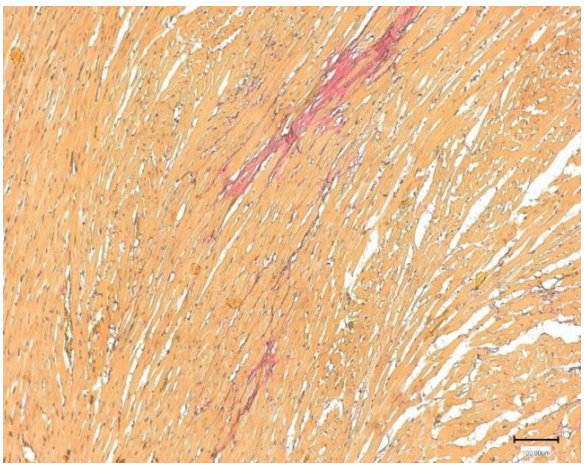

Fig. 3: Myocardium of the second experimental group rats: 30 days after the administration of adrenaline. A. Fuchsinophilic collagen fibers in endomysium and perimisium. Van Gieson's stain, x40.

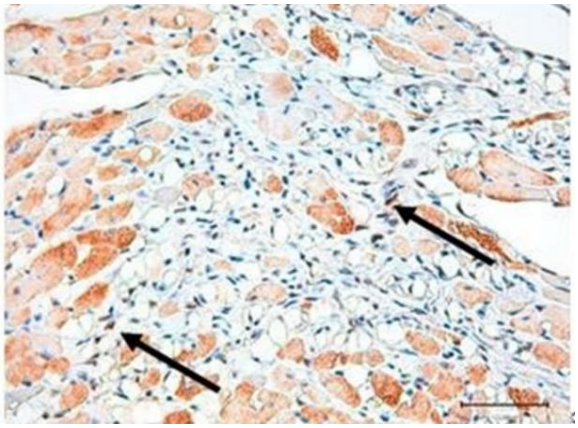

Fig. 4: Myocardium of the first experimental group rats. Gata 4+ cells, detected. Hematoxylin stain, additional application, x100.

In the first experimental group, 30 days after administration of adrenaline, Gata 4+ cells, like in the second experimental group, were detected in the responsive area (Fig. 5). MMP 9+ cells were in single quantities in the remodeling area and the subepicardial space (Fig. 6).

In the second experimental group, where glycyrrhizinic acid was applied, Gata 4+ cells were localized in the subepicardial space and myocardial remodeling foci, i.e., in the areas of granulation tissue, near the blood vessels (Fig. 7) in contrast to the first experimental group rats' myocardium. MMP 9+ cells were detected in the subepicardial space only, and they were not available in the responsive area of the myocardium. TIMP 2+ cells were detected in myocardial interstitium in the remodeling area, and they also concentrated near the blood vessels.

The morphometric assessment of the antigen density in the responsive areas of the rat myocardium, that is, in areas of its remodeling and inflammation 30 days after the adrenaline administration, made it possible to confirm the qualitative assessment of alternative and recovery processes quantitatively (Table 1).

A reliable increase in Gata 4+ cells by 13 and 14 times was detected in the first and second experimental groups, respectively, compared to the control one, but the groups did not differ in this indicator from each other.

No reliable differences between the experimental groups regarding the number of MMP 9+ were revealed as well. As compared to the control group, there was only a slight increase in the number of these cells. In this study, the cytokine MMP 9+ was practically not detected in the responsive area of the myocardium.

It was found in the study of TIMP 2+ cells that their number in the first experimental group did not differ from the control one. The amount of the given cells in the second experimental group reliably increased compared to the control one by 27.5 times. Therefore, glycyrrhizinic acid stimulates the expression of TIMP 2+ cells in the myocardium.

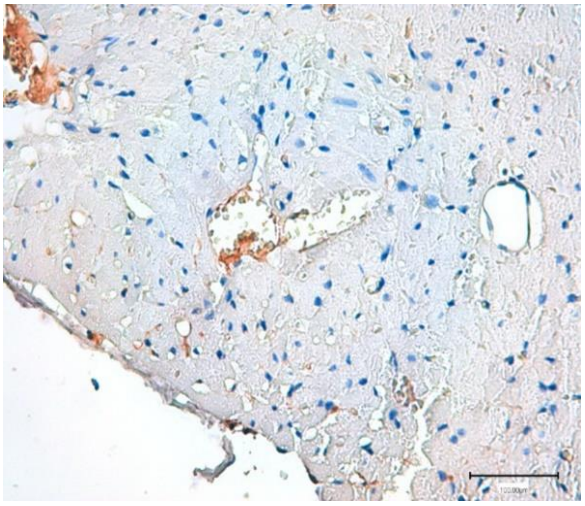

Fig. 5: Myocardium of the control group rats. MMP 9+ cells, detected. Hematoxylin stain, additional application, x200.

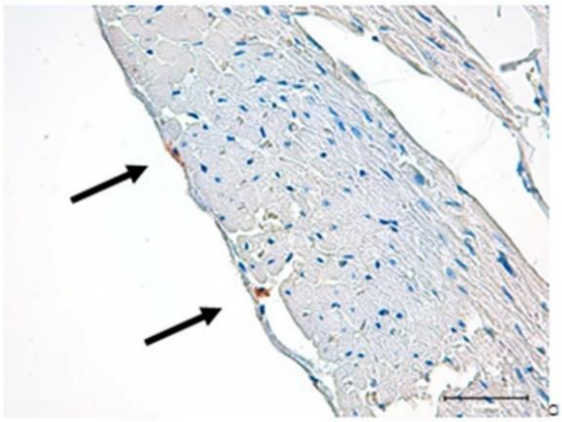

Fig. 6: Myocardium of the first experimental group rats. MMP 9+ cells in the subepicardial space. Hematoxylin stain, additional application, x100.

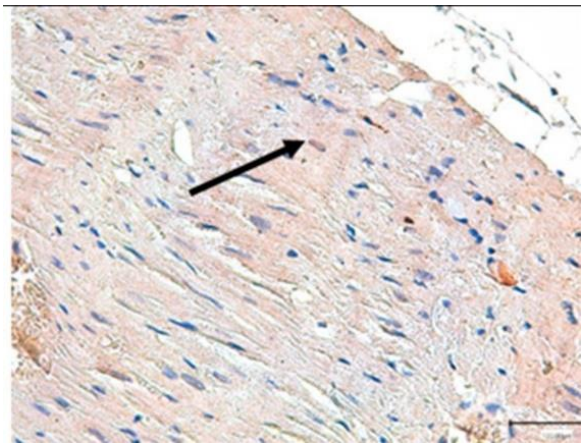

Fig. 7: Myocardium of the control group rats. Timp-2+ cells, detected. Hematoxylin stain, additional application, x200.

The expression of TIMP 2+ is stimulated by stromal cells or cells that are part of the inflammatory exudate after using glycyrrhizinic acid in the second experimental group. In this regard, in addition to the cardioprotective effect of TIMP 2+, the role of cellular cardiomyogenesis is not denied. Since Gata 4+ was found in the responsive area of the damaged myocardium in both experimental groups, but not in the control group, cardiomyogenesis in the second experimental group occurs due to the antiapoptotic effect, cellular and intracellular regeneration, i.e., cardiomyocyte hypertrophy, which is much more preferable for formation of scar in the infarcted areas.

DISCUSSION

Histological and immunohistochemical studies on laboratory animals proved cardioprotective and antiapoptotic effects of glycyrrhizic acid. Other researchers got the same results (Buttros *et al.*, 2009; Di Paola *et al.*, 2009; Akdogan and Sen, 2010; Zhai *et al.*, 2012; Janahmadi *et al.*, 2015; Cai *et al.*, 2017).

According to our data, myocardial infiltration by macrophages and Anichkov cells prevailed in rats of the second experimental group one day after administration of adrenaline. The Anichkov cells are often found in the foci of myocardial damage, as a result of which they are possibly suggested to participate in the regeneration in quality of "myocytes" (Jitesh and Geetha, 2017; Gatiyatullin *et al.*, 2018).

Numerous areas of cardiosclerosis of the left ventricular wall were identified 30 days after the use of adrenaline in the first experimental group. That is, the alterative and fibroplastic processes associated with myocardial remodeling did not decline even after 30 days. Similar findings are described by the other authors, too (Haleagrahara *et al.*, 2011; Ybarra *et al.*, 2015; Ma *et al.*, 2018).

In the second experimental group with prophylactic use of glycyrrhizinic acid, Gata 4 cells were localized in the subepicardial space and foci of myocardial remodeling - in the areas of granulation tissue, near blood vessels. MMP 9+ cells were detected in the subepicardial spaces only and were not available in the responsive area of the myocardium. TIMP 2 cells were detected in myocardial interstitium in the remodeling area and also concentrated near the blood vessels. Whereas in the first experimental group, 30 days after the adrenaline administration, there were single quantities of MMP 9+ and TIMP 2 cells. MMP 9+ cells were in the remodeling area and the subepicardial space, while TIMP 2 cells - in the perivascular space. Similar pictures are described by the other authors, too (Lee *et al.*, 2006; Adamcova *et al.*, 2010).

According to our records, the number of TIMP 2+ cells in the second experimental group increased, compared to the control group, by 27.5 times, and the number of these cells in the first experimental group did not differ from the control group. TIMP-2 possesses the properties inherent in all TIMPs, inhibiting MMPs forming non-covalent complexes with active enzymes. Besides, the TIMP family exerts an antiapoptotic effect, enabling the growth and survivability of the tissue-specific cells (Lluri *et al*., 2008; Anversa *et al*., 2013; Safonov, 2018). Complementary to the cardioprotective effect of TIMP 2+, its role in a cellular cardiomyogenesis is not denied (Gaton *et al*., 2001). Therefore, the glycyrrhizinic acid stimulates an expression with the TIMP 2+ cells in a myocardium.

Conclusions: It should be noted that the morphological studies conducted by us were confirmed by functional intravital methods and ECG (Gatiyatullin *et al*., 2018).

The histological picture of rat myocardium with the use of glycyrrhizinic acid was characterized by signs of stimulation of the cellular and intracellular regeneration that prevents gross myocardial scarring in the damaged areas in the case of the adrenaline model of myocardial damage.

The immunohistochemical study found that glycyrrhizinic acid in the rat myocardium stimulates the increased cell expression with the tissue inhibitor of metalloproteinase-2 (TIMP 2+), which has the antiapoptotic effect. This enables the growth and survivability of cardiomyocytes, which ensures the cardioprotective effect of glycyrrhizinic acid.

Authors contribution: GVB, IRG, ENS, ARS and IRD contributed equally to the experimentation. GVB, IRG and IRD wrote and edited the article. ARS, GVB and ENS similarly designed and experimented. IRG and ENS studied scientific literature about the topic. All authors read and approved the final manuscript. The research was conducted in the laboratory of morphology, pathology, and pharmacy of Bashkir State Agrarian University.

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