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CARDIOPROTECTIVE EFFECT OF ENKEPHALINS UNDER IMMOBILIZATION STRESS

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Objective: The aim of this study was to investigate the cardioprotective effect of dalargin, a synthetic leu-enkephalin.

Methods: The induction of myocardial infarction in rats, which were kept on a diet with excess fat and calcium/sodium salts for two months, by the use of immobilization stress. The experimental results indicated that the applied model allowed to induce the development of myocardial infarction within one three days, which was confirmed by electrocardiography, enzyme-linked immunosorbent assay and histological examination.

Results: Pre-treatment of rats with dalargin had no prevented myocardial infarction, however, it increased the resistance to immobilization stress and reduced infarction-induced myocardial lesions. Simultaneous administration of naloxone, an opiate receptor antagonist, together with dalargin eliminated its cardioprotective effect in experimental animals.

Conclusion: The use of synthetic leu-enkephalin dalargin significantly reduced the risk of myocardial infarction caused by excessive neuromuscular stress. The dalargin effect on the myocardium was mediated by opiate receptors.

Key words: myocardial infarction; immobilization stress; dalargin.

The diseases of cardiovascular system comprise one of the biggest problems of modern medicine. The most common forms of cardiovascular disease include hypertension, atherosclerosis, coronary heart disease and myocardial infarction [1]. There are many common links in the etiopathogenesis of cardiovascular diseases. Thus, the pathogenesis of ischemic heart disease is based on the impaired microcirculation in the heart muscle due to atherosclerotic vascular damage. Myocardial infarction occurs when the level of heart muscle supply does not correspond to the increased functional load on the bloodstream. Myocardial infarction is an acute irreversible ischemia, mostly due to coronary artery thrombosis with insufficient compensatory function of the collaterals, resulting in the necrosis of a certain portion of the heart muscle. All myoglobin-bound and physically dissolved oxygen is consumed during 8–10 seconds in the ischemic area, when the later occurs due to absolute or relative insufficiency of coronary blood flow [2]. The main factors increasing the risk of myocardial infarction are the following: 1) imbalanced nutrition, in particular, with the excess of fats and sodium/calcium salts; 2) increased carbon dioxide concentration and decreased oxygen concentration in the air inhaled by a person; 3) hypodynamia and prolonged stay in a confined space, usually attributed to the occupational activities; 4) infectious diseases; 5) psychological and emotional stress; 6) excessive neuromuscular tension. The efforts of many researchers were aimed at creating therapeutic means and methods that can increase the resistance of the myocardium to these factors in at risk patients (i. e., those having ischemic heart disease, cardiosclerosis). This would enable minimizing myocardial infarction area and reducing the risk of complications, including lethality. A significant number

of biopharmacological studies are focused at the study of cardioprotective properties of biologically active substances of peptide nature, derived from animal and plant tissues and cells [3–6].

Noteworthy, the publications on the cardiotropic properties of low molecular weight peptide fractions derived from tissues of cold-adapted animals are scarce [7–9]. One can assume that the cardioprotective effect of those fractions, at least in part, is due to the presence of biologically active peptides in their composition. It is known that an important role in curbing stress-induced pathological processes is played by anti-stress substances, such as, i.a., endogenous opioids. Their protective effect on the cell is mediated by opiate receptors, which are found in the membranes of cardiomyocytes [10–12]. Regulatory neuropeptides play an important role in the adaptation of animals to environmental factors. Opioid peptides play, in particular, a significant role in the regulation of hibernation. Quite a number of peptides acting as endogenous regulators of seasonal physiological changes in hibernating animals are known: bombesin, opioids, neurotensin, cholecystokinin, and others. The ability to hibernate has evolved in mammals as a special condition of energy preservation in unfavourable environmental conditions. The winter (or, in some species, summer) hibernation is inevitably accompanied by depletion of energy reserves, intracellular acidosis and hypoxia, similar to those that occur in ischemia. However, despite the presence of all those potentially detrimental factors, the myocardium remains undamaged for months. While circulating opioid peptides are known to increase dramatically during deep sleep, they are potential substances which may provide resistance to hypoxia in cells and tissues in hibernating animals. This opens up the prospect of using the protective potential of opioids against ischemia or hypoxia [12, 13]. There is an evidence of participation of such neuropeptides as leu- and met-enkephalins in the maintenance of hibernation [14]. The group of leucine enkephalins also includes the synthetic hexapeptide Lei-Tyr-D-Ala-Gly-Fen-Lei-Arg, known as dalargin [12]. Kyotorphin, a Tyr-Arg dipeptide having an analgesic effect, is considered to be one of the endogenous regulators of the transition of hibernating animals from active wakefulness to hibernation, which is a kind of tolerant strategy of the adaptation to unfavorable environmental conditions [15, 16]. Although kyotorphin is unable or almost unable to interact with opiate receptors directly, it interacts with them indirectly by stimulating the release of methionine enkephalins into the bloodstream where they interact with opiate receptors, [17]. The study of cardioprotective effects of low molecular weight extracts of umbilical cord blood and tissues of newborn animals could also be a promising area of modern biopharmacology; this is supported by the studies of the biological activity of those substances [18–21]. Elevated levels of endogenous opiates (β-, α- and γ-endorphins, leucine- and methionine-enkephalins) are known to be present in the fetus blood at birth, which is considered to be a reaction that protects newborn from the pain and oxygen deficiency, i.e. potentially damaging factors of so-called intranatal hibernation. Therefore, the cardioprotective effect of those extracts and low molecular weight fractions may also be due to the presence of neuropeptides.

According to the aforementioned, the excessive neuromuscular tension is one of the common causes of myocardial infarction in at-risk patients. Given this fact, the aim of this work was to study the cardioprotective effect of enkephalins under conditions of immobilization stress.

Matherials and Methods

In this study we used our own model of myocardial infarction [22]. Provided that the mechanism of human myocardial infarction pathogenesis in almost all cases involves sclerotic changes in the heart muscle and blood vessels [23], rats were pre-kept on a special electrolyte-fat diet until they developed hyperlipidemia and a critical increase in atherogenic index.

The animals were pre-divided by their behavior: low-active — supposed to be highly sensitive to exogenous influences, highly active — supposed to be low-sensitive, and moderately active — supposed to be moderately sensitive [24]; the latter group accounted for the vast majority of tested animals and were selected for the further experiment. To that end, 60 *Wistar* rats at the age of 6 months weighing 240–270 g had been selected. Animal fats in the amount of 10% of the total feed weight were added to the standard diet for rats. Instead of water, 0.9% NaCl solution was poured into the drinkers, thus provoking excessive consumption of sodium by animals, which is harmful to the cardiovascular system. The rats received 20 mg of calcium

gluconate (PJSC "Kyivmedpreparat", Ukraine) orally per animal per day. Vitamin D_2 was also administered at 2,000 IUs per animal (PJSC "Vitamins", Ukraine). Normally, ergocalciferol favors the absorption of calcium by bone tissue, but in conditions of hypervitaminosis it causes excessive calcium accumulation in the blood [25]. There is a known positive association between high serum calcium levels and the incidence of cardiovascular diseases, including myocardial infarction [26, 27]. In particular, the risk of coronary artery calcification increases, which contributes to the formation of atherosclerotic plaque. It was also borne in mind that systemic inflammation may be one of the reasons for the initiation of proatherogenic modification of blood lipoproteins, and vascular calcification [28]. To induce the systemic inflammatory process during the 8^{th} week animals were twice injected intraperitoneally with the bacterial lipopolysaccharide "Pyrogenal" (N. F. Gamaliia RIEM, Russia) with the interval of three days, at a dose of 5 μg of dry weight of the substance per animal. The development of systemic inflammation was monitored by the rectal temperature at a depth of 1 cm. Within three days after the second injection of «Pyrogenal», a steady increase in body temperature, by approximately 1.5 degrees (39.2–39.7 °C), was registered in all rats. During the implementation of the model in rats, their blood samples were periodically collected from the tail vein to study the lipid profile. Starting from the 58^{th} day of the experiment, the stressful impact was applied on the rats. To that end, animals were subjected to immobilization stress, by immobilizing the four limbs and lower jaw in the abdominal position for 3 hours daily, with the break of 24 hours.

The animals were divided into 6 groups. Group 1 consisted of animals kept on a standard balanced diet (normal group), and group 2 consisted of the rats, in which the state of blood hyperlipidemia was reproduced by keeping them on a special diet. Animals from groups 1 and 2 were not subjected to immobilization stress. The immobilization stress to the group 1 rats was not applied due to the fact that in those completely healthy animals the risk of myocardial infarction due to the stress was much lower than in rats from the other groups, and therefore the duration of stressful impact should be many times longer. This could lead to the death of the animals due to the stress-induced ulcers of the stomach and intestines. Rats of groups 1 and 2 were used to determine the serum content of cardiac troponin I in normal (group 1) and in hyperlipidemic animals, i.e. before the applying of the stressful impact (group 2). In addition, rats from group 2 were used for histological examinations of the impact of the pathogenic diet only (without immobilization stress) upon the myocardium state. Rats from group 3 were injected with 200 μl of 0.9% NaCl immediately before the immobilization. Group 4 consisted of rats, which received dalargin in the form of 0.25% solution of the pharmaceutical substance ("Peptidnyie Tekhnologii", RF) at a dose of 100 μg/kg body weight immediately before the immobilization. Group 5 consisted of rats, which, together with dalargin, were injected with naloxone, an inhibitor of opiate receptors, in the form of naloxone hydrochloride ("Sigma-Aldrich", USA) at a dose of 500 μg/kg body weight. Groups 1–3 included 10 rats each; groups 4 and 5 included 5 rats each. An electrocardiogram was registered in rats immediately after immobilization, and every hour during the immobilization and before the release of the animal. The cardiography used three standard (I, II, III) and three augmented (aVR, aVL, aVF) leads. Cardiograms were analyzed for abnormalities indicative of the occurrence of acute cardiovascular pathology. Such deviations were as follows: the appearance of a negative Q-wave, a significant decrease or absence of the R-wave (QrS or QS complexes), displacement of the ST complex relative to the isoline, high-amplitude «coronary» T-waves. The detection of these abnormalities in several leads was considered as the reason for a preliminary diagnosis of acute coronary syndrome, and the stressful impact on the animal was stopped. Eighteen hours later, blood sample was collected from the rat's tail vein, and serum was prepared from it, in which the content of cardiac troponin I was determined by enzyme-linked immunosorbent assay using the "Rat Cardiac Troponin I (cTn-I) ELISA kit" (Cusabio Technology LLC, USA). The increase of the serum cardiac troponin I in rats, that was at least 2-fold higher than the predetermined average value for the animals kept on a pathogenic diet but not exposed to immobilization stress (group 2) provided a basis for the diagnosis of a myocardial infarction. After that, the animal was excluded from the experiment by decapitation under ether anesthesia, its heart was removed and fixed in 10% neutral formalin (pH 7.4), followed by tissue processing and embedding in paraffin. Histological sections of 5–7 μm thickness

were cut and stained with hematoxylin and eosin [29]. Slide mounts were photographed and analysed using an "Axio Observer Z1" microscope (Carl Zeiss, Germany) in bright field mode. For comparison, histological samples were analyzed from the hearts of animals kept on pathogenic diet but not exposed to immobilization stress.

Experimental data were processed statistically using the statistical module of the "Excel" software (Microsoft, USA). Differences between the samples were estimated using Student's two-sample t-test. The data were presented as «sample mean ± sample standard deviation» ($\bar{X} \pm s$).

Results and Discussion

The results published earlier [21] showed that rats kept on a diet rich in fats and sodium/calcium salts for two months developed a hyperlipidemia. The level of total cholesterol in the blood from rats used for the heart infarction modeling exceeded that in animals kept on a standard diet by almost a quarter (3.32 ± 0.17 vs. 2.67 ± 0.19), and the content of low-density lipoprotein was almost three times higher (1.34 ± 0.09 vs. 0.49 ± 0.08). As a result of such changes in the lipid profile, the atherogenic index was twice as high as normal (1.84 ± 0.35 vs. 0.91 ± 0.13).

During the first minutes of immobilization stress the cardiograms of all animals without exception showed signs of severe tachycardia. Analysis of the cardiograms of animals from the control group (group 3) 1 hour after the onset of stressful impact revealed the changes in the width and amplitude of the T-wave in 9 out of 10 animals. In five rats, the T-wave was reduced or smoothed, and in four rats it was biphasic. Such changes in the cardiogram indicate the occurrence of myocardial ischemia.

Two hours after immobilization, the cardiogram of one of the rats showed a negative Q-wave in combination with a positive high-amplitude "coronary" T-wave (a sign of transmural infarction), on the basis of which a preliminary diagnosis of "acute coronary syndrome" was made, and this animal was released and transferred to a cage. Six animals had T-wave changes: four rats showed a negative symmetrical "coronary" T-wave, and two others had elevations above the ST-segment isoline. These changes could indicate unstable angina, myocardial damage, or even necrosis, so the immobilization of those animals was stopped. Cardiograms of other animals had signs of myocardial ischemia (T-wave was broadened and lowered, and was smoothed in one rat). An hour later, another rat was pre-diagnosed with acute coronary syndrome. The reasons were the same as in the first case: a negative Q-wave in combination with a positive high-amplitude "coronary" T-wave. No changes allowing to diagnose acute coronary syndrome were detected in the cardiograms of the two other rats, and after 3 hours of immobilization stress they were returned to the cages (after the application of the stress, all rats were kept in separate cages), and the next day they were repeatedly subjected to immobilization stress. However, the QS complex (with negative Q- and no R-wave) was detected in the cardiogram of one of those rats, which occurred in the first minutes after immobilization. That rat was released, and its blood was immediately tested for troponin I. In the remaining animal, the pathological changes in the cardiogram, which allowed to pre-diagnose an acute coronary syndrome (negative symmetric «coronary» T-wave) were detected three hours after the onset of the repeated immobilization stress.

Administration of dalargin to rats significantly increased the resistance of their hearts to excessive neuromuscular load, as evidenced by the increased duration (by 1.5–2 times) of their ability to resist the immobilization stress before the acute coronary syndrome was registered (Fig. 1). Simultaneous administration of dalargin and naloxone, the opiate receptor antagonist, to the animals (group 5) eliminated the protective effect of the leu-enkephalin in that test. This was evidenced by both the reduction of the time during which animals from those groups were able to resist immobilization stress until they were preliminary diagnosed with an acute coronary syndrome (Fig. 1); and the increase in cardiac troponin I in the blood to the levels found in control (group 3) animals. Micromorphological analysis also supported these findings (see below).

In the group of rats treated with dalargin before the onset of stress impact (group 4), the acute coronary syndrome was diagnosed in two cases during the second immobilization stress, and in three cases during the third immobilization stress. Preliminary diagnosis was based on the following abnormalities in the cardiograms: negative Q-wave in combination with positive high-amplitude "coronary" T-wave (3 cases), or lowered R-wave and the elevation above the ST segment isoline (2 cases).

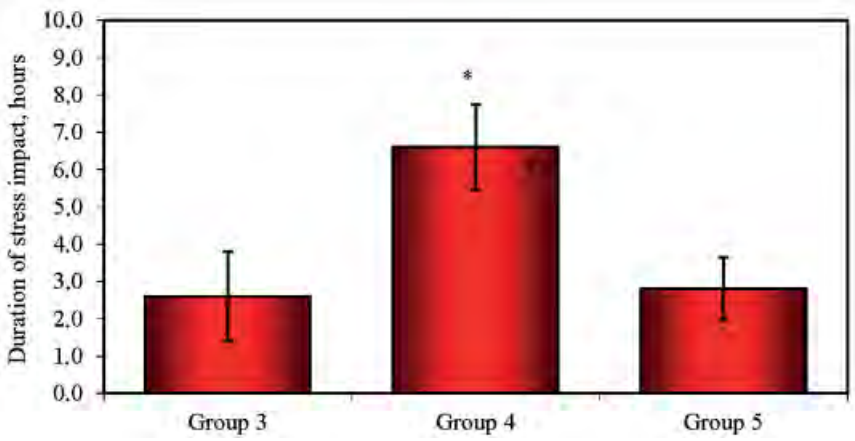

Fig. 1. **The effect of dalargin and naloxone on the total duration of rats resisting the impact of immobilization stress until the moment of registration of acute coronary syndrome signs in the cardiograms**
Note: * — statistically significant differences compared to the group 3 and group 5, $P \leq 0{,}01$.

It is known that QS and QrS cardiogram complexes may indicate large focal transmural myocardial infarction. Sera cardiac troponin I levels (Fig. 2) in the rats from all groups which were preliminarily diagnosed with acute coronary syndrome were significantly increased in all animals without exception, as compared to both the intact animals and animals kept on a pathogenic diet but not exposed to stress. This allowed us to refine the diagnosis and suppose that those animals had suffered a myocardial infarction (the preliminary diagnosis was an "acute coronary syndrome"). It should be noted that animals that were not exposed to stressful impact after being kept on a pathogenic diet also had a significant increase in this indicator compared to intact animals, but those changes were much less pronounced (16.2 ± 1.9 vs. 6.62 ± 0.29, $P \leq 0.05$). Lower (38.19 ± 1.09 vs. 54.08 ± 4.01; $P \leq 0.05$) levels of cardiac troponin I in the blood of rats injected with dalargin (group 4), compared with animals from the control group (group 3) may indicate a smaller heart damage.

Histological examination of heart tissue showed that keeping animals on a diet with high fats and calcium/sodium salts caused changes in the myocardium and blood vessels which increase the risk of myocardial infarction. Thus, the microphotographs clearly demonstrated the signs of myocardial dystrophy in the form of small intermittent groups of slightly hypertrophic or atrophic cardiomyocytes (Fig. 3, *a*). Myocardial muscle fiber bundles were thinned due to the atrophy, or demonstrated a wavy deformation. Cross sections of some cardiomyocytes showed that the cytoplasm contained small round whitish vacuoles which were similar to fat droplets. The areas with poor blood supply, and the foci of venous-capillary congestion occured intermittently. It could be seen that large and small intramural coronary arteries had uneven wall thickening due to mild coronary sclerosis and initial stages of perivascular cardiosclerosis. Clusters of lymphocytes around blood vessels, and focal infiltration of the myocardial stroma were also detected. Apparently, both atherosclerotic and myocardial forms of cardiosclerosis were present, as injection of bacterial liposaccharide ("Pyrogenal") induced systemic inflammation in the animals.

Because the aforementioned pathological abnormalities in the cardiograms in rats from all groups occurred in the leads I and aVL, and in their reciprocal leads III and aVF, those findings indicated the infarction located in the lateral wall of the left ventricle and the thrombosis of the anterior descending artery.

Histological examinations confirmed that all rats subjected to immobilization stress had a large focal transmural myocardial infarction. Myocardial tissues demonstrated similar pathological changes: focal hemorrhages, congested vessels, loss of striated muscle fibers (individual muscles took the form of so-called "minced meat"), and vascular thrombosis (Fig. 3, *b*, *c* and *d*). Necrotic muscle cells in the infarction area lacked nuclei. Blood flow in the necrotic area was completely absent, and congestion in the veins and sinuses, and blood stasis in the capillaries were registered in peri-infarct zone. Areas of intermuscular hemorrhage have been found in many tissue samples. A small focal parenchymal dystrophy of cardiomyocytes, and clusters of lymphocytes

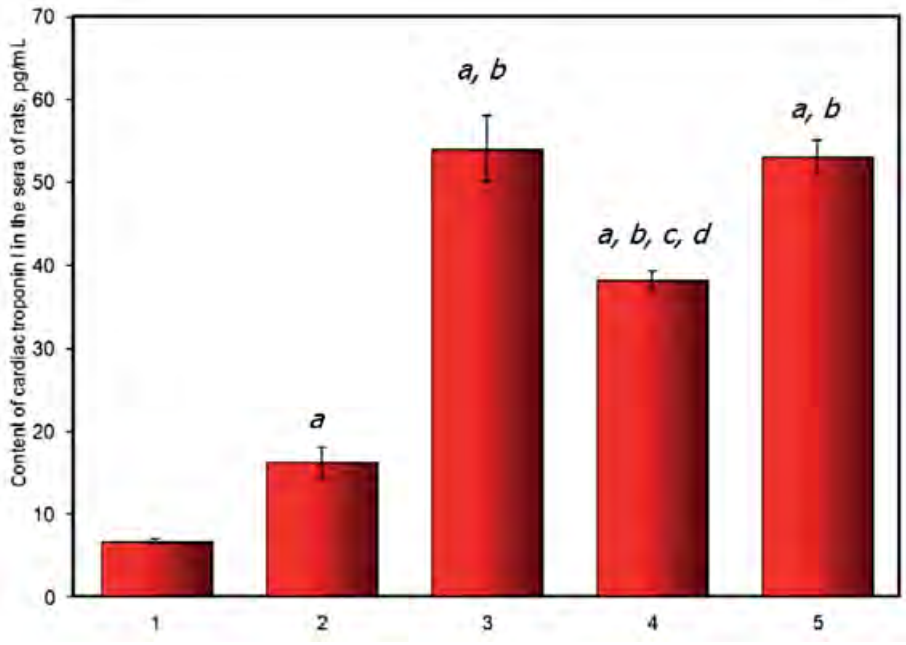

Fig. 2. **The content of cardiac troponin I in the sera of rats**

Notes:

1) Numbers on the abscissa denote groups of experimental animals;

2) *a* — statistically significant differences compared to the group 1, $P \leq 0.01$; *b* — statistically significant differences compared to the group 2, $P \leq 0.01$; *c* — statistically significant differences compared to the group 3, $P \leq 0.01$; *d* — statistically significant differences compared to the group 5, $P \leq 0{,}01$

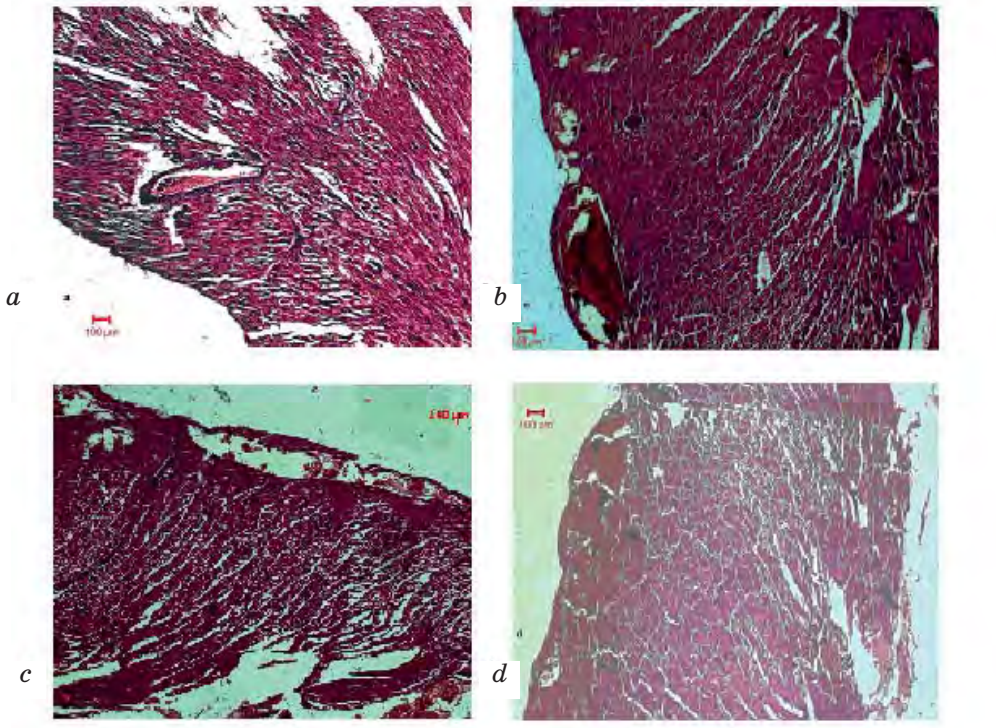

Fig. 3. **Left ventriclular tissue sections from rat hearts, stainined with hematoxylin-eosin:**

a — the animal was on a pathogenic diet, but not exposed to immobilization stress (group 2); *b* — the animal with developed myocardial infarction as a result of immobilization stress (group 3); *c* — the animal received dalargin before the application of immobilization stress (group 4); *d* – the animal received naloxone and dalargin simultaneously (group 5)

were seen. In the samples from rats which didn't receive dalargin (Fig. 3, *c*) before the onset of immobilization stress, or received dalargin and naloxone simultaneously (Fig. 3, *d*), the organized thrombi in epicardial coronary artery and small subendocardial arteries were clearly visible, as well as the destruction of cardiomyocytes in the infarction area, and formation of collaterals in peri-infarct zone. In the rats which received dalargin without concomitant administration of naloxone prior to immobilization stress (Fig. 3, *c*), the resorptions and recanalizations of the thrombi in epicardial coronary artery and in individual small subendocardial arteries were significantly more frequent. The collaterals in the peri-infarct zone were also formed oftener.

Many previous studies have shown that dalargin acts on the cells through opiate receptors [30, 31]. Therefore, the inhibition of this opiate receptor agonist by naloxone, an opiate receptor antagonist, was rather predicted.

Thus, based on the results obtained, we can conclude that enkephalins possess a cardioprotective effect. The use of dalargin, a synthetic leu-enkephalin, significantly reduced the risk of myocardial infarction due to excessive neuromuscular stress.

Animal studies were performed in accordance with the Law of Ukraine "On Protection of Animals from Cruel Treatment" (№3447-IV of 21.02.2006) in compliance with the requirements of the Institution's Bioethics Committee, which are consistent with the provisions of "The European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Strasbourg, 1986), as well as the recommendations "Bioethical expertise of preclinical and other scientific studies conducted on animals" (Kyiv, 2006).

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КАРДІОПРОТЕКТОРНА ДІЯ ЕНКЕФАЛІНІВ ЗА УМОВ ІММОБІЛІЗАЦІЙНОГО СТРЕСУ

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Мета. Вивчення кардіопротекторної дії синтетичного лей-енкефаліну даларгіну.

Методи. Розвиток інфаркту міокарду у щурів, яких протягом двох місяців утримували на дієті з надлишковим вмістом жирів та солей кальцію і натру, ініціювали шляхом застосування іммобілізаційного стресу. Результати експериментів свідчать про те, що застосована модель дозволила протягом одного-трьох днів викликати розвиток інфаркту міокарда, який було підтверджено методами електрокардіографії, іммуноензимнгого аналізу та гістологічним.

Результати. Попереднє введення піддослідним тваринам даларгіну, хоча й не запобігало інфаркту, підвищувало витривалість до впливу іммобілізаційного стресу та зменшувало ступінь ураження міокарда. Введення піддослідним тваринам разом з даларгіном антагоніста опіатних рецепторів налоксону усувало кардіопротекторну дію.

Висновки. Використання синтетичного лей-енкефаліну даларгіну істотно зменшує ризик інфаркту міокарду внаслідок надмірного нервово-м'язового навантаження. Дія даларгіну на міокард опосередкована опіатними рецепторами.

Ключові слова: інфаркт міокарда; імобілізаційний стрес; даларгін.