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# Low-Intensity Laser Radiation as a Modulator of Physiological Regeneration in Hyperadrenalemia.

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**ABSTRACT**

The low intensity laser radiation (LILR) is successfully used in clinical practice and nowadays is increasingly applied in regenerative medicine. Regeneration is closely associated with the processes of inflammation, in the pathogenesis of which the microcirculation disorder is one of the essential stages. On this point, studding of the erythrocytes morphology and their membrane structure under the action of LILR on the background of the alteration of the organism is of scientific interest and practical importance. The aim of the study was to analyze LILR effect on morpho-functional parameters of erythrocytes and adrenal glands in hyperadrenalemia. The dynamics of the morphometric parameters of erythrocytes was studied by the method of phase interference microscopy. The electrophoretic mobility was analyzed by method of microelectrophoresis. The intensity of lipid peroxidation was characterized with malonic dialdehyde accumulation in cells. The histomorphological analysis of the adrenal glands was performed. LILR reduced the severity of the adrenaline effect on erythrocytes, which was manifested in a decreased amount of the pathological forms of the red blood cells, higher erythrocytes electrophoretic mobility and lower level of lipid peroxidation. The histological analysis of the adrenal tissues showed a protective LILR effect against destructive adrenalin action. The mechanism of LILR action is realized at both cellular level through the laser radiation effect on erythrocytes membranes, and systemic level through the activation of stress-realizing systems of the organism with subsequent limitation of inflammatory response.

**Keywords**: low intensity laser radiation, phase interference microscopy, erythrocytes, adrenal glands, damage to the body.

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## INTRODUCTION

The low intensity laser radiation (LILR) is successfully used in clinical practice as an antiphlogistic, analgesic and regenerative agent. These effects of LILR are explained by its capability to affect different cell regulatory systems. Thus LILR can induce ATP production and changes in cytochrome-c-oxidase activity [1, 2], also it can regulate nitrogen oxide synthase (iNOS) level [3] and protein kinase C activity [4]. Finally, it can activate porphyrins and their derivatives with further ROS production [5], which leads to tumor cell growth inhibition and cell death [6]. LILR-induced redox dependent signaling modulates expression of proinflammatory cytokines [7, 8] and increases activity of heat shock proteins [9].

Nowadays LILR is developed as an antiphlogistic treatment combined with mesenchymal stem cells (MSC) therapy. MSC therapy is proved to be a promising strategy that improves tissue regeneration but its effectiveness is considerably limited by inflammatory microenvironment. LILR (λ = 660 nm, 4-8 J/cm2) is shown to inhibit the lipopolyssacharide-induced TLR-dependent cytokine (IL-1β, IL-6, IL-8) production [10].

To date there is no generally accepted concept about mechanisms of LILR effect on biological objects. Moreover organism susceptibility to laser exposure of intensity lower than 1 mW is doubted. It is considered that low intensity radiation does not derive any detectable functional changes in a healthy organism, so such changes may be manifested only in a damaged organism [11].

Any damage of the organism is accompanied by the inflammatory reaction carried out by a dynamic self-adaptive control system with stereotypic kinetics, which is independent on the damaging factor (mechanical or thermic trauma, circulatory disorders, infection, endotoxins or exotoxins), though the damaging factor may determine the reaction peculiarities [12]. The inflammatory reaction is followed by regeneration processes. Mechanisms of inflammation and reparative regeneration are evolutionarily related to the mechanisms of physiological inflammation and physiological regeneration, and both are typical protective-adaptive processes which occur in the absence of any complications in uncomplicated cases.

The disorder of microcirculation including the blood rheology is one of the most considerable stages in the inflammatory process [13]. The inflammatory develops through ischemia-reperfusion cycle with microcirculation disorder. Shortening of the ischemia stage duration by any means will improve disease outcome. Therefore, an analysis of red blood cells (RBC) status seems to be relevant because of erythrocytes capability to transport oxygen and affect rheological properties of the blood. It should be taken into account that trauma, hemodynamic disorder and pain syndrome provoke stress of various intensity leading to the transitory hyperadrenalinaemia. In addition, brain tumor or inflammation predominantly in diencephalic areas, which affect the process of adrenalin secretion, pheochromocytoma and adrenocortical insufficiency may be accompanied by hyperadrenalinaemia. Therefore the purpose of this study was to analyze the influence of LILR on morphofunctional parameters of RBC and adrenal glands in case of hyperadrenalinaemia.

## MATERIALS AND METHODS

40 white non-pedigree pubescent rats weighing 180-200g were examined. Adrenaline toxemia was created with intraperitoneal injection of adrenalin hydrochloride (0,1 mg/kg). Animals were assigned into 4 groups with the following treatment schemes: group 1 (“adrenaline group”) received adrenalin injection (0,1 mg/kg), group 2 (“adrenaline-LILR group”) received the same injection and after 30 min were exposed to LILR, group 3 (“LILR group”) received no injection but was exposed to LILR, and group 4 (“intact group”) was comprised by control intact animals. The research were carried out according to principles established in Universal Declaration of animal rights as well as according to the Order of the Ministry of the Health of Russia №119n of April 1, 2016 “Approval of the suitable laboratory practice”. The study was approved by the Commission on Bioethics of Lobachevsky University.

The rat placed in an open chamber was exposed with LILR at the occipital region for 10 minutes from a distance of 2-5 mm. The therapeutic laser apparatus “Uspekh” (“Voskhod”, Russia) working on pulse frequency 415 Hz at 0,8 0,9 µm wavelength (minimum value of average power density in the plane of the output window was 193 µW/cm$^2$) was used as a LILR source.

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The cell morphology was studied with laser interference microscope MIM-340 (Ekaterinburg, Russia) equipped with the 30× objective (NA=0.65) and laser at 650 nm with power not exceeding 2 mW. The frame size was 195×145 µm. Images were captured with CCD camera VS-415U (NPK Videoscan, Russia) with resolution 782×582 pixels. The total acquisition time for one image was 10 seconds. Reconstruction of the phase images was carried out using the phase step method (WinPhast software, USA), further image processing was performed with FIJI and Microcal Origin software (Microcal Inc., USA). At least 100 cell were analyzed to study RBC morphology in every sample.

The intensity of lipid peroxidation was characterized by presence of malondialdehyde MDA in RBC [14]. The MDA concentration was measured by method based on its ability to react with two molecules of thiobarbituric acid at 90-100°C with formation of colouredtrimethine complex.

The RBC electrophoretic mobility was determined by micromethod [15], recording the time of RBC passing 100 µm distance in Tris-HCl buffered solution, pH 7.4, at a current of 8 mA. RBC electrophoretic mobility value was defined using the equation: $U= S/T \cdot H$, where S distance to which the cells moved, T time, H– gradient of electric potential. The value of potential gradient was determined from the the equation: $H=I/g \cdot \chi$, where I – amperage, g – chamber cross section, $\chi$ – electrical conductivity of the media.

The histologic specimens for optical microscopy were prepared by fixing in 10% neutral formalin solution for 72–96 hours, dehydration in alcohols with increasing concentration and subsequent paraffin-embedding. The 7 µm thick slices were obtained with SM2000R microtome (Leica, Germany) and stained with haemotoxylin and eosin. Specimen examination was carried out with DM1000 microscope (Leica, Germany), video images were captured using DFC290 camcorder (Leica, Germany).

Statistical analysis was performed using ANOVA (BIOSTAT software). The differences were considered to be significant at $p < 0,05$.

## RESULTS AND DISCUSSION

Morphometric studies of RBC surface topography showed typical discocyte profile and uniform distribution of intracellular structures in a group of intact animals (Fig.1). The RBC membrane had a smooth surface. After adrenalin administration with or without LILR exposure the changes of phase height of the cells were detected that reflects various physiological processes in the cells. It was noticed that the decrease of discocyte quantity correlates with increasing rates of reversibly and irreversibly changed RBCs (Table 1) The typical 3D phase portraits of the cells and results of the cell measurements are shown in figures 1-3.

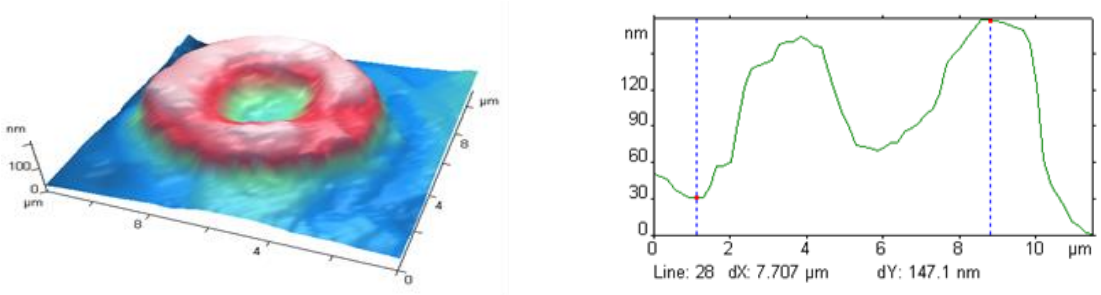


**Fig 1:3D phase portrait of a discocyte. Phase height of the discocyte is 147 nm, diameter is 7.8 µm.**

In the “adrenaline-LILR” and “adrenaline” groups the RBC morphological changes one hour after adrenaline administration were represented by clearly defined poikilocytosis. Also increasing rate of RBC pathological forms predominantly echinocytes (Fig.2) was registered. RBCs had a heterogeneous phase height along the cell perimeter. Comparison of RBC profiles in the intact and experimented groups indicated that the RBC phase height in treated groups is more significant than the reference value.

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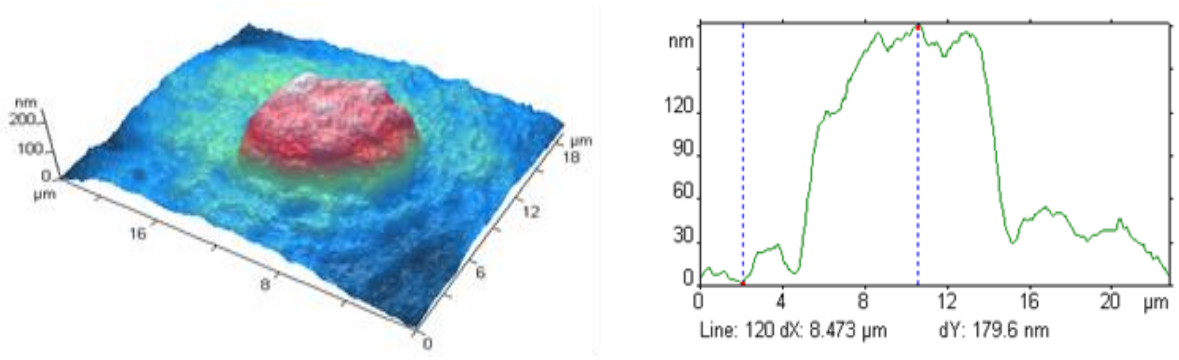


**Fig 2: 3D phase portrait of an echinocyte. Phase height of the echinocyte is 179 nm, diameter is 8.6 µm.**

Relative predominance of stomatocytes in the "adrenaline-LILR" group was demonstrated one hour after alteration in comparison to group which received only adrenaline injections (Table 1). However after a day this ratio was reversed though the total quantity of pathologically changed erythrocytes continued to increase in both groups. Phasometric images of RBCs in "adrenalin" group revealed that increased rates were detected not only for stomatocytes but also for echinocytes represented mainly by spheroechinocytes (Fig. 3). At the same time "adrenaline-LILR" group showed predomination of the types I and II echinocytes.

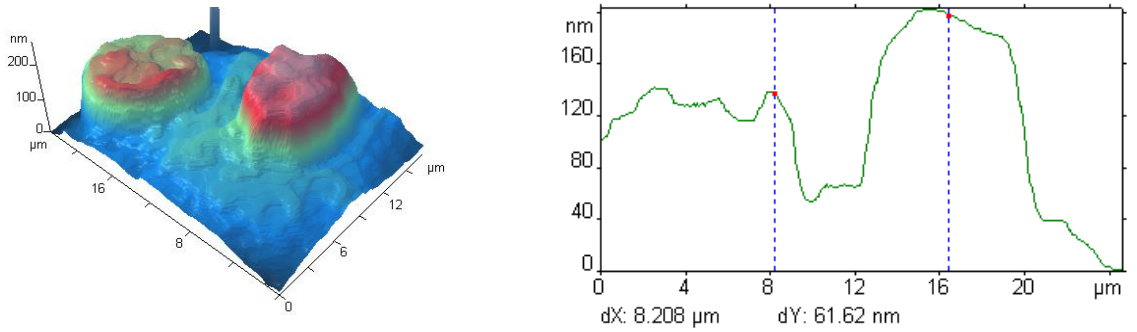


**Fig 3: 3D phase portrait of echinocyte and spheroechinocyt. Phase height of the echinocyte and spheroechinocyt are 74 nm (left) and 132 nm (right), diameter is 8.6 µm, diameters of 9.1 µm and 7.13 µm respectively.**

After a week the morphometric characteristics of RBCs in the "adrenaline-LILR" group returned to ones the intact group (Table 1), and at the same time in adrenalin administered group an increased quantity of small-diameter spherocytes remained (Fig.4). LILR itself did not derive any considerable changes in the erythrocyte morphology, though comparison with intact animal group indicated a slight increase in discocyte quantity due to decrease in fractions of echinocytes, stomatocytes and degenerative changed erythrocytes (Table 1).

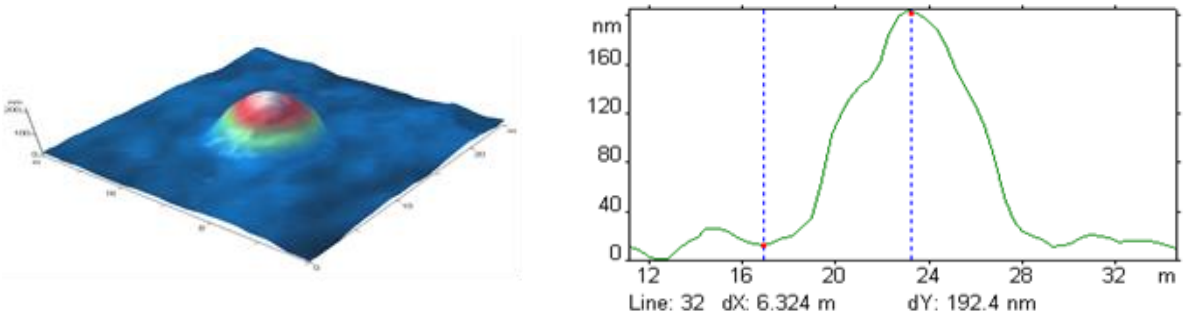


**Fig 4:3D phase portrait of a spherocyte. Phase height of the spherocyte is 192 nm, diameter is 6.5 µm.**

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**Table 1: The morphology of RBCs in blood smears of intact and altered rats (%).**

| Group | Time after treatment | The morphology of RBC | | | |
|---|---|---|---|---|---|
| | | Discocytes | Echynocytes | Stomatocytes | Degenerative |
| Intact | 1 hour | 86.0±0.7 | 11.0±0.7 | 2.5±0.3 | 0.5±0.3 |
| | 1 day | 83.5±0.3 | 13.0±0.7 | 2.5±0.3 | 1,0±0 |
| | 1 week | 82.5 ±1.8 | 12.5±0.4 | 3.5±1.1 | 1.5±0.4 |
| LILR | 1 hour | 85.0±0.7 | 11.0±0.2 | 3.0±0.7 | 1.0±1.0 |
| | 1 day | 83.0±0.4 | 14.0±0.2 | 2.0±0.7 | 1.0±0 |
| | 1 week | 86.5±0.3 | 10.5±0.3 | 2.5±0.4 | 0.5±0.4* |
| Adrenalin | 1 hour | 36.5±2.5* | 49.0±2.1* | 10.0±0.2* | 4.5±0.3* |
| | 1 day | 49.5±2.6* | 37.0±2.4* | 10.0±1.4* | 3.5±0.4* |
| | 1 week | 77.0±1.4* | 13.5±0.3 | 7.0±0.7* | 2.5±0.3* |
| Adrenalin + LILR | 1 hour | 28.0±2.1* | 54.5±1.6* | 13.5±1.1* | 4.0±0.2* |
| | 1 day | 57.5±2.6* | 32.0±2.8* | 6.5±1.1* | 4.0±1.4* |
| | 1 week | 82.5±1.4 | 11.5±0.7 | 5.0±0.7 | 1.0±0.3 |

* statistically different from intact group, $p<0.05$.

The increase in the percentage of pathological forms of RBCs in adrenalin administered groups may be explained by changes in membrane structure derived by receptor binding to stress-realizing agent (adrenalin). High level of catecholamines and erythrocyte adrenore activity lead to the Na+/K+-ATPase inhibition [16], which in its turn causes calcium accumulation in erythrocyte cytosol, which underlies membrane lipid domains disorder. Also changes in RBC-membrane electrophoretic band composition such as decrease in relative amount of proteins in bands 3,6,7 and increase in proteins of band 2.3 were registered [17, 18]. These changes drive membrane structural and functional disorders.

LILR effect may be caused by photostimulation due to photon absorption by chromatophores [19] and molecular oxygen. Resultant primary formation of reactive oxygen species (ROS) drives defensive mechanisms such as reactivation of metal-containing antioxidant enzymes [20]. Superoxide dismutase (SOD) and catalase are able to intercept ROS which promote inflammatory response. The obtained data on LILR involvement in RBCs response to adrenalin treatment are in agreement with the reported effects of LILR. We suppose that LILR initially activates the oxidation processes in adrenalin treated group leading to more pronounced erythrocyte modification manifested in stomatocytes formation. Then, the antioxidant processes are activated, and the shape of the cells return to normal, with decrease in amount of echinocytes and spherocytes.

The hypothesized mechanism of LILR effects is confirmed by studies of the MDA concentration and RBC electrophoretic mobility. MDA concentration growth was registered in the adrenaline administered group for the whole period of observation, whereas the LILR exposure provoked the MDA level decrease. LILR exposure in combination with adrenalin injection determined a progressive decrease of MDA concentration after initial accumulation. Similar effect was shown for RBC electrophoretic mobility. The electrophoretic mobility in adrenaline administered group decreased up to the end of the experiment. The reverse effect was registered in LILR-exposed group. RBC electrophoretic mobility in “Adrenaline+LILR” group was characterized by two phases: a decrease followed by an increase (Table 2).

An increase in RBC negative charge determines rheological blood properties and microcirculation improvement. In respect that high level of RBC aggregation and subsequent blood viscosity augmentation are an important integral effect in acute adrenalin intoxication, LILR may be considered as a corrective agent in hyperadrenalemia.

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**Table 2: MDA concentration and RBC electrophoretic mobility in studied groups**

| Index | Period after adrenalin injection | Intact | LILR | Adrenalin | Adrenalin + LILR |
|---|---|---|---|---|---|
| RBC electrophoretic mobility, μm cm $B^{-1}c^{-1}$ | 1 hour | 1.23 ± 0.06 | 1.59±0.08*# | 1.12 ± 0.04* | 1.15 ± 0.08* |
| | 24 hours | 1.32 ± 0.06 | 1.46 ± 0.07# | 1.10 ± 0.06* | 1.41 ± 0.02# |
| | 1 week | 1.22 ± 0.07 | 1.54 ± 0.05*# | 1.07 ±0.05* | 1.36 ± 0.09# |
| MDA,nmol/ml | 1 hour | 1.29 ±0.22 | 0.48 ±0.61*# | 4.14±0.75* | 4.45 ±0.37* |
| | 24 hours | 1.49 ±0.44 | 1.64 ±0.26# | 3.41 ±0.81* | 3.04 ±0.19* |
| | 1 week | 2.09 ±0.53 | 1.69 ±0.67# | 3.61±0.28* | 2.79 ±0.25# |

* - statistically different from "Intact" group, p< 0.05
# - statistically different from "Adrenalin" group, p< 0.05.

Our findings suggest that LILR can have both a direct effect on blood cells and indirect effect on neuroendocrine brain structures. For example, the RBC electrophoretic mobility is not only an indicator for RBC electrokinetic potential and accordingly the electronegativity of cells, but it is also a marker of the organism stress reaction [21]. The decrease of RBC electrophoretic mobility accompanies the activation of sympathoadrenal system, whereas its increase is characteristic for activation of hypophysialadrenal system and is attributed to limitation of stress reaction [22]. The latter was observed under LILR exposure.

Since the stress reaction is realized through adrenal glands we performed the histopathologic analysis of adrenal cortex.

Adrenal glands of the intact animals have clearly defined cortex and medullary substance. The organ is encapsulated in thin connective tissue. In zona glomerulosa, the small-sized endocrinocytes are assembled into spherical structures of coiled glomeruli. In zona fasciculate, the bigger polygonal endocrinocytes form parallel cords oriented perpendicularly to the epinephros surface. The branching epithelial cords comprised of small cells form a net in zona reticularis. Small vacuoles are evident in cell cytoplasm. The nuclei of the endocrinocytes are big with well-defined nucleoli and optimal ratio eu- to heterochromatin. The border of zona reticularis and zona glomerulosa is comprised by a zone of small cells with big nuclei and more basophilic cytoplasm. This intermediate (sudanophobic) zone is responsible for the regeneration of cortical substance. Numerous hemocapillaries are located between the cell cords and flat nuclei of endotheliocytes are clearly seen (Fig. 5A).

The adrenal medulla consists of big round-shaped cells assembled in clusters and cords. The nucleoli are well-defined. The vessels of adrenal medulla are plethoric (Fig. 5B).

No significant morphological changes of adrenal glands in both "Adrenaline" and "Adrenalin+LILR" groups were observed one hour after the adrenalin injection. The organ retained clearly zoned. The epinephros capsule was not defibrated. Vacuolization was registered in both cortical (zona fasciculata) and medullar endocrinocytes. The intermediate zone is well-defined.

One day after adrenalin injection substantial hemocapillary expansion and plethora enhancement were registered (Fig. 5 C, D). The vacuolization of endocrinocytes in cortical (zona fasciculata and zona glomerulosa) and medullar substance was observed. The border of zona glomerulosa and reticular zone lost its clarity. Local vacuolization of cells in zona glomerulosa and hemocapillary plethora were still detected one week after adrenalin administration (Fig. 5 G, H).

Morphological changes in epinephros of "Adrenalin+LILR" animals exposed to LILR had a slightly different tendency. The capillary plethora was detectable one day after the exposure (Fig. 5 E, F). The vacuolization of endocrinocytes in zona fasciculata was insignificant, though it was more pronounced in

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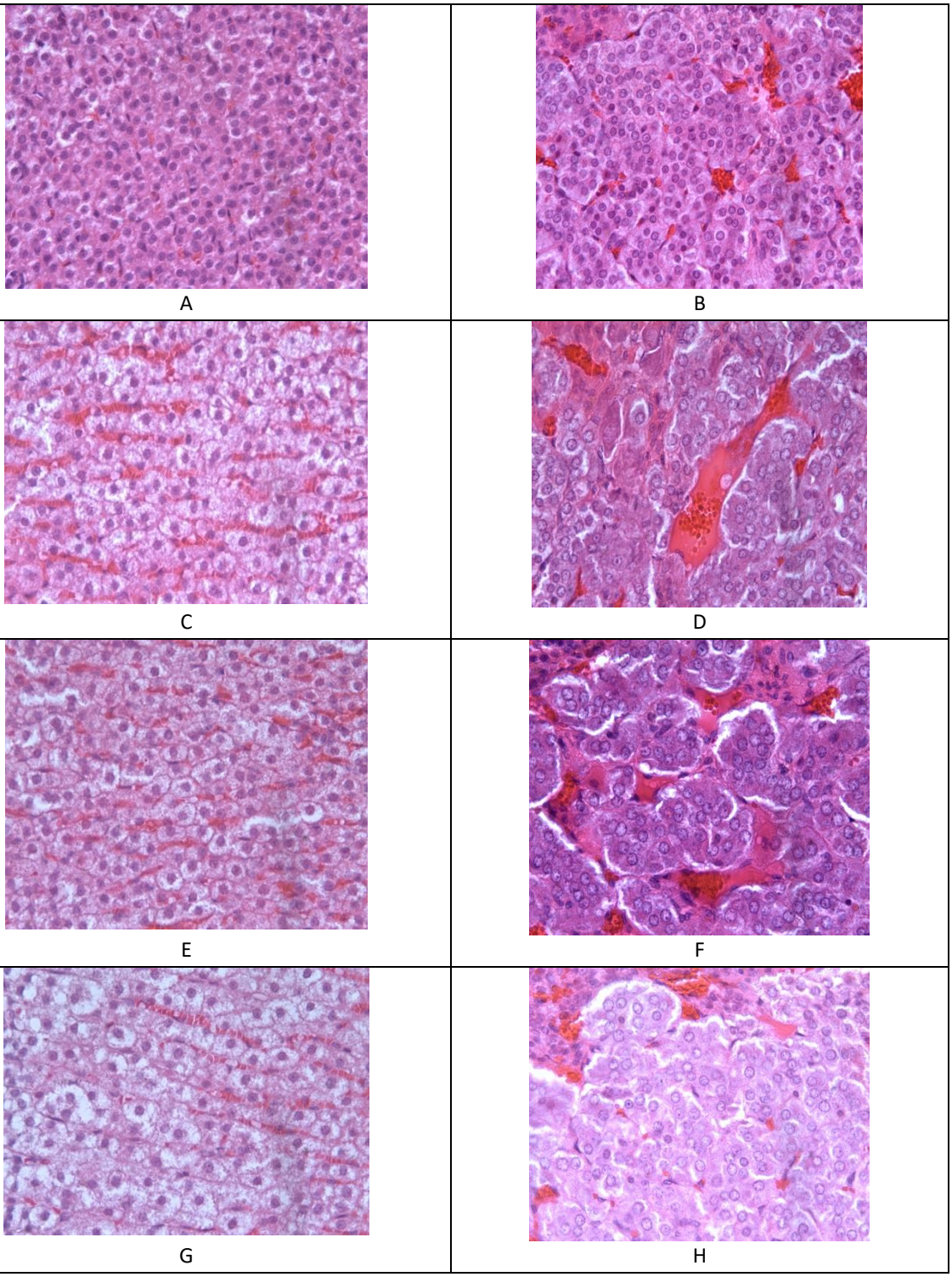
medullar cells. One week after treatment, the morphological state of epinephros in treated animals did not differ from that in intact group (Fig.5. I, J).



A B C D E F G H

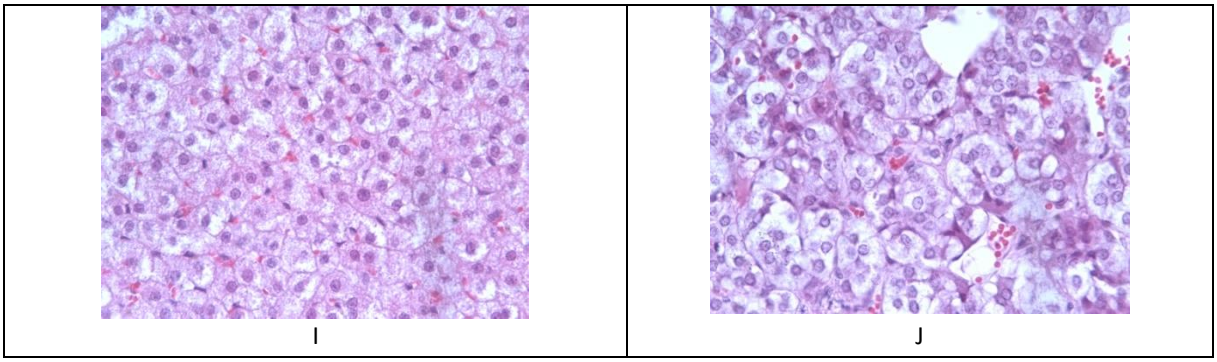



**Fig 5: The structure of cortical substance (zona fasciculata) (A, C, E, G, I) and adrenal medulla (B, D, F, H, J). A, B – intact animals. C, D – 1 day after adrenalin injection. E, F – 1 day after adrenalin injection followed by LILR treatment. G, H – 1 week after adrenalin injection. I, J – 1 week after adrenalin injection followed by LILR treatment. Hematoxylin and eosin staining. Magnification, 400×.**

## CONCLUSION

Thus, the obtained data indicate that LILR has corrective effect in hyperadrenalemia. The mechanism of LILR action, apparently, is realized, firstly, at cellular level, that is manifested in the modification of the erythrocyte membrane promoting improvement of microcirculation and blood rheology, and secondly, at the system level through the activation of stress-realizing systems of the organism with subsequent limitation of inflammatory response. The revealed LILR effect must be taken into account when elaborating remedial strategies, which should include both specific and non-specific influence on various stages of inflammatory reparative reaction to enforce its homeostatic character and to normalize the pathological deviations.

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