INTRODUCTION
International Diabetic Federation (IDF) notes that about 425 million individuals suffered from diabetes mellitus (DM) in 2017 and this amount will rise to 629 million in 2045 [1]. Chronic hyperglycemia as the main link in DM pathogenesis leads to systemic vessels and nerves lesion with chronic bone complications development consequently. Complex general and local biological and biochemical changes occur in bone tissue restoration process in DM. They depend on the bone’s blood supply, patient’s age, general condition of body, and quality and type of treatment [2]. Bone’s fracture is always accompanied by soft tissue damage. Tissue regeneration is determined by genetic and epigenetic factors. The epigenetic factor consists of many components; hormonal status is one of the key ones. The slowing down of bone reparative processes during diabetes mellitus is caused by many mechanisms. In particular, glycosylation end products formation causes big effect on bone strength [3]. Also, diabetic polyneuropathy can lead to increased bone resorption, and micro- and macroangiopathy - disrupt the blood flow to the bones. From literature data it is known that wound healing disorders in the first phase of inflammation cause impaired fibroblast proliferation and collagen synthesis. Delayed wound healing in diabetic patients may be due to a defect in the inflammatory response [4, 5]. Insulin deficiency has a great effect in the inflammation phase and less effect on collagen synthesis, as confirmed by the results of studies on tissue cell cultures. In vitro studies, decrease in calcification level and ossification of the newly formed tissue and disruption of cartilage formation under conditions of hormone deficiency were found. A special role in bone wound healing disorders has bone callus vascularization. Adequate microcirculation, through enhanced oxygenation, ensures the normal functioning of osteoblasts [6, 7]. Despite the prevalence of diabetes, the question of hyperglycemia effect on reparative osteogenesis at all its stages remains un-studied. The literature data are numerous and contradictory.

THE AIM
The aim of our study was to evaluate influence of hyperglycemia on reparative osteogenesis after perforated tibial fracture in rats.

MATERIALS AND METHODS
We conducted an experimental study on white rats, to study the effect of hyperglycemia at the stage of reparative osteogenesis after perforated tibial fracture. To study the histological features, 2 groups of rats weighing from 70 to 200 g were formed.
RESULTS AND DISCUSSION

In the control group, a complete reparative bone regeneration had occurred with the formation of full bone structure, ensuring restoration of bone's anatomical shape and function, as evidenced by morphometric and immunohistochemical research methods.

In the first phase, surrounding tissues and bone tissue disintegration, an increase of periosteum, periosteum cells and osteocytes proliferation, which is necessary for transition to the second phase, was detected. Also we found large-focal lymphoid infiltration and newly formed small-caliber vessels. The activity of eNOS expression depends on the level of calcium in the cytosol of endotheliocytes, which, in our opinion, also indirectly argues in favor of normal bone healing. Expressed eNOS expression was determined, and iNOS expressed moderately.

In the second phase, tissue organization, lymphocytic infiltration, edema and cell proliferation were observed. It was especially seen in connective and cartilage tissues, which was confirmed by an increase of MMP-9 and Ki-67 expression. At the same time, the expression of TGF-β was moderate, as was the expression of all vascular endothelium markers (Fig. 1).

In the third phase, massive bone marrows merged into a compact substance with wide bone canaliculi of primary osteons. In rats, a clear cortical layer was determined, the periosteum was clearly differentiated. We observed sub-sidence of inflammatory process, as well as the processes of angiogenesis. The calcified structures were clearly oriented, the areas of excessive regenerate bedding were in the process of resorption, which was confirmed by strong expression of TGF-β.

In the study of hyperglycemia group in the first phase, extensive zones of hemorrhages were observed in fracture zone and surrounding soft tissues, sometimes with the accumulation of hemosiderin granules. In many small and medium vessels were determined near-wall thrombi and focal congestion. The periosteum fibrous layer was markedly loosened. In the periosteum was found uneven plethora. When compared with the control group, its width was significantly increased (p = 0.03), due to edema and wrinkling of the structure. As in the control, near the fracture site in a compact substance, lacunae with resorption foci and rare osteons were found, but their number is lower than in the control (Fig. 2).

In osteons, numerous osteocytes were observed, their number tended to decrease. Bone cells and the inner layer of the periosteum proliferated, which was confirmed by moderate expression of Ki-67, in some fields of view the expression of this marker was weak. The proliferation index was 2.8 ± 0.1 (p = 7.9892E-11), and it was significantly reduced compared with the control group.

During immunohistochemical study with TGF-β, its expression was not observed. In soft tissues, virtually diffuse lymphoid infiltration was detected, which was confirmed by strong expression of CD3 and CD20 (Fig. 3).

MMP-9 expression, in contrast, was moderate, both in connective tissue and in cartilage, which was not observed.
When assessing vessel formation, weak expression of CD34 was detected, VEGF marker was found in small areas and also expressed weakly, while expression of CD31 was not detected at all. ENOS expression was reduced, however, iNOS expressed moderately.

In the second phase of reparative regeneration, beginning of soft bone callus formation was observed. Unlike healthy rats, osteoid tissue was formed in 40.0% (n = 2) of observations, and in 60.0% (n = 3) there was a fibrous structure of osteoid tissue and cartilage patches. The border between the fibrous layer of periosteum and connective tissue of paraosteum looked blurred. The periosteum was significantly (p = 0.04) wider than in the control group. As in the first phase in hyperglycemia rats, it was observed edematous. The cell density of periosteum inner layer tended to decrease, while the density of osteocytes was significantly reduced (p = 0.06) when compared with the control group. Lacunas with optically empty resorption

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**Fig. 1.** A. The osteons structure in bone near the fracture site in control group rat in the first phase of reparative osteogenesis. Stained with hematoxylin and eosin, x100

B. Weak expression of TGF-β of rat bone in the control group in control group rat in the first phase of reparative osteogenesis. Indirect immunoperoxidase method using TGF-β, x400

**Fig. 2.** The structure of rare osteons in bone near the fracture site in hyperglycemia group in the first phase of reparative osteogenesis. Stained with hematoxylin and eosin, x100

**Fig. 3.** Strong CD20 expression in soft tissues near fracture site in hyperglycemia group in the first phase of reparative osteogenesis. Indirect immunoperoxidase method using CD20, x400

**Fig. 4.** Strong MMP-9 expression in hyperglycemia group in the third phase of reparative osteogenesis. Indirect immunoperoxidase method using MMP-9, x400
zones were observed in several fields of view in a compact bone substance. Outside the fracture, formed and, in some places, collapsing osteons with cells resembling osteocytes were detected.

Comparing to healthy rats, cell proliferation of periosteum and osteogenic tissue was less observed. The proliferation index was significantly reduced comparing with the control \( (p = 1.4082E-08) \). Lymphocytic infiltration was lower than in the first phase, however, it still remained large-focal with a tendency to merge foci. Active expression of CD3 and CD20 was observed.

During evaluating of angiogenesis, we observed mild CD34 expression and weak VEGF expression. IHC studies with CD31 also showed weak expression. At the same time, strong expression of iNOS was determined, and the expression of eNOS decreased.

In the third phase, in microscopic preparations of hyperglycemia rats, the onset of solid callus formation was observed. In the space between fragments, a small mesh of bone trabeculae was found, mainly from lamellar bone tissue. The width of the periosteum averaged 149.5 ± 3.2 μm. Periosteum inner layer cell density and the number of osteocytes tended to decrease, when compared with the control group, but when compared with rats of the same group, the second phase, on the contrary, tended to increase. The index was also significantly lower than in healthy rats \( (p = 7.0767E-07) \). Expression of MMP-9 was moderately active in cartilage and connective tissue (Fig. 4).

IHC studies with TGF-β showed moderate expression in several fields of view. In hyperglycemia group inflammatory process subsided, but infiltration still remained focal with a tendency for lymphocyte accumulation to be perivascular.

Immunohistochemistry method showed strong CD34 expression in the vascular endothelium. When IHC assay with VEGF, expression of this marker remained moderate, as in the second phase, CD31 expressed weakly. In IHC studies with eNOS and iNOS markers, their expression level was about the same.

**CONCLUSIONS**

The results demonstrated that in hyperglycemic rats, there was a delay in the callus formation, a decrease in proliferation and ossification, and a slowdown in the processes of angiogenesis. This is confirmed by an increase in MMP-9 expression in connective tissue, a decrease in TGF-β expression in all phases, an increase in the expression of CD3 and CD20 and a marked decrease in the expression of all vascular markers. Due to hyperglycemia, incomplete blood supply to the tissues occurs, necrosis of bone and soft tissues develop in the area of the fracture, the reparative reaction slows down considerably and manifests itself in the development of fibrous and, less commonly, cartilage tissue.

**REFERENCES**


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