ISSN 0030-5987. Orthopaedics, traumatology and prosthetics. 2021. № 4

УДК 616.748-007.23:616.758-005.4]-003.93:611.018.1"712.4"](045)

DOI: http://dx.doi.org/10.15674/0030-59872021463-69

The influence of regenerative technology processes in leg after traumatic ischemia (experimental study)

A. T. Pidlisetsky [1, 2], O. V. Dolhopolov [1], S. I. Savosko [3], O. M. Makarenko [4]

[1] SI «Institute of Traumatology and Orthopedics NAMS of Ukraine», Kyiv
[2] Communal non-profit enterprise ENT «Lviv Regional Hospital for War Veterans and Repressed named after Yu. Lypa». Ukraine
[3] Bohomolets National Medical University, Department of Histology and Embryology, Kyiv. Ukraine
[4] Interregional Academy of personnel Management, Kyiv. Ukraine

Post-traumatic muscle ischemia results from severe injury and can lead to muscle dysfunction. Therefore, patient management and treatment are very significant in all periods of injury. New methods are performed, especially using regenerative technologies to avoid complications and improve long-term outcomes. Objective. To determine histological changes in the muscles of the injured limb after traumatic ischemia after injection of platelet-rich plasma, Bone marrow stem cell concentrate (BMAC), and Stromal vascular fraction (SVF) prepared from adipose tissue on the 5, 15, and 30 days. Material and methods. Experiments were conducted on rabbits (Chinchilla breed). A tourniquet imposed on a lower limb, from the middle third of the thigh to the ankle joint. After 6 hours, the tourniquet was removed. The animals were divided into four groups: control, platelet-rich plasma, bone marrow stem cell concentrate, and stromal vascular fraction prepared from adipose tissue—histological muscle changes provided by Tescan Mira 3 LMU (Czech Republic) in scanning transmission electron microscopy. Results. On the 5th day after the experiment were no significant histological changes in muscles but in the contrary on the 15 days after experiment in BMAC and SVF groups detected new muscle fibers formation in necrotic areas and myonucleus organization. On the 30th day new angiogenesis was detected around muscle fibers. Platelet-rich plasma group characterized by massive connective tissue formation in necrotic areas. Conclusions. Necrosis and progressive muscle hypotrophy are unavoidably for this type of injury. It was shown that BMAC and SVF could stimulate regeneration and angiogenesis. Key words. Traumatic ischemia, necrosis, histological changes in muscles.

Тактика ведення та лікування пацієнтів із післятравматичною ішемією м'язів кінцівок є визначальною на всіх етапах, тому розроблюють новітні методи лікування зі застосуванням клітинних технологій для попередження розвитку ускладнень і покращення віддалених результатів. Мета. Визначити в експерименті гістологічні зміни в м'язах ушкодженої кінцівки після травматичної ішемії за умов введення збагаченої тромбоцитами плазми, аспірата кісткового мозку та стромально-васкулярної фракції жирової тканини. Методи. Роботу виконано на 65 кролях породи Шиншила вагою 4,2–4,5 кг. Тваринам накладали джгут на ліву нижню кінцівку від середньої третини стегна до надп'ятково-гомілкового суглоба, який через 6 год знімали. Після цього тварин розділили на 4 групи: 1-ша — ішемія-контроль, 2-га — введення збагаченої тромбоцитами плазми, 3-тя — введення клітин аспірата кісткового мозку, 4-та — стромально-васкулярної фракції жирової тканини. На 5, 15, 30-ту доби по 5 тварин із кожної групи виводили з експерименту та виконували гістологічні дослідження. Результати. На 5-ту добу після моделювання в усіх групах кролів виявлено набряк м'язової тканини, дегенерацію та фрагментацію м'язових волокон у субфасціальній ділянці. На 15 і 30-ту доби в 3-й і 4-й групах зафіксовано формування нових м'язових волокон на місцях некрозу, появу міотубул із міоядер. На 30-ту добу в групі контроль-ішемія домінувала атрофія м'язових волокон. Висновки. Травматична ішемія кінцівки кролів протягом 6 год спричинює дегенерацію та прогресування гіпотрофії м'язових волокон скелетних м'язів гомілки. Введення збагаченої тромбоцитами плазми, аспірата кісткового мозку та стромально-васкулярної фракції жирової тканини не призводить до відновлення м'язової тканини в субфасціальних ділянках, але позначається на частковому збереженні м'язових волокон глибоких міонів м'яза. Ключові слова. Кролі, травматична ішемія, нижня кінцівка, некроз, гістологічні зміни в м'язах.

Key words. Traumatic ischemia, necrosis, histological changes in muscles

Introduction

Skeletal muscle atrophy is persistent with severe limb nerve injury and traumatic ischemia, and limb function is significantly impaired. Even with early and successful microsurgical recovery of peripheral nerves, limb blood vessels, and adequate postoperative therapy, it is impossible to predict with high probability the degree of recovery of limb muscles whose atrophy may be irreversible, with fibrosis, contractures, and adipose tissue invasion. Under conditions of atrophy of muscle fibers after various injuries, there is a tendency to increase the degree of irreversible morphological changes, even after treatment, limb dysfunction is inevitable [1, 2].

Skeletal muscles are known to have the potential to regenerate in response to various types of injuries, such as trauma, ischemia, and some types of muscular dystrophy [2–5]. However, the structural basis and time course of recovery in skeletal muscle are insufficiently studied. It is believed that the regeneration of muscle tissue can be stimulated through the use of autologous cell technology. In particular, administration of bone marrow-derived mesenchymal stromal cells (MSCs) to patients with critical limb ischemia has been shown to stimulate angiogenesis and preserve ischemic lesions in about 80 % of cases [6]. Studies of the effect of bone marrow-derived MSCs have shown their stimulating effect on the proliferation and migration of endothelial cells and differentiation of myoblasts [7]. However, it should be noted that about 98 % of cells in the bone marrow aspirate are a population of hematopoietic cells, and the remaining 2 % are endothelial precursors and some other cells [8, 9].

As an alternative to isolated MSCs, a concentrated autologous bone marrow aspirate derived from adipose tissue cells and platelet plasma concentrate have been suggested in traumatology to stimulate tissue regeneration after traumatic injuries [10].

We have previously identified damage to the peripheral nerve of the limb, structural and biochemical changes in skeletal muscle after traumatic ischemia, significant atrophy of muscle fibers with fibrotization, and cell reorganization along individual muscle fibers, which may be an early manifestation of regeneration [11]. Morphometry of damaged muscle fibers did not allow to fully assess the recovery processes without and after the introduction of suspensions of autologous cells, although it is a convenient way to linearly measure muscle fibers, the time course of their atrophy. Evaluation of biochemical changes in muscles indicates a violation of redox processes and the rapid accumulation of lipid peroxidation products, which can have a cytotoxic effect on damaged cells and, consequently, provoke permanent disorders [12].

In this study, we additionally used transmission electron microscopy to analyze the reactions of satellite cells in damaged areas of muscle. Based on published findings, these changes have not previously been compared after traumatic ischemia and administration of platelet plasma and cell aspirates. All this indicates the urgency of the problem of studying the morphological basis of skeletal muscle regeneration after traumatic ischemia and identifying the activation of regenerative processes or undesirable consequences of the use of tissue technology in traumatology.

The aim of the study: to investigate the regeneration of leg muscles after traumatic ischemia and the introduction of platelet plasma, aspirate of bone marrow cells and adipose tissue.

Material and methods

The experiments were performed on 65 rabbits of the Chinchilla breed weighing 4.2–4.5 kg. The animals were simulated mechanical ischemia of the left hind limb. To do this, a medical elastic tourniquet (5.5 cm wide) was applied from the middle third of the thigh to the ankle joint, thereby immobilizing the limb and causing impaired vascular perfusion. After 6 h, the tourniquet was removed and divided into 4 groups of 15 individuals each: Group 1 — ischemia-control, Group 2 — introduction of platelet plasma concentrate, Group 3 — aspirate of bone marrow cells, Group 4 — stromal-vascular adipose tissue fractions. 5 rabbits from each group were removed from the experiment on the 5^{th}, 15^{th} and 30^{th} day. An additional 5 intact rabbits were used for morphometric study. Animals were anesthetized with sodium thiopental at a dose of 60 mg/kg (intraperitoneally).

All animal manipulations were performed in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, No. 123, Council of Europe, L222, 24.08.1999 and the Commission on Bioethics of the Institute of Traumatology and Orthopedics (Minutes No. 4 of 10.12.2021).

Preparation of platelet plasma concentrate

Blood was collected from the ear vein of a rabbit in the amount of 5 ml, which was placed in a special tube to obtain a concentrate of platelet mass, followed by centrifugation at 760 g for 8 minutes The resulting concentrate was evacuated to a 5 ml syringe and inserted into the posterior deep space of the shin.

Obtaining aspirate of bone marrow cells

Autologous aspirate of bone marrow cells was obtained from the proximal thigh of rabbits. A bone trocar (diameter 10 G) was inserted into the proximal thigh, and a 2 ml syringe was used to aspirate 2 ml of bone marrow. The aspirate was separated through a Tulip Emulsifier™ filter, and the anticoagulant dextrose citrate ACD-A was added. The resulting aspirate was centrifuged at 760 g for 8 min and aspirated with 1 ml of the upper plasma layer and the cell layer (55,800 cells/ml).

Obtaining stromal-vascular fraction of adipose tissue

With access of up to 2 cm, 5 mg of abdominal omentum was collected and mechanically ground to a suspension, which was homogenized by passing the systems into two connected syringes through a 1 mm hole. Then it was centrifuged at 760 g for 8 minutes

Platelet plasma concentrate, bone marrow aspirate cells, and adipose tissue suspension were injected into the middle third of the calf muscle.

Histological studies

Muscle fragments of animals with cell and plasma injection sites were fixed in 10 % formalin solution on phosphate buffer (pH = 7.4) for 24 h at 4° C. The samples were then washed, dehydrated in a solution of isopropanol with chloroform (70–99.8 %) (1:1) and poured into paraffin. On a Thermo Microm HM 360 microtome (Thermo Fisher Scientific, USA), 6 μm thick sections were made, dewaxed, and stained with hematoxylin and eosin. Muscle fiber morphometry (diameter, μm) was performed using Carl Zeiss software (AxioVision SE64 Rel.4.9.1) (Carl Zeiss Microscopy, LLC, USA) and BX 51 microscope (Olympus, Japan).

Transmission electron microscopy (TEM)

Muscle samples were fixed in a 2.5 % solution of glutaraldehyde in phosphate buffer followed by 1 % OsO_4 fixation, dehydrated in ethanol and acetone, and sealed in epoxy resin (Epon 812, Araldite 502). Ultrathin sections of epoxy blocks were obtained on a Reichert-Jung ultratome (Ultracut E, Germany). Sections were fixed on tungsten mesh and contrasted with 2 % uranyl acetate solution and lead citrate. The samples were examined on a scanning electron microscope Tescan Mira 3 LMU (Czech Republic) in transmission microscopy mode (STEM).

Statistical processing was conducted in Origin Lab 8.0 software. The indicators are presented as M ± m, where M is the average value, m is the standard error. The groups were compared using the non-parametric Kruskal-Wallis test. The differences were considered significant with $p < 0.05$.

Results and discussion

According to the results of histological studies of the subfascial muscle in the control group of animals with ischemia, in groups after administration of platelet plasma concentrate, bone marrow aspirate cells and stromal-vascular fraction of adipose tissue on the 5th, 15th and 30th days revealed structural disorders in muscle fibers, connective tissue of endomysium and perimysium.

In the ischemia-control group (Fig. 1, a, b, c) edema, a significant decrease in the thickness or fragmentation of muscle fibers and their atrophy on the 30th day were recorded. On the 5th day, the interstitial space increased due to swelling between the muscle fibers. Simultaneously, fibers with signs of atrophy and additional nuclei were detected (Fig. 1, a). In endomysium and perimysium, stratification of collagen fibers due to edema, areas with mononuclear infiltration, stasis of capillaries were revealed. Areas of cellular detritus and macrophages with phagosomes were detected by TEM, active phagocytosis and utilization of these products were observed (Fig. 2, a).

After the administration of platelet plasma on the 5th day, as in the ischemia-control group, changes in the density and shape of muscle fibers, their separation due to endomysium edema were determined. In some muscle fibers, an increase in the number of nuclei along the fiber contour was recorded, which can be considered as a manifestation of regeneration (Fig. 1, d). TEM showed destructive changes of fibroblasts of perimysium — lysis of nuclei, vacuolation of cytoplasm. The appearance of macrophages was determined (Fig. 2, d). Skeletal muscle regeneration is known to involve a symbiosis of macrophages and myoblasts [3].

The administration of bone marrow cell aspirate did not improve the structure of degeneratively altered muscle fibers due to ischemia. Deep disintegration of muscle fibers (Fig. 1, g), proliferation of fibroblasts between muscle fibers, violation of histoarchitectonics of endomysium (Fig. 2, g) were noted.

After the introduction of the stromal-vascular fraction of adipose tissue into the muscle area, destructive disorders of muscle fibers were also observed, but between them on the 5th day there were mononuclear cells and fibroblasts (Fig. 1, k). Longitudinal bands of nuclei have been found in muscle fibers, which is a manifestation of regeneration. Macrophages with phagosomes predominated in the areas

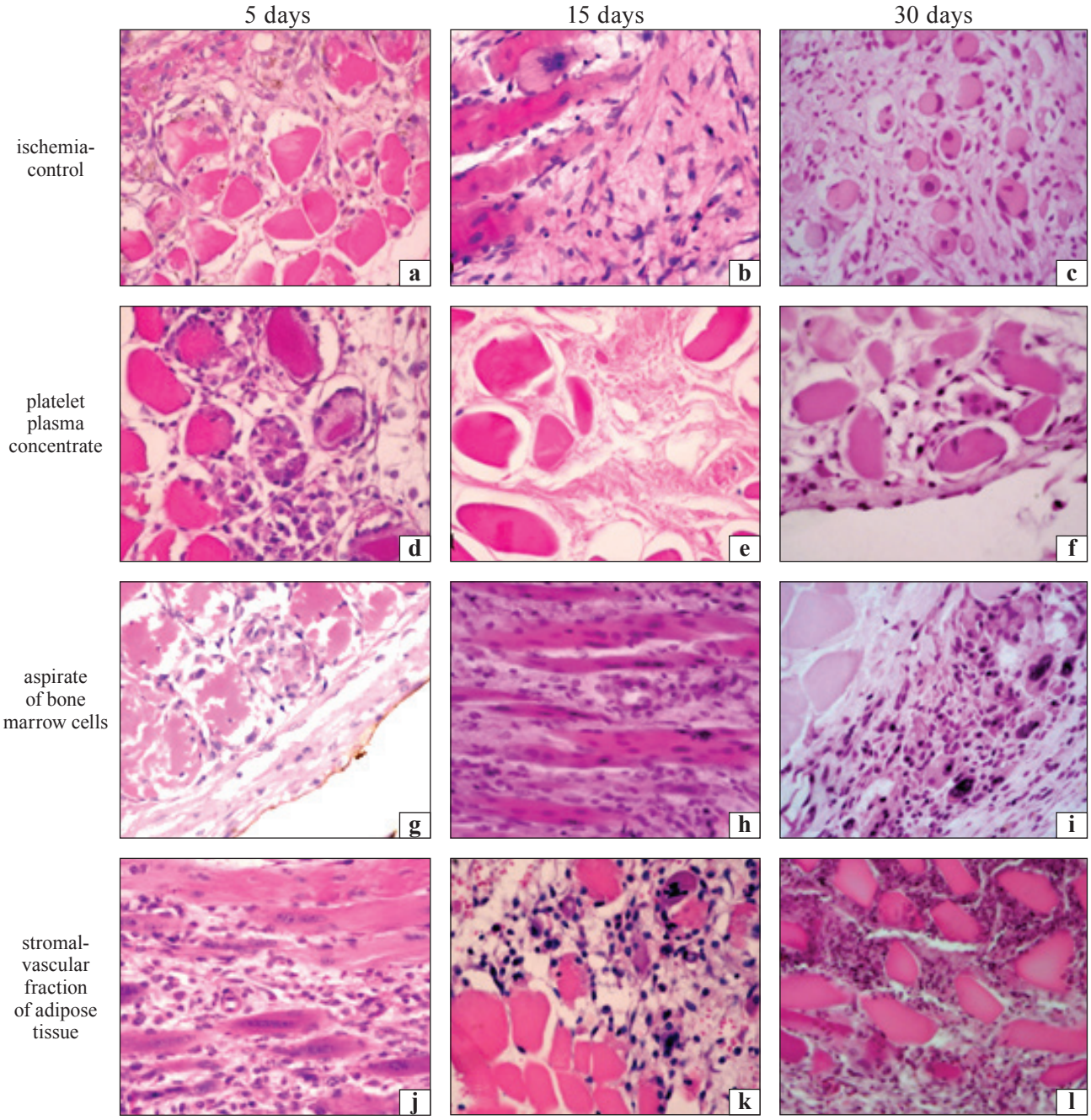

Fig. 1. Structural changes in the subfascial area of skeletal muscles of the leg of rabbits with ischemia of the limb (a–c) and the introduction of platelet plasma concentrate (g–e), aspirate of bone marrow cells (g–k), stromal-vascular fraction of bone tissue (l–o). Degeneration of muscle fibers in all groups on the 5th day and for all periods in the 4th group (l–n); inflammatory infiltration (n); appearance of groups of nuclei in damaged fibers (c, e, f, i, k). H&E stain (40x10)

of destruction, elimination from the interstitial space of cellular detritus and lipid granules derived from adipocytes were recorded (Fig. 2, d).

Fifteen days after the simulation of ischemia, similar degenerative changes were observed in all series of the experiment, namely: thinning of muscle fibers with lumbar striation on longitudinal sections, central location of nuclei, signs of atrophy. The edema persisted, which led to the separation of muscle fibers (Fig. 1, e, m). Groups of muscle fibers without nuclei were detected after the introduction of platelet plasma concentrate, which also indicates their atrophy (Fig. 1, e). There was a remodeling of endomysium and perimysium with the formation of loose connective tissue (Fig. 1, b, i).

According to electron microscopic examination in the control-ischemia group and after the introduction of platelet plasma concentrate, areas with fibroblasts and collagenogenesis were registered (Fig. 2 b, e). After the introduction of the aspirate of bone marrow

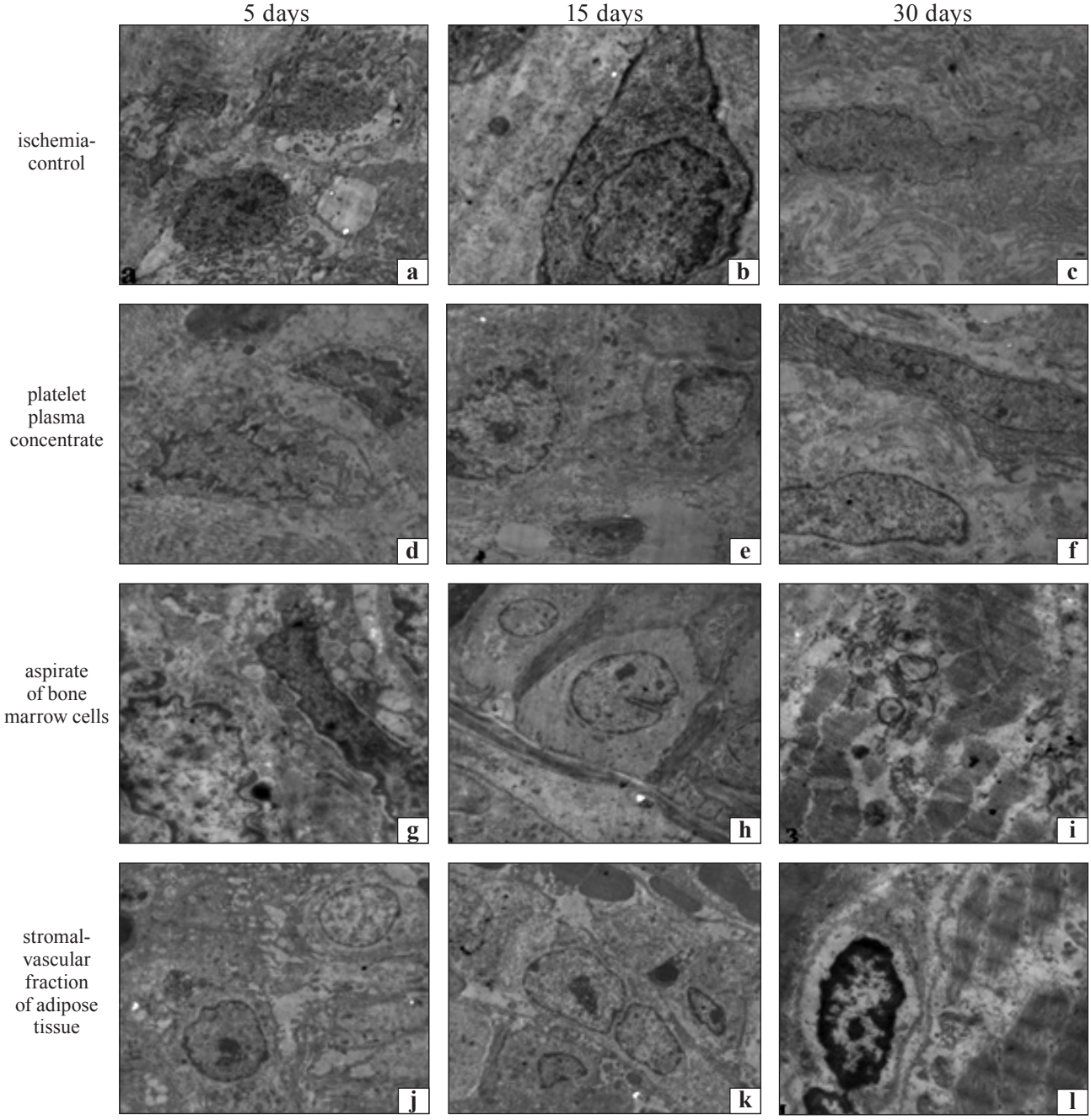

Fig. 2. Areas of shin muscle damage with limb ischemia (a–c) and subsequent administration of platelet plasma concentrate (d–f), bone marrow cell aspirate (g–k), stromal-vascular fraction of adipose tissue (l–n). Macrophages on the 5th day in all groups; satellite cells on the 15th day (i, m); increase in collagen density (g); lamellar atypical bodies in muscle fibers (k). Contrast with osmium oxide and uranyl acetate TEM. Magnification 60,000 (g, k, n); 30,000 (d, e, f); 22,000 (a–c, i)

cells and adipose tissue cells, areas with bands of satellite cells were recorded, and the formation of myotubules (Fig. 2, i, m) was observed in some places, which is a sign of regeneration. Myoblasts had large nuclei, they were dominated by euchromatin, and no myofibrils were registered in the cytoplasm. According to scientific information, myoblastic differentiation begins with the activation of resident satellite cells [13].

On the 30th day, degenerative changes in all series of the experiment prevailed in the muscles. Fibrosis was observed in different areas of perimysium and endomysium. Significant atrophy of muscle fibers was recorded in the ischemia-control group (Fig. 1, c), and after the introduction of adipose tissue cells, additional inflammatory infiltrates were noted (Fig. 1, o). Regenerative changes were associated with cell proliferation, the presence of satellites and

macrophages. Deep muons show the appearance of extra nuclei along muscle fibers, which is also a manifestation of regeneration. In the process of electron microscopic examination for this period of observation showed an increase in the number of fibroblasts and collagen (Fig. 2, c, f) with atypical lamellar structures in muscle fibers, which is a manifestation of autophagy (Fig. 2, k). Myofibrils with sarcomere boundaries were recorded in structurally preserved muscle fibers, although the density of fibrils in actin-myosin complexes was reduced (Fig. 2, n).

According to the results of morphometric study, the muscle fibers of the deep zone on the 30th day after ischemia of the limb had a much smaller diameter compared to the intact group (41.9 ± 2.5) μm vs. (81.5 ± 1.4) μm ($p < 0.05$). After the introduction of platelet plasma, the width of muscle fibers was greater than in the 1st group of the experiment by 1.7 times, but did not reach the control values (70.5 ± 1.8) μm against (81.5 ± 1.4) μm ($p < 0.05$). An increase in the width of muscle fibers was also recorded in a series with the introduction of bone marrow cells (65.2 ± 2.2) μm, with stromal-vascular fraction of adipose tissue (45.7 ± 1.7) μm. These values were also significantly smaller ($p < 0.05$) compared with the intact group.

It is known that significant damage to skeletal muscle tissue is characterized by low reparative capacity, which leads to research on the use of various technologies to stimulate regeneration [14]. However, the peculiarities of the use of cell technology and platelet-enriched plasma require in-depth study, because so far only partial recovery of muscle tissue has been reported using them [15]. Improvement of inflammatory condition, reduction of fibrous scar size and recovery have been demonstrated in PRP-treated affected muscles [16]. When platelet plasma concentrate was used for all periods of the experiment, we observed degenerative changes in muscle tissue with the formation of areas of fibrosis. After administration of bone marrow cell aspirate and stromal vascular fraction of adipose tissue, an increase in reparative manifestations was observed, although in the 4th group inflammatory infiltration developed, which is an undesirable consequence of the use of cells derived from adipose tissue.

Thus, histological and electron microscopic studies have led to several important conclusions. First, 6-hour mechanical ischemia results in degenerative changes in the skeletal muscles of the limb with pronounced manifestations in subfascial muons, and deep muon muscles suffer from malnutrition, which is manifested by a significant decrease in muscle fiber thickness. Second, the use of tissue technology only partially prevents the progression of destructive muscle changes in the long term. Ischemic damage to superficial muons in the subfascial area is irreversible. The administration of bone marrow aspirate cells, stromovascular adipose tissue fraction, and platelet plasma concentrate did not result in muscle regeneration. Destructive changes in muscle fibers, edema, fibrosis, endomysium, and perimysium persist by the end of the 30-day follow-up period. However, on the verge of necrosis, individual muscle fibers are regenerating, which, according to electron microscopy, has been detected since the 15th day. The frequency of administration of the studied factors is debatable and it is likely that several injections would help to improve the results, which requires further research. It is known that the consequences of traumatic muscle ischemia depend on the state of blood microcirculation, but these data are not covered in this study.

Conclusions

Traumatic ischemia of the limbs of rabbits for 6 hours causes the development of degenerative changes with the progression of malnutrition of skeletal muscle fibers of the shin muscles. The introduction of platelet plasma, bone marrow aspirate and stromal-vascular fraction of adipose tissue does not lead to the recovery of muscle tissue in subfascial areas, but affects the partial preservation of muscle fibers of deep muscle myons, as revealed by morphometric studies on the 30th the day of the experiment.