Reparative Reactions of the Skeletal Muscles in Early Aging OXYS Rats with Toxic Metabolic Injuries Caused by Bupivacaine

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Comparative analysis of reparative reactions in the anterior tibialis muscles of OXYS and Wistar rats after intramuscular injections of bupivacaine was carried out for evaluating the regeneratory potential of skeletal muscles under conditions of chronic disorders of oxidative metabolism. Lumpy degradation of lesion foci and mononuclear cell infiltration predominated on day 1; after 3 days myoblast proliferation and fusion processes were maximally pronounced; after 7 days maturing myotubules created groups of fibers with central nuclei. At the ultrastructural level, regeneration of the muscle fibers was associated with signs of activation of their nuclei and of satellite cell nuclei. Lesser (in comparison with the control) mean cross-section areas of the muscle fibers on day 14 after the start of the experiment indicated that the regeneration processes were not completed by this term. The proliferative potential of myogenic precursor cells was retained in hereditary dysfunction of mitochondria in OXYS rats.

Key Words: *OXYS rats; skeletal muscles; regeneration; bupivacaine; electron microscopy*

The processes of physiological and reparative regeneration of the skeletal muscle fibers largely depend on the population of resident satellite cells, the main sources of postmitotic nuclei in the skeletal muscles [10]. Despite high efficiency, the replication potential of this cell system obviously has certain limitations. A progressive decrease in the count and proliferative activity of satellite cells during aging and in some chronic degenerative processes was demonstrated on clinical material and in experiments in cell cultures and *in vivo* [12,15]. However, there is still no universal opinion whether it is an important limiting factor in regeneration of muscle fibers (MF) under conditions of chronic disease of the skeletal muscles [6,13]; all studies in this direction concerned mainly muscular dystrophies.

The model of genetically determined myopathy in early aging OXYS rats [1] offers interesting potentialities for the studies of these aspects. The parameters of development of this pathological process render it certain similarity with the so-called "mitochondrial myopathies" [4], and one of the causes of reduced compensatory hypertrophy and increased degeneration of skeletal muscles, which we detected in 9-month-old animals [5], could be probable reduction of the proliferative potential of myogenic precursor cells, caused by exhaustion of their pool in repeated degeneration-recovery cycles [7] or by changed microenvironment because of metabolic disorders and restructuring of the skeletal muscle cells.

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We hypothesized that the first signs of reduction of the regeneratory potential *in vivo* could be detected under conditions of the extreme strain of reparative reserve of skeletal muscles exposed to an extreme damaging factor. Bupivacaine (BC) was chosen due to its capacity to induce rapid degeneration of MF, which is often used in studies of the skeletal muscle regeneration [14].

We studied the regeneratory potential of skeletal muscles with consideration for the special physiological context, created by the previous strain of the hemostatic mechanisms and adaptive restructuring of the skeletal muscle fibers in early aging OXYS rats [1,3].

MATERIALS AND METHODS

Experimental group consisted of 38 OXYS rats and 38 Wistar rats aged 6 months (215-340 g); intact animals of the same age (5 for each strain) served as the control.

Bupivacaine (anecaine, Pliva) was injected (0.5% solution, 0.2 ml) via a fine needle into the central area of the anterior tibialis muscle. The animals were sacrificed after 3 h, 1, 3, 7, and 14 days. The contralateral muscle was taken for control in all animals; in 2 cases, 0.2 ml sterile isotonic saline was injected into it at each term of the experiment.

Muscle preparations were resected in the "tension at rest" status [4]. Muscle fragments for photo-optic study were fixed in 10% neutral formalin, processed by the standard method, and embedded in paraffin with consideration for the MF orientation. Paraffin sections were stained with hematoxylin and eosin in combination with Perls and van Gieson reactions with post-staining of elastic fibers with Weigert resorcin-fuchsin, and PAS reaction was carried out. Semithin (1 μ) sections were prepared by the standard method [4] and stained with Schiff's reagent and Azur II. The study was carried out under a Leica DM 4000B universal microscope. Microphotographs were made with a Leica DFC 320 digital camera using Leica QWin software.

Ultrathin sections were made on an LKB III ultratome, contrasted with uranyl acetate and lead citrate after Reynolds, and examined under a JEM 1010 electron microscope.

The mean cross-section area of MF was measured in stereotypically selected transverse paraffin and semithin sections of the center of the anterior tibialis muscle by stereological methods using a test lattice.

The data were statistically processed using Student's t test; the differences were considered significant at $p<0.05$.

RESULTS

Light microscopy of somatic muscles showed that the resorptive phase of the reparative reaction was clearly manifest as early as 24 h after the start of experiment; lumpy degradation of lesion foci and pronounced mononuclear cell infiltration (both processes were usually subtotal) predominated. Numerous macrophages were seen in the foci of lesions. However, active phase of the regeneratory reaction culminated 3 days after the start of the experiment. The main events of this stage were almost complete resorption of necrotic MF and maximally pronounced proliferative phase of the regeneratory reactions.

Zones of necrotic fibers with signs of degradation and almost complete absence of the nuclei were seen in the preparations. Fields of cell infiltration presented by macrophages (including siderophages) and, to a considerable degree, by proliferating myoblasts (Fig. 1, *a*) fusing into fine basophilic myotubules (Fig. 1, *b*) surrounded these zones. Macrophages and myoblast-like cells filled the profiles of empty sarcolemmal sacs, groups of larger myotubules with central nuclei forming bundles.

The architecture of muscle tissue closer to the zone of red fibers was in general preserved. Solitary fine myotubules were adjacent to the MF with normal structure (Fig. 1, *b*). Sometimes myoblasts fusing into a symplast united the preserved fragments of the same MF.

After 7 days, the architecture of muscle tissue started to recover. No irreversibly damaged MF were seen in the majority of preparations. A characteristic feature was maturation of myotubules forming groups of immature MF (Fig. 1, *c*) and scattered fine, but already less basophilic striated fibers with central clear nuclei. Endomysial strata in the zone of growing MF contained well-developed capillary network without signs of stasis and an appreciable number of macrophages and fibroblasts (Fig. 1, *d*).

However, despite general trend of the transition of the regeneratory reaction into the phase of young fiber maturation, zones of "protracted" reparation were seen in some preparations, with fields of proliferating myoblasts and immature myotubules surrounding isotropic remnants of MF, absorbed by macrophages. Capillaries in infiltration zones were in a state of stasis, foci of hemorrhages were detected. Significant differences in the width of sarcomers and contracture injuries were detected in MF adjacent to these foci.

After 14 days, the general architecture of the skeletal muscle was virtually restored. Small fibrils lost basophilia in comparison with the previous term and were less easily differentiated from the

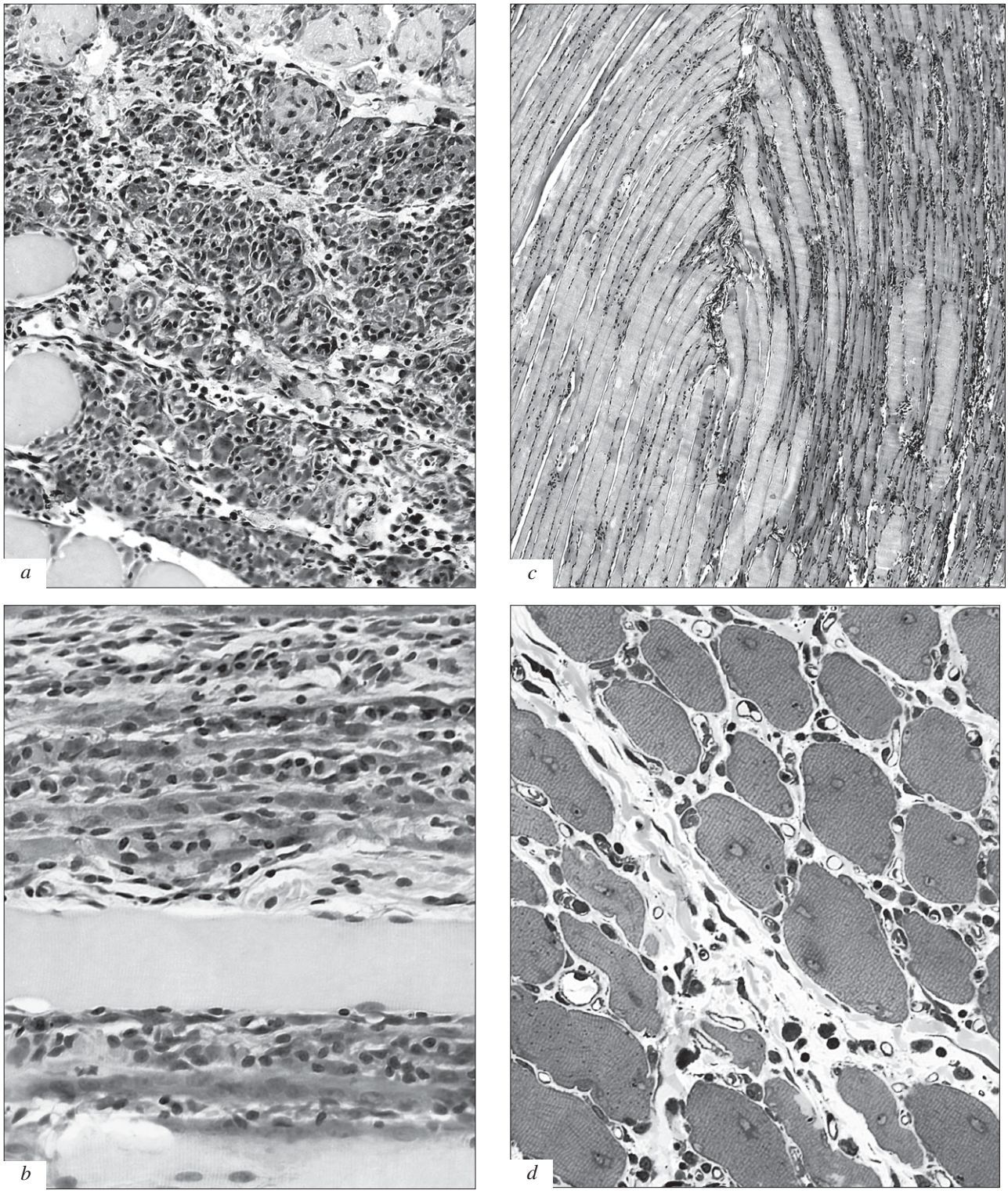

Fig. 1. Reparative reactions of skeletal muscles in OXYS (*a*, *d*) and Wistar rats (*b*, *c*) 3 (*a*, *b*) and 7 days after injection of BC (*c*, *d*). *a*) cell infiltration (macrophages and proliferating myoblasts) near the zone of necrotic MF, ×250; *b*) intact MF surrounded by infiltration cells and new myotubules, ×500; *c*) regenerating MF in the external zone of the anterior tibialis muscle, ×80; *d*) immature MF with central nuclei, numerous capillaries in the endomysium; ×500. *a-c*: hematoxylin and eosin staining; *d*) semithin section, Azur II staining.

background, but they did not yet reach normal size and constituted an appreciable part of the entire population (Fig. 2, *a*). About 20-40% cells retained the central nuclei.

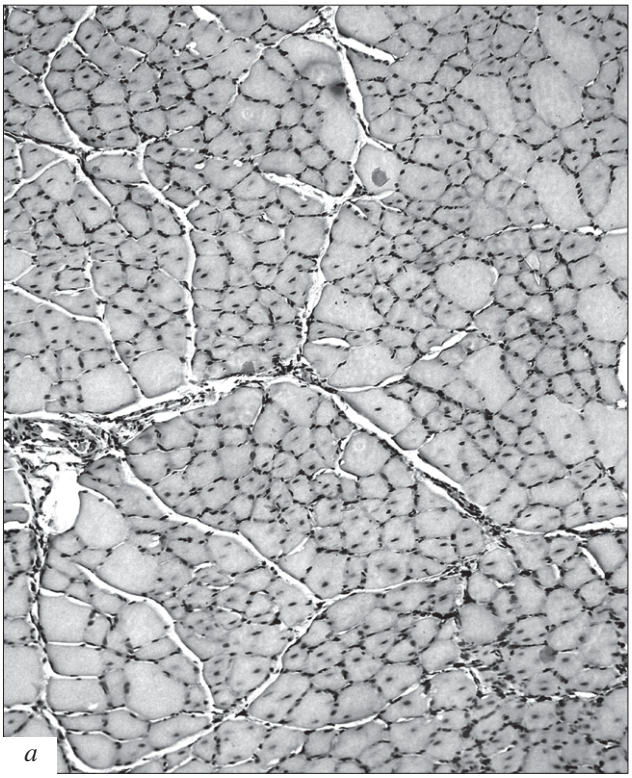

Fig. 2. Reparative reactions of skeletal muscles 14 days after injection of BC. *a*) anterior tibialis muscle in OXYS rat. Fine fibers with central nuclei constitute an appreciable part of MF population. Hematoxylin and eosin staining, ×150; *b*) mean cross-section area of MF of the anterior tibialis muscle in Wistar and OXYS rats. Light bars: control; dark bars: experiment.

The efficiency of regeneratory reactions in OXYS and Wistar rats was compared by measuring the mean cross-section area of MF in stereotypically selected transverse sections from the anterior tibialis muscle on day 14. The mean values in Wistar and OXYS rats were significantly below the control, *i.e.* regeneration processes were not completed during this period in both groups.

The absence of significant differences between the experimental (regenerating) groups (Fig. 2, *b*) indicates retained regeneratory (proliferative and repopulating) potential of myogenic precursor cells in OXYS rats with chronic disorders of oxidative metabolism. The trend to MF hypertrophy (larger mean cross-section area) in OXYS rats from the control group corresponded to the results obtained previously in studies of other muscles [5].

Electron microscopy showed that MF regenerate at the intracellular and cellular levels throughout the experiment and their regeneration is associated with activation of nuclei in MF and satellite cells.

Activated status of the nuclei of the subsarcolemmal location manifested by an increase in their surface area due to scalloped nucleolemma, increased size and number of the nuclear pores, and appearance of 2-3 large nucleoli. Numerous small mitochondria with compact matrix and numerous cristae accumulated in the perinuclear zone at one or both poles of the nuclei, free ribosomes appeared, dilated tubules of the cytoplasmic reticulum were seen, and fine chaotically oriented filamentous structures were forming.

Direct ultrastructural signs of satellite cell activation were observed in some cases. At rest these were sharply thinned cells located between the basal plate and sarcolemma, containing flat nucleus with small marginal lumps of heterochromatin and solitary cytoplasmic organelles. The first structural signs of satellite cell activation were the appearance of the nucleolus and hyperplasia of the granular endoplasmic reticulum (Fig. 3, *a*). Later these cells acquired a barrel shape because of enlarged nucleus and mitochondrial hyperplasia, which provided the induction of protein-synthesizing function and phenotypical modification with the formation of elongated cytoplasmic processes.

Chains of nuclei in maturing myotubules and immature MF were located in the central part of the fiber, with a “train” of small mitochondria at their poles (Fig. 3, *b*). The myofibrillar system was represented by fine myofibrils, sometimes not quite leveled, because of which their Z-bands formed a wavy pattern.

Reparative regeneration of skeletal muscles is a multistaged process, the key events are resorption of cell detritus, activation, proliferation, differentiation, and fusion of myogenic precursor cells, revascularization, and innervation [8]. The prevalence and efficiency of individual phases and the entire process in general depend on the type and

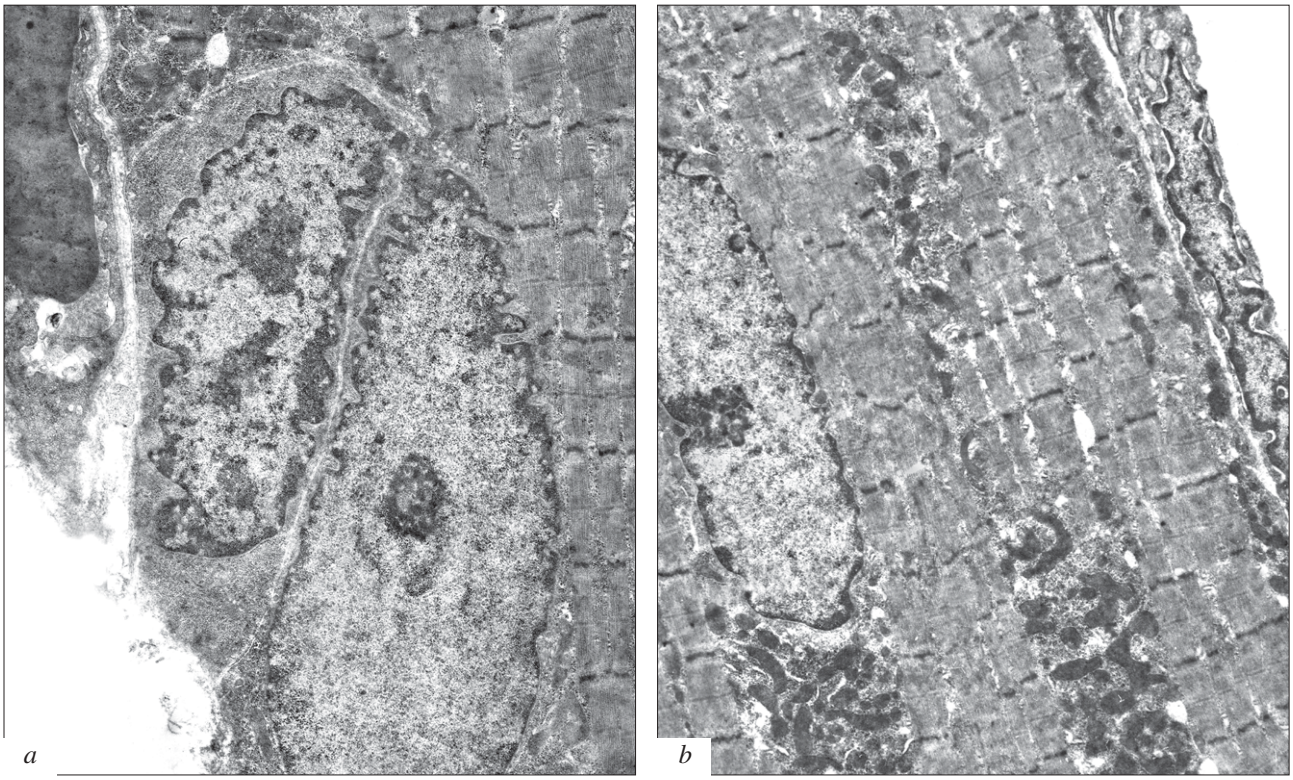

Fig. 3. Ultrastructural characteristics of reparative reactions of the skeletal muscles of OXYS (*a*) and Wistar rats (*b*) 7 days after BC injection. *a*) activation of satellite cell: scalloped nucleus, widened nuclear pores, formation of nucleoli. Similar changes in the myocyte nucleus, ×5000; *b*) a fragment of young MF. Central location of the nucleus, formation of intracellular regeneration zones, ×3000.

severity of the destructive factor, microenvironmental factors, proliferative and repopulating potentials of the satellite cells. Bupivacaine caused acute disorders in energy and calcium homeostasis [11], leading to rapid development of a wide spectrum of degenerative changes in MF [2]. However, the satellite cells were resistant to these effects [14] and hence, proved to be capable of providing subsequent recapitulation of the myogenesis program. It seems that BC exhibited no appreciable destructive effect on the natural microenvironment, essential for full-value regeneration of muscle tissue (the vessels, nerve endings, and basal membranes, forming the directing carcass for proliferating myoblasts and growing myotubules) [9]. This was confirmed by findings in semithin sections, when several fiber-tubules were surrounded by remnants of the basal membrane.

On the whole, normal dynamics of restoration of muscle tissue architecture after massive injury in early aging OXYS rats suggests that the imbalance between the destructive and reparative processes in these animals (augmenting MF degeneration at the age of 9 months) [1] is largely due to failure of the compensatory mechanisms inside the MF, disturbed regulatory interactions, and hence, insufficient level of "physiological" regeneration, but not reduction of the proliferative potential of precursor cells. Full-value regeneratory reaction in response to extreme damage indicates that significant reparative reserves are retained under these conditions and prompts the possibility of its stimulation [9] in order to support the compensatory processes and prevent the changes in the skeletal muscles, associated with aging and mitochondrial defects.

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