**RESPOSTA DA REGENERAÇÃO PÓS-TRAUMÁTICA DO MÚSCULO ESQUELÉTICO
À MEDICAMENTAÇÃO DE PELÓIDE HÚMICA****SKELETAL MUSCLE POSTTRAUMATIC REGENERATION RESPONSE TO HUMIC
PELOID MEDICATION****ПОСТТРАВМАТИЧЕСКАЯ РЕГЕНЕРАЦИЯ СКЕЛЕТНЫХ МЫШЦ В УСЛОВИЯХ
ПРИМЕНЕНИЯ ГУМИНОВОГО ПЕЛОИДОПРЕПАРАТА**

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RESUMO

Uma das tarefas relevantes da morfologia atual é a investigação do potencial regenerativo dos tecidos e a pesquisa de novos medicamentos que melhorem a eficiência do processo de recuperação. Atualmente, os especialistas clínicos se concentram em medicamentos baseados em compostos naturais. No entanto, tal influência de medicação nos processos que ocorrem dentro dos tecidos lesados ainda não é identificada. O objetivo deste estudo foi investigar a resposta da regeneração pós-traumática do tecido muscular esquelético à medicação de pelóide húmica, com base em ácidos húmicos modificados por íons zinco. A extração de ácido húmico foi realizada por meio de procedimento de patenteado. O estudo incluiu ratos de laboratório Wistar com hiperextensão do músculo fêmur frontal. As preparações foram estudadas por meio de microscopia, microscopia eletrônica e autoradiografia. A avaliação das preparações histológicas mostrou que, sob exposição ao medicamento pelóide, os processos de catabolismo das fibras musculares são inibidos, o edema intersticial fica restrito à área lesionada, o crescimento dos vasos na área dos capilares danificados é estimulado, os macrófagos migram, e a área e a duração da inflamação pós-traumática diminuem. Além disso, a ingestão de medicamentos de pelóide encurta o comprimento dos estágios de histogênese reparadora do tecido muscular esquelético: mio-satélites são ativados mais precocemente do que no grupo de controle, mioblastos e mioplastos são detectados, a separação de áreas sarcoplasmáticas nucleares de fibras musculares parcialmente lesadas é estimulada, miotúbulos aparecem entre 3 a 5 dias antes do que no grupo controle. No geral, a eficiência de regeneração do tecido muscular aumenta em 21%. Os resultados obtidos permitem concluir que a medicação pélvica baseada em ácidos pelóides húmicos modificados por íons zinco influencia positivamente a estimulação do processo de regeneração. Isso levará a uma investigação mais aprofundada das substâncias húmicas: fúlvica, himaomelânica, humina e ácidos húmicos de pelóides como medicamentos e sua implementação na prática clínica.

Palavras-chave: *tecido muscular esquelético, pelóides, regeneração reparativa.*

ABSTRACT

One of the relevant tasks of current morphology is the investigation of tissue regenerative potential and research for new medications that improve recovery process efficiency. Nowadays, clinical specialists focus on medications that are based on natural compounds. However, such medication influence on the processes that occur within the injured tissues is still not identified. The purpose of this study was to investigate skeletal muscle tissue posttraumatic regeneration response to humic peloid medication, based on humic acids modified by Zinc ions. Humic acid extraction was carried out by means of patent procedure. The study included laboratory Wistar rats with hyperextension of front femur muscle. The preparations were

studied by means of light and electronic microscopy and autoradiography. Histologic preparations evaluation showed that under peloid medication exposure the apolexis processes within muscle fibers rupture are inhibited, interstitial edema becomes restricted by the injured area, vessel growth into the area of damaged capillaries is stimulated, macrophages migrate and the area and duration of posttraumatic inflammation decrease. Additionally, peloid medication intake shortens the length of skeletal muscle tissue reparative histogenesis stages: myosatellitocytes are activated earlier than in the control group, myoblasts and myosimplasts are detected, the separation of nuclear sarcoplasmatic areas from partly injured muscle fibers is stimulated, myotubules appear 3 – 5 days earlier than in control group. Overall, muscle tissue regeneration efficiency increases by 21%. Obtained results allow us to conclude that peloid medication based on humic acids modified by Zinc ions positively influence the stimulation of the regeneration process. This will lead to further investigation of humic substances: fulvic, humatmelanic, humin and humic acids of peloids as medications and their implementation in clinical practice.

Keywords: *skeletal muscle tissue, peloids, reparative regeneration.*

АННОТАЦИЯ

В современной морфологии одной из актуальных проблем является изучение регенераторных возможностей тканей и поиск средств, улучшающих эффективность восстановительных процессов. В настоящее время внимание клиницистов все чаще привлекают средства, созданные на основе природных соединений, однако их влияние на процессы, происходящие в поврежденных тканях до сих пор неизвестны. Целью исследования является изучение посттравматической регенерации скелетной мышечной ткани в условиях использования пелоидопрепарата гуминовых кислот, модифицированных ионами цинка. Извлечение гуминовых кислот из пелоидов осуществляли по запатентованной методике. Объектом исследования были лабораторные крысы линии *Wistar*, которым проводили перерастяжение передней мышцы бедра. Материал изучали с помощью методов световой и электронной микроскопии, а также автордиографии. Изучение гистологических препаратов показало, что в условиях воздействия пелоидопрепарата процессы распада в области разрыва мышечных волокон ингибируются, в зоне повреждения ограничивается интерстициальный отек, стимулируется прорастание в зону повреждения кровеносных капилляров, миграция макрофагов, уменьшается площадь и продолжительность посттравматического воспаления. Одновременно применение пелоидопрепаратов способствуют ускорению стадий репаративного гистогенеза скелетной мышечной ткани: в более ранние сроки, чем в контроле активизируются миосателлитциты, обнаруживаются миобласты, миосимпласты, стимулируется отделение от частично поврежденных мышечных волокон ядерно-саркоплазматических территорий, по сравнению с контролем на 3 – 5 суток раньше появляются миотубы. Итоговая эффективность восстановления мышечной ткани увеличивается на 21%. Полученные результаты позволяют сделать вывод о положительном стимулирующем влиянии пелоидопрепарата гуминовых кислот, модифицированных ионами цинка на процессы регенерации. Это позволит продолжить дальнейшую разработку субстанций гуминового ряда: фульвовых-гуматомелановых – гуминовых – гумусовых кислот пелоидов в качестве лекарственных препаратов и рекомендовать их применение в клинической практике.

Ключевые слова: *скелетная мышечная ткань, пелоиды, репаративная регенерация.*

INTRODUCTION

Skeletal muscles that provide not only body movements but also a number of vital functions are influenced by numerous factors that may lead to muscle tissue damage and its malfunctioning.

Muscle tissue regeneration and responsiveness issues are widely discussed in classic histological literature (Mauro A., 1961; Carlson, 1973; Schmalbrach Y., Hallhammer U., 1977; Klishov A. A. 1971; Danilov R. K., 1982; Schudlo N.A. *et al.*, 2014). There are detailed descriptions of the origin, means and stages of

muscle tissue regeneration (Tulaeva O. N., 2003; Nepomnyaschikh L. M., Bakarev M. A., 2005; Yamschikov N. V. *et al.*, 2011; Chernova O. N. *et al.*, 2015), however, it is still acute to develop new means of regeneration process stimulation. Research of new ways of synthetic processes stimulation is being carried out in different directions. Numerous works are dedicated to the study of different physical impact factors (Plaksina L. N., Ukhov Y. I., 2001; Bulyakova N. V., 2004; Tulaeva, Bovtunova S. S., 2004), exercise load factors (Kuznetsov S. L., Papas E.A., 1999; Morozov V. I. *et al.*, 2006), biotic factors (Stadnikov A. A., Shevlyuk N. N., 2006) and a number of

pharmacologic factors (Terekhov A. Y., 2005; Ushabev O. I. *et al.*, 2007; Balaev T. A., 2011; Rabovskaya L. A. *et al.*, 2011).

Over the recent years, biologically active preparations have been intentionally used in clinical practice to stimulate the tissue recovery, to supplement the consumed macronutrients and to manage the most important organism functions selectively during major physical loads and injuries (Plechewa D. V. *et al.*, 2018; Dmitriev A., Kalinichev A., 2017).

Medication stimulatory effect improves blood flow and metabolism in tissues and activates granulation and epithelization. Traditionally, among pharmacological means of recovery multivitamin and macronutrient preparations are used. Medications that stimulate reparative regeneration are used in the form of creams, ointments, and gels. It should be noted that most of them contain nonindifferent to the organism substances as the main ingredient like steroids, anabolics, vitamins, and hematogenesis stimulators that can lead to different allergic reactions and other negative responses (Bachmaier M. *et al.*, 2015).

Alternative medications without the mentioned side effects are based on natural compounds. The high biological activity of peloids is well known. Their humic compounds are described as a group of complex natural organic compounds that are formed as a result of biochemical transformations of animal and plant residues under the influence of climate factors and microorganisms. Theoretical and applied medicine is particularly interested in sulfide muds, which are characterized by a long period of biota biological activity in humid conditions, weak-base solution, and negative redox potential of the mud solution. Humic compounds content is highest in low mineralized silt sulfide muds. In the group of humic substances the most fluent acid fraction is extracted; these humic acids are divided by water solubility into humic and fulvic acids depending on the solution acidity (Platonov V. *et al.*, 2014; Kuzminova E. V. *et al.*, 2015).

Broad spectrum therapeutic effects of humic acids are explained by their structural polymorphy. They are high-molecular polydisperse polyheterofunctional systems that include an aromatic ring and aliphatic peripheral fragments. Humic acids are characterized by polymolecularity, stochasticity, and structural disarray. Their macromolecules include electron donating and electron accepting substituents

that are capable of forming charge-transfer complexes (Ubashev I. O., 1997; Verba O. Yu., 2005).

Electrolytic activity is an important property of humic peloid medication that allows reducing ion balance inside the damaged body cells, which leads to normalizations of biopotentials (Khasanov V. V., 2006).

The developed peloid medications based on biologically active natural compounds that come in various formulations (solutions, suppositories, ointments), are neutral to the organism, possess high biological activity, and do not provoke allergic reactions. It was proved experimentally that humic acids are not toxic. No negative reactions were reported when administered orally at the dose of 15 g/kg of tested animals (Avvakumova N.P. and Krivopalova N.A., 2012).

Due to their structural organization, humic acids are considered to be a perspective group of compounds that can be modified by different bioelements and, thus, the desired biological effect can be obtained from the received complexes. The developed formulations, as purified concentrates of main active substances of peloids, are made more active by means of adding components that provide additional target activity (Buzlama B. C., Shabunin S. V., 2007; Avvakumova N. P., Krivopalova M. A. *et al.*, 2012).

There is no data in the scientific literature on skeletal muscle tissue posttraumatic regeneration response to humic peloid medication, based on humic acids modified by Zinc ions. The choice of Zinc compounds in the present study is based on active participation of this bioelement in the synthesis of more than 40 metalloenzymes. It should be noted that Zinc containing medications show antimicrobial activity and immunomodulatory effect; their administration activates the cell and tissue growth (Romanteeva Y. V., 2006; Obukhova O. V., 2016).

Scientific data allows predicting the therapeutic activity of humic acid peloids, modified by Zinc ions, on skeletal muscle tissue posttraumatic regeneration.

The purpose of this study was to investigate skeletal muscle tissue posttraumatic regeneration after experimental hyperextension of m.biceps femoris underexposure of humic acid peloids, modified by Zinc ions.

MATERIALS AND METHODS

Humic acid is extracted from peloids by means of patented procedure (Avvakumova N. P. *et al.*, 2011).

Air dry weights were diluted in a minimal volume (0.01 mol/dm³) of sodium hydroxide solution and further kept for 2 hours on 35-40°C water-bath. The obtained solution was filtered through a paper filter (white strip) and neutralized with a hydrochloric acid solution (0.01 mol/dm³) to pH 7.36. Zinc humate was obtained by adding the estimated volume of 1% zinc chloride solution so that the obtained solution contained 5 mg of Zinc ions for 1 g of humic acids. The obtained solution of zinc humate was diluted with distilled water to get 0.1% zinc humate solution. Peloid formulation is a transparent liquid of red-brownish color with a slight specific smell.

The medication was tested on 130 – 150g white Wistar rats. Ether anesthesia was applied, and rats' skin was cut 1.5 cm along the front femur muscle after that 1/3 of the front femur muscle was separated by means of dissector and fixed with Bülroth's forceps. Further dosed extension of the separated part of the muscle was carried out by extending the forceps jaws up to 3 cm. The extension was stopped when the muscles lost resistance to jaws extension, which generally happened after 2.5 – 3 mins. The wound was closed with corner caprofile stitches. The first test group of rats was administered 1 ml of the tested medication s/c in the thigh for 6 days. The rats in the control group didn't receive the medication.

The probes for histological assay were taken on the 3, 5, 7, 11, 15, 20 and 30th day, the samples were placed into 10% formalin, poured with paraffin and iron hematoxylin stained with haematoxylin and eosin according to Van Gieson's method. The relative area of necrosis and area of muscle tissue within the regenerated was measured by the stereometric net (Avtandilov G. G., 1973).

DNA synthetic activity was detected by a histoautoradiographic method using ³H thymidine (specific radioactivity 240 tB, foreign trade association Isotope). The autoradiographs were developed in amid developing solution.

For submicroscopic examination, the samples were prefixed in 2.5% glutaraldehyde solution with 0.1 M phosphate buffer and fixed in 1% osmium tetroxide. After dehydration, the samples were covered with araldite. Ultrathin

sections were contrasted by 2.5% uranyl acetate solution. The sections were studied under electron microscope Hitachi-9.

RESULTS

Standard processes were observed after the muscle's injury in the control group: muscle fibers necrosis, activation of non damaged or partly damaged muscle fibers accompanied by myosatellitocyte release, myoblasts, and myoblasts formation and further development of new muscle fibers.

Muscle hyperextension is followed by local alteration of muscle fibers. Generally, injured areas go along muscle fibers with very few single fibers remaining not damaged. Muscle injury leads to major interstitial edema development and necrotic contraction nodes formation in muscle tissue. Zenker's degeneration is spread in muscle fibers within the first day after the injury. Since hyperextension leads to non-uniform damage, the necrosis is observed in several sites along muscle fibers, and single fragments keep their structural integrity for some time. The spaces within the necrotic fibers are filled with leukocytes and macrophages that provide necrotic tissue lysis and clear the spaces from cell debris (Figure 1).

Pyknotic nuclei appear in the damaged muscle fibers, myofibrils adhere and become homogenous. Myofibrillar protein coagulation leads to protein fusion that is shown ultrastructurally as electron-dense areas. Such areas are surrounded by macrophages that phagocytize necrotic muscle fibers (Figure 2).

On the 3rd day after the injury, myosatellitocytes are activated in partly untouched fibers. The myosatellitocytes are well-seen light-optically and electronic microscopically due to a large light nucleus and their disposition under the basal membrane of a muscle fiber (Figure 3).

Muscle tissue destruction processes continue up to 7 – 9 days in the control group, muscle fibers necrosis area is being filled with phagocytes, blood capillaries grow in between muscle fibers, activated fibroblasts quickly fill in interstitial space with collagen fibers. Simultaneously, reparative muscle tissue histogenesis processes begin. Myosatellitocytes that separated from muscle fibers differentiate into myoblasts, which are divided mitotically.

As a result, post-synthetic myoblasts merge into myosimplasts, which start synthesizing contractile proteins and assembling myofibrils, and by the 9th day after the injury, myosimplasts look like long thin

tubes with light nuclei in the center (Figure 4).

Further differentiation involves myosimplasts transformation into myotubules and young muscle fibers. Fiber sarcoplasm is filled with myofibrils and mitochondria in between them first loosely and later more densely (Figure 5).

Interstitial edema remains in the control group up to 20th day from the experiment beginning. Collagen fibers fill the spaces between young muscle fibers forming a connective tissue scar by the 30th day.

Test group observation showed that under zinc humate exposure necrotizing processes at the site of muscle fibers rupture were significantly inhibited. The area of necrotic changes decreases by 9.7% in comparison with the control group. The duration of muscle fibers degradation shortens by 3-4 days in comparison with the control group. At the same time, tissue macrophages migration and function is stimulated. As a result, this increases necrotic products resorption. Interstitial edema is limited, numerous capillaries grow into the damaged area on the 3rd day, and the duration of posttraumatic inflammation shortens (Figure 6).

In its turn, early clearance of the muscle wound from necrotic debris is beneficial for myogenic elements development. Partly untouched basal membranes of the damaged muscle fibers act as a guide frame for the developing muscle fibers.

Muscle fibers degeneration processes are followed by regeneration processes. Myoblast, myosimplasts, myotubules and muscle fibers formation was observed in both animal groups according to the reparative histogenesis stages.

Zinc humate administration stimulated earlier myosimplasts synthesis in comparison with the control group. Myoblasts and separate myosimplasts were detected on the 3rd day at the site of hyperextension and necrosis of muscle tissue. Myosimplasts in the test group, as in the control group, had oxyphilic cytoplasm and contained up to 3 – 5 light round nuclei. Myosimplasts appear in damaged areas as well as in partly damaged or nondamaged muscle fibers (Figure 7).

Thus, peloid medication administration promotes earlier myotubules formation (by 3 – 5 days) in comparison with the control group. Myotubules are thin, long, multinuclear symplasts with centrally located nuclei and peripherally oriented myofibrils. Zinc humate administration seems to stimulate biosynthetic processes in myosimplasts.

Under test medication exposure, the formation of young muscle fibers results from the differentiation of myotubules on the 7th day

after the experimental hyperextension. By contrast, in the control group, this process starts on the 10th day. Young muscle fibers, like in the control group, are assembled incoherently on the edges of the regenerate being formed (Figure 8). Developing loose fibrous tissue forms the central part of the regenerate.

Additionally, peloid medication administration stimulates the separation of nuclear sarcoplasmic areas from partly damaged muscle fibers (Figure 9), which also seem to be a part of the myosimplast structure.

Besides, in the test group the processes of myosatellitocytes activation are prolonged, even on the 9th day from the beginning of the experiment there are still seen DNA-synthesizing nuclei in muscle fibers (Figure 10).

As a result, by the 30th day after hyperextension zinc humate administration leads to the increase of newly synthesized muscle tissue by 21.5% in comparison with the control group.

DISCUSSION

The kinetic data obtained by the limited solution volume method made it possible to determine the optimal impregnation time of a macroporous polymeric carrier with technical TAA, that is 25-35 h. The presence of a highly volatile diluent (acetone) contributes to the reduction of the extractant layer formation rate on the carrier surface, thereby decreasing the sorption characteristics over time. On the contrary, the temperature increase leads to the increase in the impregnation rate, since the extractant viscosity reduces without the injection of additional components of the organic phase (diluent). This can be confirmed by the rate constants, calculated according to the data of linearized integral kinetic curves with the aid of a pseudo-second-order model.

The impregnation process takes place in the diffusion region, since the apparent activation energy of the process is relatively low (37 ± 4 kJ/mol). However, the kinetic data, described with a high degree of correlation by means of the pseudo-second order and Elovich models, is indicative of the contribution of TAA physical adsorption on the carrier and the possible interaction of the extractant molecule with two sorption centres of the carrier.

To shorten the impregnation time, it is reasonable to carry out the process of obtaining impregnates at an elevated temperature.

CONCLUSIONS:

The results obtained in this study show that peloid medication administration in posttraumatic period decreases degradation processes in the damaged muscle fibers and activates the processes of skeletal muscle tissue reparatory regeneration. As a result, muscle, as an organ, increases its regeneration efficiency.

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Figure 1. Control preparation. 3rd day. Necrotized muscle fibers, the damaged area is filled with leukocytes. Haematoxylin-eosin staining. Ob.40, ok. 10.

Figure 2. Control preparation. 3rd day. Ultrastructure of the damaged muscle fiber, a macrophage is observed nearby. x7000

Figure 3. Control preparation. 5th day. The ultrastructure of a myosatellitocyte (Msc) that is partly separated from the damaged muscle fiber (MB). x7000

Figure 4. Control preparation. 9th day. Young myosymplast is located between necrotized muscle fibers. Haematoxylin-eosin staining. Ob.40, ok. 10.

Figure 5. Control preparation. 15th day. Myotubule ultrastructure. x7000.

Figure 6. 3rd day of the experiment. Test preparation (zinc humate administration). The damaged area is densely vascularized. Tissue macrophage mass release. Haematoxylin-eosin staining. Ob. 40. ok. 10

Figure 7. 7th day of the experiment. Test preparation (zinc humate administration). Myosin plasmid is seen in partly damaged muscle fibers. Haematoxylin-eosin staining. Ob. 40. ok. 10

Figure 8. 7th day of the experiment. Test preparation (zinc humate administration). Newly formed myosin plasmids and young muscle fibers. Haematoxylin-eosin staining. Ob. 40. ok. 10.

Figure 9. Test group. 7th day. Ultrastructure of a partly damaged muscle fiber (MB); nuclear sarcoplasmic area (Яст) is being separated; nearby lies separated myoblast (M6). x7000.

Figure 10. 7th day of the experiment. Test preparation (zinc humate administration). DNA-synthesizing cells within activated muscle fibers. Iron haematoxylin staining. Ob. 90. ok. 10.