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Effect of Hyperbaric Oxygen Treatment on Myogenic Transcriptional Factors in Regenerating Rat Masseter Muscle

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A B S T R A C T

Hyperbaric oxygen (HBO) treatment was shown to be beneficial as an additional treatment for ischemic muscles in crush injuries and ischemia-reperfusion injuries. The purpose of this study was to assess the influence of hyperbaric oxygen treatment on transcriptional myogenic factors during muscle regeneration. Those factors (MyoD, myf5, myogenin, mrf4) are essential for determination and differentiation of skeletal muscle tissue and together with several other factors control gene expression during myogenesis. The process of regeneration in rat masseter muscle was provoked with injection of local anesthetic bupivacaine hydrochloride. Following injection, the animals were treated once daily in hyperbaric chamber from one to ten days and than sacrificed. Immunohistochemical and Western blot analysis of frozen masseter muscle samples showed a transient upregulation of myoD and myogenin transcriptional factors in the muscles of hyperbaric oxygen treated rats and of rats that have not been treated after the injury. HBO treatment had no effect on the expression of MyoD and myogenin transcriptional factors in the regenerating rat masseter muscle.

Key words: *hyperbaric oxygen treatment, rat muscle, regeneration, myogenic regulatory factors*

Introduction

As hyperbaric oxygen (HBO) treatment was shown to be favorable as an additional therapy for ischemic muscle injuries[1–5], various experimental models served to examine the influence of HBO on the course of muscle repair following some other types of muscle injuries or conditions[6–11]. Skeletal muscles consist of bundles of contractile myofibres containing hundreds of peripherally located post-mitotic nuclei. In spite to this fact, these organs have the capacity of plastic adaptation to functional demands as well as an ability to regenerate itself following injury. The cells responsible for providing myonuclei for postnatal growth or repair are mononucleated satellite cells. They become activated and then divide leading to the formation of new myofibers or to repair of existing ones. Myogenic lineage progression of satellite cells and their myoblast progeny is regulated by various transcriptional factors, including muscle specific basic helix-loop-helix (bHLH) regulatory factors. The family of bLHL myogenic factors consists of MyoD, myf5, myogenin and mrf4. These factors contribute to the transcriptional activation of muscle genes during commitment and differentiation of muscle fibers. They are involved in the process of muscle regeneration either on the protein[12–16] or on the transcriptional level[13,17–19].

The aim of this study was to analyze the influence of hyperbaric oxygen treatment on the expression of MyoD and myogenin during regeneration of rat masseter muscle.

Material and Methods

Animals and experimental procedure

Male Sprague-Dawley rats weighing 200–250 g, used in this study, were kept in standard cages at constant temperature and were given water and food ad libitum throughout the experimental period. The study was approved by the Ethics Committee of the Medical Faculty of

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the University of Rijeka. The animals were anaesthetized with an intraperitoneal injection of ketaminhydrochloride (0.1 mg/g body weight) and xylazinhydrochloride (0.02 mg/g body weight). Regeneration was induced by injection of local anesthetic bupivacaine hydrochloride into the masseter muscle an incision overlying the masseter muscle was done and up to 1mL of 0.5% bupivacaine was injected into the superficial part of the muscle. Thereafter the rats were divided in two groups: the hyperbaric and the non-hyperbaric group. After the bupivacaine hydrochloride injection, the experimental hyperbaric group of animals underwent HBO treatment. They were placed in hyperbaric experimental chamber in which pure oxygen was administered at 2.2 ATA pressure in a sixty-minute session. The sessions started on the day of surgery and were conducted once daily. Animals were sacrificed after 1, 2, 3, 4, 5, 7 or 10 days of treatment. Rats in the non-hyperbaric group received no further treatment after bupivacaine injection and were kept in cages until they were sacrificed after 1, 2, 3, 4, 5, 7 or 10 days. The oxygen concentration in the hyperbaric chamber maintained ≥98.5% (Dräger Oxymeter). Sodium carbonate crystals were used to reduce the accumulation of CO_2. All rats were killed by the ether inhalation. The masseter muscle was removed and quickly frozen in isopentane cooled by liquid nitrogen. The masseter muscles of intact rats served as controls to the experimental muscles, i.e. to hyperbaric and non-hyperbaric muscles. The muscles were stored at –80°C until further analysis. For histological analysis, serial 8 μm-thick transverse sections were cut and stained with haematoxylin and eosin (HE).

Protein extraction and western blotting procedures

For MyoD and myogenin immunoblotting, the total muscle protein containing cytoplasmatic and nuclear fractions was extracted from masseter muscles by pulverization of frozen samples. The samples were immersed in a homogenizing buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS. 0.1mM phenylmethylsulfonylfluoride was added at the time of use at 4°C. The samples were incubated for 30 min at 4°C. Each homogenate was transferred to microfuge tubes and centrifuged at 13000 rpm for 20 min at 4°C, and the supernatant was obtained. An aliquot of the supernatant was used to determine the protein concentration using Bredford method, and the remainder of the supernatant was stored at –80°C for subsequent Western blotting analysis. Optimal loading concentration for immunoblotting was found to be 85 μg and 75 μg per sample for MyoD and myogenin, respectively. To verify MyoD and myogenin protein bands two control standards were ran simultaneously in each gel: a molecular weight marker (Precision Protein Standards) and protein isolated from neonatal rat muscles (highly expressing both myogenic regulatory factors). Proteins were denaturated by boiling for 3 min after adding 5% solution for denaturation (500 μL Leammli, 25 μL 2-mercaptoethanol). Proteins were separated in either a SDS-10% (MyoD) or 12.5% (myogenin) polyacrylamide gel. The proteins were transferred to nitrocellulose membranes for 3 h at 70 V. The membranes were immersed in blocking solution containing 0.5% nonfat dry milk (Bio-Rad), dissolved in TBS (pH 7.46), for 1 h. Then the membranes were incubated either with the monoclonal mouse anti-MyoD (1:400) (DakoCytomation) or monoclonal mouse anti-myogenin (1:400) (DakoCytomation), diluted in blocking solution overnight at 4°C. After that, the membranes were washed 6×10 min in TBS with 0.1% Tween 20 and incubated for 1 h at room temperature with a secondary antibody (rabbit anti mouse conjugated with peroxidase 1:2000) (DakoCytomation). Then the membranes were washed 5x with TBS with 0.1% Tween 20 for 1 h. The antibody bindings were visualized with DAB (diaminobenzidin, 0.1 mg/mL), 0.02% hydrogen peroxide and 0.03% CoCl. The quantity of blotted proteins was evaluated by scanning densitometry.

Immunohistochemistry

For the MyoD and myogenin, the slides were fixed in 2% paraformaldehyde for 10 min, washed in TBS, and treated with 3% H_2O_2 in TBS with 0.1% Tween 20 (TBST) for 5 min. Then the cryosections were washed in TBS for 5 min. After that the slides were immersed in blocking solution (10% normal swine serum in TBS with 0.2% bovine serum albumin – TBSTB) for 20 min. Afterwards, the slides were incubated for 60 min with the primary mouse monoclonal antibody anti-MyoD (DakoCytomation) or primary mouse monoclonal antibody anti-myogenin (DakoCytomation) diluted 1:50 in TBST. The sections were washed 2×5 min in TBST, treated with the secondary biotinylated antibody for 15 min and with streptavidin conjugated to peroxidase for 15 min (LSAB+ Kit, DakoCytomation, ready-to-use). Finally, the sections were washed in TBS, visualized with DAB for 10 min. After rinsed in water, the sections were mounted in glycerol gelatin medium.

Statistics

All data are expressed as means and SE. The results within each group and between groups were compared using a two-way analysis of variance and the Tukey-Kramer multiple comparisons post hoc test. The level of significance was set at $p<0.05$.

Results

Histology of regenerating muscle

The first day after the bupivacaine injection, muscle fiber degeneration and the presence of cellular infiltration was evident. On the second and third day, necrotic muscle fibers were phagocytozed by macrophages and during the following days, the amount of these cells decreased. Sporadic islets of preserved fibers were dispersed over the cross section. On the fourth day, small new myofibres with central nuclei began to appear becoming more numerous and larger for several subse-

quent days but ten days after injury, still remained smaller than undamaged fibers. Space among fibers became narrower (Figures 1–3).

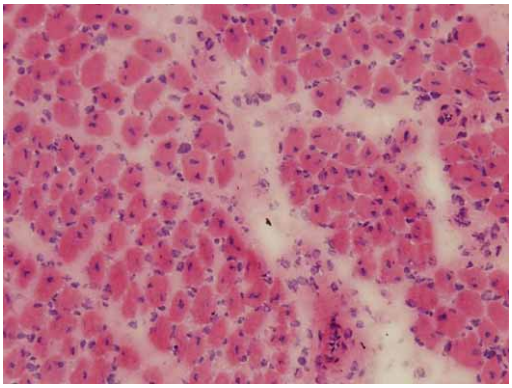

Fig. 1. Cross-section of masseter muscle seven days after bupivacaine injection. Small regenerating muscle fibers with central nuclei. (H&E staining; original magnification: ×400).

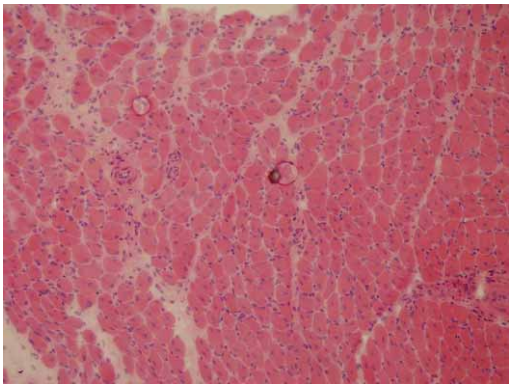

Fig. 2. Cross-section of masseter muscle ten days after bupivacaine injection. Small regenerating muscle fibers with central nuclei. (H&E staining; original magnification: ×200).

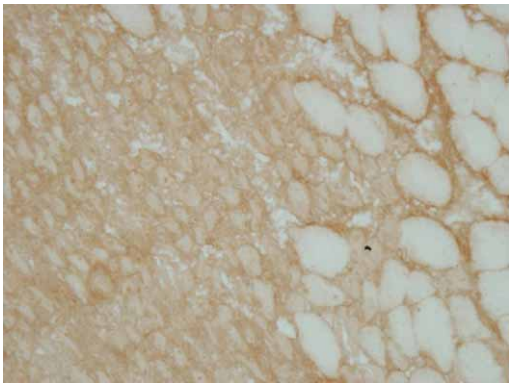

Fig. 3. Immunohistochemical staining with anti-MyoD antibodies. Masseter muscle on the seventh day after bupivacaine injection. No MyoD positive nuclei. Muscle fibers in regenerating portion on the left side are smaller than undamaged muscle fibers on the right side. Original magnification: ×400.

MyoD and myogenin protein expression

MyoD and myogenin proteins were not detectable in undamaged masseter muscles using either the Western blot analysis or the immunohistochemical method. Their expression was examined during sequential ten days of the regeneration process in the masseter muscles. The level of MyoD protein expression in regenerating masseter muscle determined by Western blot analysis increased on the second day. The highest level of expression was observed on the third day and than declined towards the 5th day (Figure 4). The immunoreactive band for myogenin protein was detected two days after injury, on the 3rd day the level of expression was the highest and then diminished towards the fifth day (Figure 5). There were no significant differences in the MyoD and myogenin expression levels in the masseter muscles between hyperbaric treated group and damaged but not hyperbaric treated group.

On the second day after the regeneration was provoked, MyoD positive cells, presented by the immunohistochemical method with anti-MyoD antibodies, became visible in the regenerating regions. Day after, the number of MyoD positive nuclei was even higher. Thereafter it diminished and on the seventh day was not present. The myogenin positive cells were observed on the second day after bupivacaine hydrochloride injection, and were more abundant on the third day, thereafter declining. MyoD and myogenin positivity was present in the regenerating portions, but also rare positive cells were seen between and in undamaged fibers adjacent to regenerating portion (Figure 6).

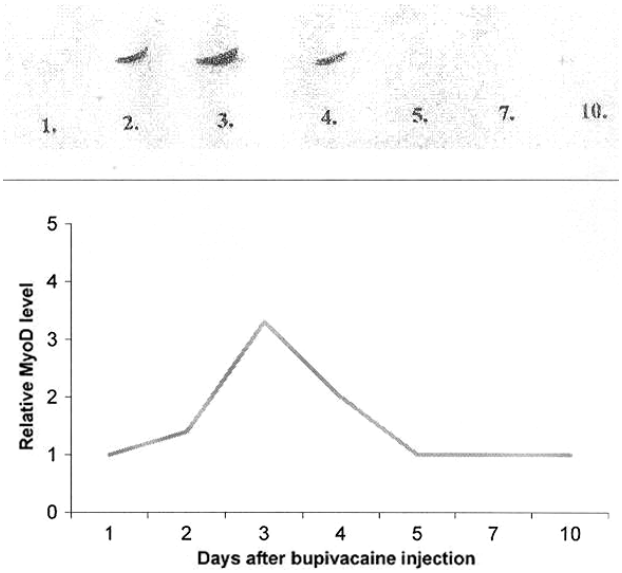

Fig. 4. MyoD expression determined by Western blot in regenerating masseter muscle of untreated rats. The levels of MyoD protein expression in the masseter muscles after 1–5, 7 and 10 days of regeneration. The immunoreactive band of ~45 kDa corresponded to the predicted molecular mass of the rat MyoD protein. The density of each band was expressed relative to the background density (considered to be 1.0).

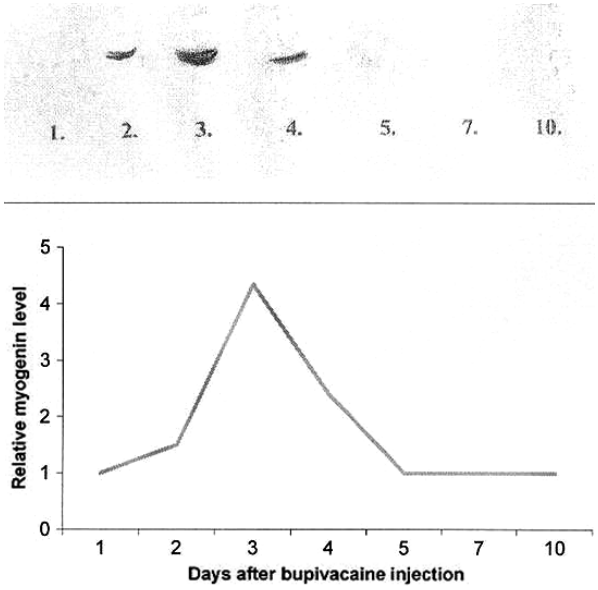

Fig. 5. Myogenin expression determined by Western blot in regenerating masseter muscle of untreated rats. The levels of myogenin protein expression in the masseter muscles after 1–5, 7 and 10 days of regeneration. The immunoreactive band of ~34 kDa corresponded to the predicted molecular mass of the rat myogenin protein. The density of each band was expressed relative to the background density (considered to be 1.0).

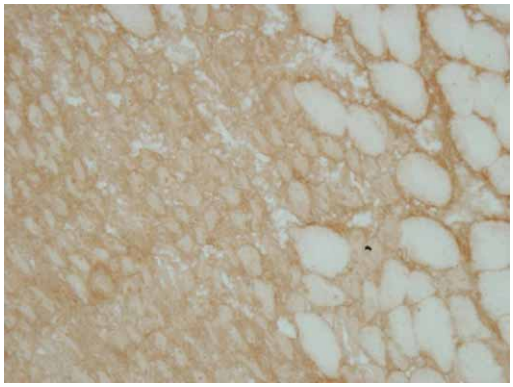

Fig. 6. Immunohistochemical demonstration of myogenin expression in cross-section of masseter muscle demonstrated on the third day after bupivacaine injection. Note the myogenin positive nuclei in regenerating portion. Rare myogenin positive cells between and in preserved muscle fibers adjacent to regenerating portion (arrows). Original magnification: ×200.

Discussion and Conclusion

Our study has shown that myogenic transcriptional factors MyoD and myogenin increased in early period of regeneration in rat masseter muscle injected with local anesthetic bupivacaine hydrochloride. The level of MyoD protein expression increased on the second day, was the highest on the third day and than declined. The myogenin protein was detected two days after injury, on the 3rd day the level of expression was the highest and then diminished towards the fifth day. These results coincide with the results of several other studies in which limb muscles were damaged myotoxically[16] or by freezing[12], although they appeared already one day after injury. Similar results with the MyoD myogenic factor got Mc Loon et al. in myotoxically damaged orbicularis oculi muscle, although its expression remained increased seven days that could be attributed to severe injury with two doses of bupivacaine[14]. According to some authors, the masseter muscle regenerate weaker than limb muscles[13,20]. The masseter muscle displayed weaker regenerative response than tibialis anterior muscle when was damaged by freeze injury; this effect might be due to lower number of myoblasts in masseter muscle[13]. But the freeze injury provokes more severe damage, while after the bupivacaine injection satellite cells remained preserved and muscle regenerate rapidly[21]. In our study, ten days after bupivacaine injury, histological assessment of injured muscle has implied satisfactory process of regeneration.

Hyperbaric oxygen treatment improved the contractile capacity and fiber size of extensor digitorum longus muscle[8] and soleus muscle[7] injured with bupivacaine injection. This effect was achieved when the treatment lasted 90 min for 25 days at 2 ATA or 14 days at 3 ATA. As we were interested in the influence of hyperbaric oxygen at the early period of myogenesis, when myogenic regulatory factors are involved, our treatments lasted up to ten days. The protocol applied in our study consisted of treatments that lasted sixty minutes at 2.2 ATA pressure in order to minimize possible oxidative stress, although the treatment applied in their study was within the range of values of the protocols commonly used in clinical treatments. As exposure to hyperbaric oxygen had mobilized stem/progenitor cells from the bone marrow and increased the number of circulating cells expressing CD34 and stem cell antigen-1 in mice[22], we presumed that hyperbaric oxygen treatment probably could enhance the activity of satellite cells as well, and therefore the expression of myogenic regulatory factors.

In conclusion, we have demonstrated that muscle regeneration caused a transient upregulation of MyoD and myogenin transcriptional factors in rat masseter muscle. The role of these transcriptional regulatory factors is important in managing a regenerative response of muscle fibers following bupivacaine hydrochloride injection. The treatments of hyperbaric oxygenation have not altered the expression of myoD and myogenin transcriptional factors in damaged rat masseter muscle.

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UČINAK TRETMANA HIPERBARIČNIM KISIKOM NA TRANSKRIPCIJSKE FAKTORE REGENERIRAJUĆEG M. MASSETER U ŠTAKORA

SAŽETAK

Tretmani hiperbaričnim kisikom su se pokazali korisnima kao dodatni tretman za ishemične mišiće u *chrus*-ozljedama i ishemijski-reperfuzijskim ozljedama. Svrha ovog rada je bila procijeniti utjecaj tretmana hiperbaričnim kisikom na transkripcijske miogene faktore tijekom mišićne regeneracije. Ovi faktori su bitni u određivanju i diferencijaciji skeletnog mišićnog tkiva, te zajedno s nekoliko drugih faktora kontroliraju gensku ekspresiju tijekom miogeneze. Proces regeneracije u m. masseter štakora izazvan je injekcijom lokanog anestetika bupivakain hidroklorida. Nakon toga su životinje bile tretirane u hiperbaričnoj komori jednom dnevno od jedan do deset dana i potom žrtvovane. Druga skupina životinja bila je žrtvovana u istim danima kao i prethodna skupina, ali bez tretmana hiperbaričnim kisikom. Imunohistokemijska i Western blot analiza na smrznutim uzorcima mišića pokazale su prolazno povećanje mioD i miogenin transkripcijskih faktora u obim skupinama životinja. Tretman hiperbaričnim kisikom nije utjecao na ekspresiju mioD i miogenin transkripcijskih faktora u regenerirajućem mišiću, m.masseter, štakora.