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# Influence of Hyperbaric Oxygen Treatment on Myogenic Transcriptional Factors of Denervated Rat Muscle

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## ABSTRACT

*The aim of this study was to determine whether hyperbaric oxygen (HBO<sub>2</sub>) treatment influences the expression of transcriptional myogenic factors in denervated rat's extensor digitorum longus muscle. Thus, expressing regulatory myogenic factors MyoD and myogenin were analyzed in denervated muscles (up to 30 days). Second group of denervated rats were afterwards treated with HBO<sub>2</sub>. Normal, innervated muscles were used as controls. Western blot analysis showed a significant upregulation of MyoD and myogenin proteins in denervated muscle during this period. Denervated muscles of rats exposed to HBO<sub>2</sub> treatment had also significant upregulation of both transcriptional factors but the treatment had not altered their expression. The immunohistochemical analysis showed MyoD and myogenin protein expression through this period in the denervated, untreated muscles and in denervated muscles of rats treated with HBO<sub>2</sub>, too. One month denervation caused a reduction in muscle fiber cross-sectional area. The treatment with HBO<sub>2</sub> had not reduced the degree of atrophy. The protocol of hyperbaric oxygenation (HBO) applied in this study had no beneficial effect either on transcriptional myogenic factors or on atrophy of denervated rat muscle.*

**Key words:** hyperbaric oxygen, denervation, myogenic transcriptional factors, rat

## Introduction

Hyperbaric oxygen (HBO<sub>2</sub>) treatment is used as a primary therapy for many years in several medical conditions and serves as an additional therapy to drugs and other therapies for different disorders, especially those caused by local hypoxia or ischemia. During HBO<sub>2</sub> treatment, the patient exposed to increased pressure, breathes pure (100%) oxygen under pressure at a higher level than the normal atmospheric pressure. That treatment increases amount of oxygen dissolved in the plasma and its delivery to the tissues. About forty percent of an adult's human body is comprised of skeletal muscles<sup>1</sup>. The musculature is often susceptible to trauma and affected by different diseases. HBO<sub>2</sub> was shown to be beneficial as an adjunctive treatment for ischemic muscles in crush injuries, compartment syndrome and ischemia-reperfusion injuries<sup>2–8</sup>, although, in some studies HBO<sub>2</sub> had no influence on healing<sup>9</sup>. The data about the effect of hyperbaric oxygenation (HBO) on the healing of myoto-

xically damaged rat muscle<sup>10–12</sup>, or on the activity of metabolic enzymes differ. Effects of HBO on fiber types of muscles from diabetic rats<sup>13</sup>, and healthy<sup>14–17</sup> or young rats<sup>18</sup> are unequal, too. Some report of no effect, the others, on the contrary found a beneficial effect of such treatment.

The regenerative capacity in adult skeletal muscles depends on the capability of quiescent satellite cells to proliferate, differentiate, and thus regenerate damaged muscle tissue. Following injury, overloading, and exacerbated stretching, these cells become activated. During this process, specific myogenic transcriptional factors are up regulated in myogenesis. These transcriptional factors are myf5, MyoD, myogenin and MRF4. In spite the unequal results dealing with expression of myogenic transcriptional factors in denervated muscles, in most of the studies MyoD or myogenin expression was found to be increased on transcriptional<sup>19,20</sup> or protein level<sup>21–26</sup>.

To our knowledge, as available from literature overview, the studies describing the influence of the HBO on denervated muscle by itself have not been found (only as an indicator of the function of crushed nerve treated with HBO<sub>2</sub>). Thus, the aim of the present study was to determine whether the HBO influences the degree of atrophy and expression of myogenic transcriptional factors MyoD and myogenin in denervated rat extensor digitorum longus (EDL) 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 given water and food *ad libitum* throughout the experimental period. The Ethics Committee of the Medical Faculty of the University of Rijeka approved the study. The animals were anaesthetized with an intraperitoneal injection of ketaminhydrochloride (0.1 mg/g body weight) and xylazinhydrochloride (0.02 mg/g body weight). Denervation was performed by removing a 5-mm segment of the right sciatic nerve high in the thigh region. The proximal and the distal stump were ligated and then sutured into surrounding muscles, far from each other to prevent reinnervation. The denervated rats were randomly divided in two main groups, denervated and denervated hyperbaric group. After being operated, rats in the denervated group received no further treatment and were kept in cages until they were sacrificed after 3, 15 or 30 days of denervation. The animals of the experimental hyperbaric group underwent HBO treatment after surgery and were placed in hyperbaric experimental chamber in which pure oxygen was administered at 2.2 atmospheres absolute (ATA) pressure in a sixty-minute session. Six rats were exposed at a time. The sessions started on the day of operation and were conducted once daily, always at the same time. The hyperbaric group was also subdivided in three groups that matched to the groups of denervated rats. The first and second group was respectively treated for three or fifteen consecutive days before being sacrificed. The rats in the third hyperbaric group were also exposed to the same hyperbaric conditions for fifteen days, but were sacrificed thirty days after denervation. Each group of animals consisted of six rats. The oxygen concentration in the hyperbaric chamber was kept  $\geq 98.5\%$  (Dräger Oxymeter). To eliminate the accumulation of carbon dioxide chamber was flushed with 100% oxygen at a rate of 4 L/min every 20 min during compression. Sodium carbonate crystals were used to reduce the accumulation of CO<sub>2</sub>. All rats were killed by the ether inhalation. The denervated extensor digitorum longus (EDL) muscle was removed and quickly frozen in isopentane cooled by liquid nitrogen. The EDL muscles of normal, innervated unoperated rats served as controls to the experimental muscles, that is denervated and denervated hyperbaric muscles. The muscles were stored at  $-80\text{ }^{\circ}\text{C}$  until further analysis. For

histological analysis, serial 8  $\mu\text{m}$ -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 EDL 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  $^{\circ}\text{C}$ . The samples were incubated for 30 min at 4  $^{\circ}\text{C}$ . Each homogenate was transferred to microfuge tubes and centrifuged at 13000 rpm for 20 min at 4  $^{\circ}\text{C}$ , and the supernatant was obtained. An aliquot of the supernatant was used to determine the protein concentration using Bradford method, and the remainder of the supernatant was stored at  $-80\text{ }^{\circ}\text{C}$  for subsequent Western blotting. Optimal loading concentration for immunoblotting was found to be 85  $\mu\text{g}$  and 75  $\mu\text{g}$  *per* sample for MyoD and myogenin, respectively. Equal loading of samples was confirmed by comparison with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) immunoblot. To verify MyoD and myogenin protein bands two control standards were run 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  $\mu\text{L}$  Leammi, 25  $\mu\text{L}$  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 70V. 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  $^{\circ}\text{C}$ . After that, the membranes were washed 6 $\times$ 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 for five times with TBS with 0.1% Tween 20 for 1 h. The antibody bindings were visualized with DAB (diaminobenzidin, 0.1mg/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<sub>2</sub>O<sub>2</sub> 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 pri-

mary 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 AEC (3-Amino-9-ethylcarbazole) staining kit or with DAB for 10 min. After rinsed in water, the sections were mounted in glycerol gelatin medium.

### Morphometry

Cross-sectional areas of muscle fibers from the muscles of three groups of animals were analyzed, normal innervated muscles, muscles denervated for 30 days and muscles denervated for 30 days but treated with HBO<sub>2</sub>. These areas were analyzed by a computer program for quantitative analysis »ISSA« (VAMS, Zagreb, Croatia). About 500–600 fibers were measured by outlining the contours of fibers.

### Statistics

Cross-sectional area of muscle fiber with standard deviation (SD) was calculated. The statistical evaluations were performed by Student t-test at  $p < 0.05$  level of significance. 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

### Muscle morphology and fiber size

Myofibers of unoperated EDL muscle displayed characteristic polygonal shape in transverse sections, stained with haematoxylin and eosin. In one month denervated muscles of the untreated and the HBO<sub>2</sub> treated rats, the reduction in the muscle fiber cross-sectional area was observed. The fibers were irregularly shaped. In normal, innervated EDL muscle, the mean cross-sectional area of fibers was  $1960.4 \pm 515 \mu\text{m}^2$  and in one month denervated untreated muscle was  $941.3 \pm 332 \mu\text{m}^2$ . The degree of atrophy was 52% and was statistically significant ( $p < 0.001$ ) comparing to the normal muscle. The mean cross-sectional area of muscle fibers from the one month denervated but HBO<sub>2</sub> treated muscles were even lower, i.e.  $928.2 \pm 307 \mu\text{m}^2$ , and was also statistically significant ( $p < 0.001$ ) comparing to that of the normal muscle.

### Western blot analysis

MyoD and myogenin proteins were not detectable in normal innervated EDL muscle using Western blot analysis. However, this analysis showed a progressive increase in MyoD and myogenin protein expression in the denervated and untreated EDL muscle. The immunoreactive bands of both proteins were detected on the 3<sup>rd</sup>,

15<sup>th</sup> and 30<sup>th</sup> day after denervation. The highest level of expression was observed on the 30<sup>th</sup> day for both, MyoD and myogenin (Figure 1). As an in denervated EDL, the bands of both proteins were detected on the 3<sup>rd</sup>, 15<sup>th</sup> and 30<sup>th</sup> day after denervation in denervated and HBO<sub>2</sub> treated EDL muscle as well. The highest expression of both myogenic factors was found on the 30<sup>th</sup> day after denervation and the HBO<sub>2</sub> treatment (Figure 2). There were no significant differences in the MyoD and myogenin expression levels in the EDL muscles between the untreated denervated rats and the denervated, but HBO<sub>2</sub> treated muscles.

### Immunohistochemistry

The immunohistochemical method with anti-MyoD and anti-myogenin antibodies applied on serial trans-

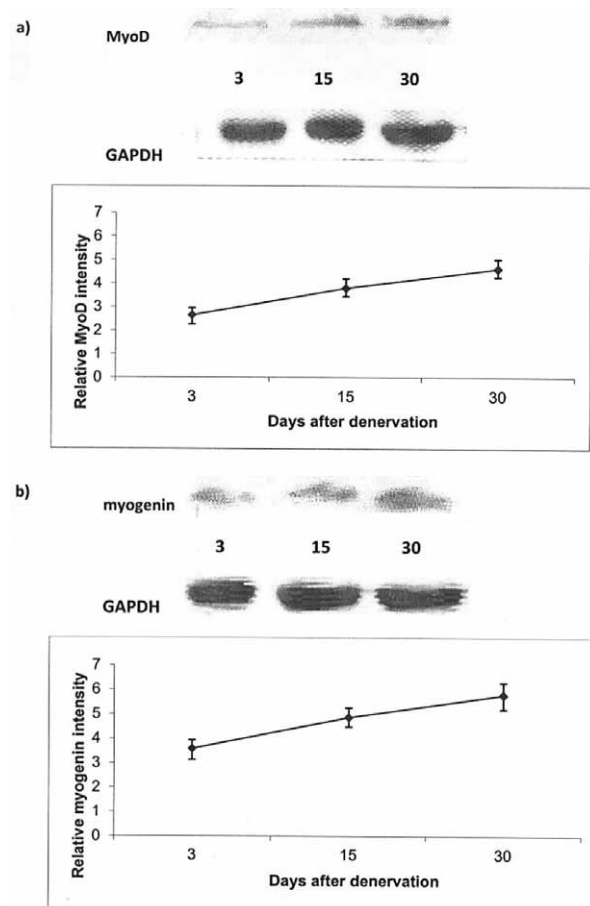


Fig. 1. MyoD and myogenin expressions determined by Western blot in denervated extensor digitorum longus (EDL) muscle of untreated rats. a) The levels of MyoD protein expression in the EDL muscles after 3, 15 and 30 days of denervation. The immunoreactive band of ~45kDa corresponded to the predicted molecular mass of the rat MyoD protein. b) The levels of myogenin protein expression in the EDL muscles after 3, 15 and 30 days of denervation. The immunoreactive band of ~34kDa corresponded to the predicted molecular mass of the rat myogenin protein. GAPDH was used as a loading control. The density of each band was expressed relative to the background density (considered to be 1.0).

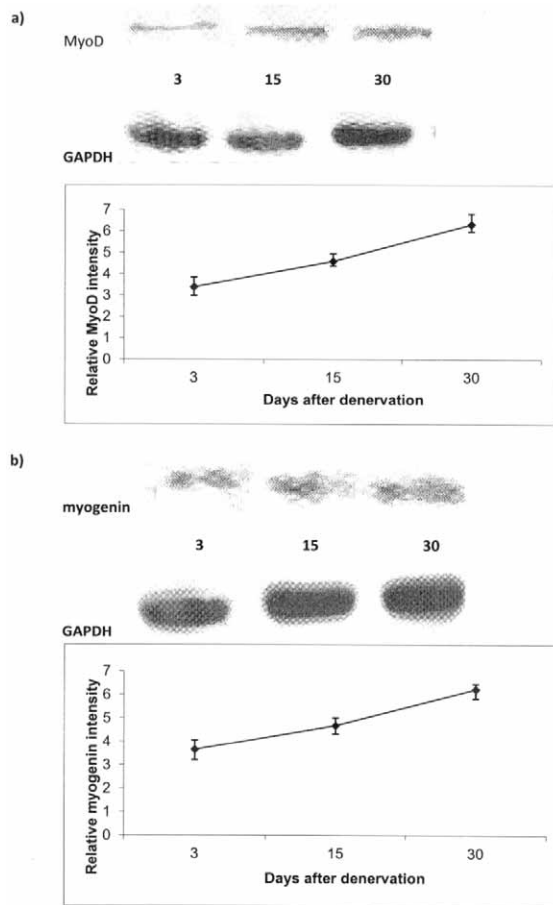


Fig. 2. MyoD and myogenin expressions determined by Western blot in denervated extensor digitorum longus (EDL) muscle with hyperbaric oxygen treatment. a) The levels of MyoD protein expression in the EDL muscles after 3, 15 and 30 days of denervation. The immunoreactive band of ~45kDa corresponded to the predicted molecular mass of the rat MyoD protein. b) The levels of myogenin protein expression in the EDL muscles after 3, 15 and 30 days of denervation. The immunoreactive band of ~34kDa corresponded to the predicted molecular mass of the rat myogenin protein. GAPDH was used as a loading control. The density of each band was expressed relative to the background density (considered to be 1.0).

verse sections of non-denervated muscles has not shown the expression of these proteins (Figure 3). On the contrary, the expression of both transcriptional factors was demonstrated in the denervated muscles and the denervated muscles of HBO<sub>2</sub> treated EDL in all three different periods after denervation (Figure 4).

### Discussion and Conclusion

Skeletal muscle undergoes rapid atrophy in response to denervation. Denervated muscle loses large percent of its weight during the first several weeks of the denervation<sup>25,27–31</sup>. Progressive diminishing of the mean cross-sectional areas of muscle fibers occurs<sup>32</sup>, although the

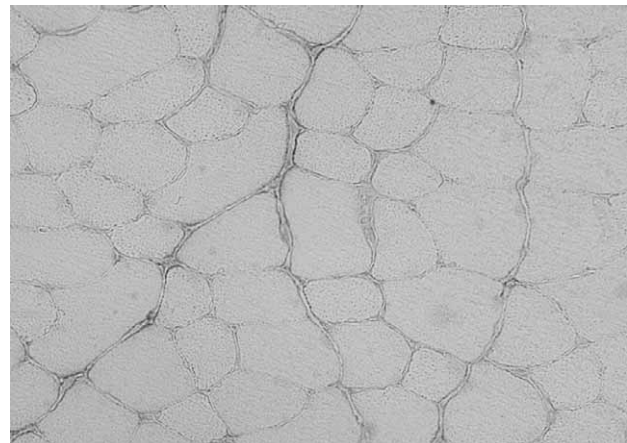


Fig. 3. Immunohistochemical demonstration of myogenin expression in cross section of innervated extensor digitorum longus (EDL) muscle. No positive nuclei. Original magnification: x400.

atrophy does not affect all fiber types equally<sup>24,33,34</sup>. In our study, we have found that the one month denervation caused a decline of the mean cross-sectional area of fibers in EDL for 52%, but HBO treatments had not additionally diminished the degree of atrophy. The findings of some other studies investigating the effect of HBO<sub>2</sub> on the muscle fiber size showed that HBO<sub>2</sub> has not influenced the degree of muscle fiber atrophy of healthy old mice<sup>17</sup>. Those animals were exposed to lower oxygen concentration and pressure (36% O<sub>2</sub> at 1.25 ATA), but 6 hours lasting sessions through two weeks. The rate of fiber size changes may depend on the pressure and duration of applied HBO exposure as demonstrated by Gregorevic et al.<sup>10</sup>. They showed that the pressure of 2 ATA in 90 minutes session during 14 days did not alter fiber size in EDL rat muscle damaged with bupivacaine injection, but prolonged exposure (25 days) or augmented pressure (3 ATA) improved the size and function of muscle<sup>10</sup>. Although the applied pressure and duration in the aforementioned study were within the range of values of the protocols used in clinical treatments, we wanted to examine the effect of lower pressure exposure, i.e. 2.2 ATA, as the standard treatment pressures used in humans are in the range from 2–2.5 ATA. Oter et al.<sup>35</sup> determined the effect of various HBO pressure modalities on the oxidative values of rat lung, brain and erythrocytes. They concluded that, although the inhalation of pure oxygen, rather than pressure causes oxidative stress. Oxidative stress in the range of HBO pressure from 2 to 2.5 ATA was relatively low as compared with that seen at 3 ATA<sup>35</sup>.

As in our previous study<sup>36</sup>, in this study we demonstrated an increased expression of myogenin and MyoD protein throughout the period of one month after denervation using either immunohistochemistry or Western blot analysis. Although the results of some other studies about the appearance of MyoD and myogenin in denervated muscles are uneven, our results are in concordance with the results of the majority of them; the expression of MyoD and myogenin mRNAs levels<sup>19–21,25,37</sup> or their

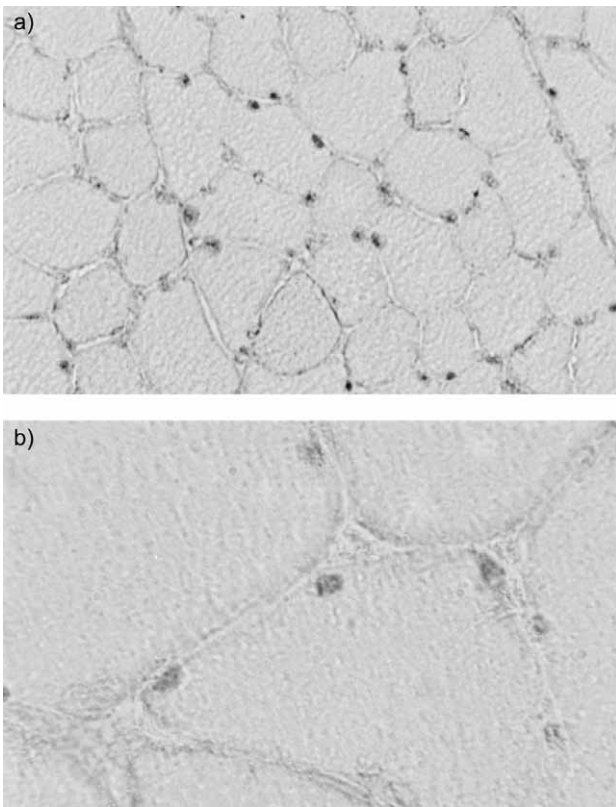


Fig. 4. a) Immunohistochemical demonstration of myogenin expression in cross section of extensor digitorum longus (EDL) muscle on the third day after denervation and hyperbaric oxygen treatment. Note the myogenin positive nuclei. Original magnification:  $\times 400$ . < b) Immunohistochemical demonstration of myogenin expression in cross section of extensor digitorum longus (EDL) muscle on the third day after denervation and hyperbaric oxygen treatment. Note the myogenin positive nuclei. Original magnification:  $\times 1000$ .

protein levels increased in early postdenervation days<sup>21,37</sup> and remained elevated even during one<sup>20,25</sup> or several months<sup>37</sup>. In our previous study<sup>36</sup> we showed that MyoD and myogenin proteins were localized in a large number of myonuclei and to a lesser extent in the nuclei of satellite cells. These data are in agreement with the findings of Hyatt et al.<sup>25</sup> and of Ishido et al.<sup>26</sup>. The latter, exploring only MyoD transcriptional factor at the protein level, found its expression in myonuclei and in satellite cells as well. They assumed its presence in myonuclei as possible protection of muscle against apoptosis<sup>26</sup>. In denervated fast muscles the levels of myogenin protein increased first few days after the nerve was cut and so it remained till the end of the first month when it began to decline gradually, and was located only in the myonuclei<sup>23</sup>. Identical localization of MyoD transcriptional factor was also confirmed in denervated tibialis anterior muscle within one week after denervation<sup>22</sup>.

The goal of this study was to find out whether the increased amount of dissolved oxygen in the blood caused by HBO<sub>2</sub> treatment can increase the expression of MyoD

and myogenin transcriptional myogenic factors. However, the HBO<sub>2</sub> treatment applied in this study has not altered the expression of both myogenic transcriptional factors in denervated EDL muscle when compared to that of the untreated denervated muscle. The understanding of signaling and regulatory processes involved in achieving the effects of hyperbaric oxygenation is incomplete<sup>38</sup>. As the hyperbaric oxygenation treatments are beneficial in the adjunctive therapy of several ischemic conditions, many experiments were done to explain the mechanisms underlying this efficiency. An improved regeneration of ischemic limb muscle achieved during the HBO<sub>2</sub> treatment was confirmed by the increase in myf5 and several growth factors<sup>2</sup>. Exploring the positive effect of HBO<sub>2</sub> treatment on wound healing in ischemic limb, it was found that HBO increased bone marrow nitric oxide and thereby augmented the release of endothelial progenitor cells in the peripheral circulation that contribute to the wound healing improving the ischemic limb perfusion<sup>39</sup>. Similar findings showed that the HBO stimulates the nitric oxide synthase (nNOS) activity and therefore increases the perivascular nitric oxide synthesis<sup>40</sup>. The main pathway for the satellite cell activation from quiescence involves the release of nitric oxide (NO)<sup>41</sup>. Elevated partial pressure of oxygen during HBO increases synthesis of NO, probably through stimulation of nNOS<sup>40</sup>. Thus our presumption was that HBO could lead to stronger satellite cell activity in the denervated muscle and therefore improve muscle regeneration. As explained in the study of Thom et al.<sup>42</sup> the mechanism by which HBO<sub>2</sub> stimulates stem/progenitor cells mobilization to the peripheral circulation evolved in mice when the protocol procedure was applied at 2.8 ATA pressure, but when it was changed to 2.0 ATA, it caused only a nominal effect<sup>42</sup>. Thus, we assume that the pressure of 2.2 ATA applied in our study, might not be sufficient to provoke stronger activation of satellite cells in denervated muscle.

In conclusion, the protocol of HBO<sub>2</sub> treatments applied in this study had not diminished the degree of atrophy of the denervated EDL muscle and had not altered expression of transcriptional factors MyoD and myogenin. Denervation is not such as an ischemic condition in the beginning, but later a substantial percentage of capillaries degenerate<sup>43</sup> and migration of satellite cells toward capillaries and small vessels occurs<sup>44</sup>. In respect to aforementioned, we speculate that in the late period of denervation the HBO<sub>2</sub> treatment could eventually be beneficial in bringing larger amounts of oxygen to the cell. But on the other side, there are large appositions of collagen between myofibres in the late phase of denervation<sup>44</sup>. Thus the ability of HBO<sub>2</sub> to enhance the collagen production<sup>45</sup> probably could deteriorate already bad condition in long-term denervated muscle.

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## UTJECAJ HIPERBARIČNOG KISIKA NA MIOGENE TRANSKRIPCijske FAKTORE U DENERVIRANOM MIŠIĆU ŠTAKORA

### SAŽETAK

Svrha je ovog rada bila odrediti utjecaj tretmana hiperbaričnim kisikom na ekspresiju transkripcijskih miogenih faktora u denerviranom m. extensor digitorum longus štakora. Regulacijski miogeni faktori MyoD i miogenin analizirani su u mišiću koji je bio denerviran tijekom 30 dana bez tretmana s hiperbaričnim kisikom i u denerviranom mišiću, ali tretiranom hiperbaričnim kisikom. Western blot analiza je pokazala signifikantno povećanje MyoD i miogenin proteina u denerviranom mišiću tijekom cijelog perioda. U denerviranim mišićima štakora koji su bili tretirani hiperbaričnim kisikom također je došlo do pojačane ekspresije obaju transkripcijskih faktora, no, hiperbarični tretmani nisu promijenili njihovu ekspresiju. Imunohistokemijska analiza pokazuje ekspresiju MyoD i miogenin proteina kroz ovaj period u denerviranom netretiranom mišiću, a također i u denerviranom mišiću štakora tretiranih hiperbaričnim kisikom. Denervacija koja je trajala mjesec dana, dovela je do smanjenja površine poprečnog presjeka mišićnog vlakna, a tretman hiperbaričnim kisikom nije smanjio stupanj te atrofije. Protokol tretmana hiperbaričnim kisikom primijenjen u ovoj studiji nema povoljan utjecaj na transkripcijske miogene faktore i na atrofiju denerviranog mišića u štakora.