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Effects of hyperbaric oxygen therapy on rabbit skeletal muscle during extremity lengthening

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Abstract
Introduction Extremity lengthening through distraction osteogenesis is limited by the surrounding skeletal muscle and neurovascular structures rather than the bone itself. The purpose of this study is to evaluate the effects of hyperbaric oxygen therapy on skeletal muscle during distraction osteogenesis.
Materials and methods Twenty New Zealand white rabbits were randomly divided into two groups. Right tibia of all rabbits was distracted at a rate of 0.125 mm per 6 h (0.5 mm/day) for 10 days with circular external fixator. Experimental group rabbits ($N = 10$) underwent 2.5 ATA hyperbaric oxygen therapy for 2 h everyday for 20 days, control group rabbits ($N = 10$) did not receive any corresponding treatment. Skeletal muscle perfusion was evaluated with scintigraphy before and after the distraction period. Serum CPK, LDH and AST levels were measured before and after the distraction period. All animals were killed on the 27th day. The right tibias of all animals were removed and tibialis posterior muscle was harvested for histopathologic and histomorphometric assessment with light and electron microscopy.
Results Skeletal muscle perfusion was decreased in the control group in comparison with pre-distraction level ($P = 0.008$). However, no significant decrease was observed in the experimental group ($P = 0.678$). There were no statistical differences in serum CPK, LDH and AST levels between groups ($P = 0.340$, $P = 0.077$, $P = 0.796$). The mean area of the muscle fibers was measured as $398.66 \pm 9.16\ \mu^2$ in the experimental group and $349.44 \pm 5.76\ \mu^2$ in the control group ($P = 0.000$) with light microscopy. Mild fibrosis was observed in connective tissue component of muscle tissue in control group. An average of 26 myofibrils (20–32) was counted in a 16-cm^2 unit area in experimental group and 50 myofibrils (35–65) in the control group with electron microscopy. Enlargement in the sarcoplasmic reticulum, degenerative changes in nuclear cytoplasm and increase in myofibril diameter were observed in the control group, which was not observed in the experimental group
Conclusion Results of this study suggest that HBO treatment alleviates the detrimental effects of distraction on skeletal muscles and preserves its ultrastructure.

Keywords Distraction osteogenesis · Hyperbaric oxygenation · Skeletal muscle

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Introduction

Distraction osteogenesis is an effective technique used to stimulate the new bone formation in a controlled fashion, and it is widely used in the treatment of bony loss, pseudoarthrosis, chronic osteomyelitis, limb length discrepancy and biologic reconstruction after wide tumoral resections and deformity [1, 7, 14, 16, 20, 22, 27]. One of the major obstacles in extremity lengthening is the limited adaptation of muscles transpassing corticotomy level to distraction process. Though new bone formation could be achieved with success, soft tissue maladaptations including muscle weakness and loss of range of joint movements may lead to unfavorable results [14]. Degenerative changes in muscles such as necrosis and fibrosis have been shown in experimental lengthening models [5, 17, 25]. However, contradictory studies advocating skeletal muscle histiogenesis have also been reported [6, 13, 14, 24, 28, 29].

Hyperbaric oxygen (HBO) therapy has been proved to have beneficial effects on skeletal muscle after various injury models [2, 11, 12, 21, 30]. Consequently, we hypothesized that HBO therapy may alleviate the detrimental effects of distraction process on muscles. Thus, clinically it may prevent muscle weakness and loss of range of joint movement.

Materials and methods

Twenty adult New Zealand white rabbits weighing an average of 1.8 kg (range 1.5–2.0 kg) were included in the study. The animals were fed a standard laboratory diet and water and kept under 12 h day/night cycle. Rabbits were housed separately in the standard cages in a temperature-controlled room (20–22°C). Before initiation of the study, approval from the Local Ethics Committee was obtained. The study was carried out in the "Center for Experimental Animals" at the same institution. Rabbits were randomized into experimental and control groups, each consisting of ten animals.

The surgical technique (Day 1)

A measure of 50,000 IU/kg per day Penicillin G, procaine (Procilin®, Fako, Turkey) was administered intramuscularly for infection prophylaxis 1 day before the surgery and continued for 3 days. Inhalational anesthesia with N_2O/O_2 and Sevoflurane (Sevorane; Abbott, Queenborough, England) (1–2 vol%) at a tidal volume of 15–20 ml/kg and at a rate of 30–35 cycles/min was used for the operation. Right cruris of the rabbit was sterilized using standard technique. Circular external frame (CEF) made up of two 5/8 C-rings of 40–45 mm diameter was fixed to metaphysis of tibia proximal to osteotomy site and diaphysis distal to the osteotomy site. The rings were connected to each other by three rods and the distance between two rings was 4 cm. The frame was fixed to tibia by two Kirschner wires of 1-mm diameter, which were inserted at 45°–60° angles to each other. Wires were tensioned by 30–35 N. After fixation of CEF frame, skin subdermis, and periosteum were exposed with an anteromedial longitudinal incision and and a transverse osteotomy was performed on proximal metaphyseal region by an osteotome. Subsequently the periosteum and skin were closed properly (Fig. 1).

Muscle scintigraphy (Day 5 and 27)

On the fifth postoperative day, all rabbits were sedated with 10 mg/kg ketamine hydrochloride (Ketalar®, Eczacibasi, Istanbul, Turkey) i.m. and then 3mCi Technetium-99m methoxy-isobutyl-isonitrile (Tc-99m-MIBI) (Medi-Radiopharma, Budapest, Hungary) was injected into the ear vein of each rabbit to evaluate the perfusion of the skeletal muscles in both lower extremities. Thirty minutes after the injection, the subject was positioned laterally on the imaging table of the gamma camera (GE, Millennium, Milwaukee, WI, USA) equipped with low-energy high-resolution collimator and planar acquisition of 10 min was initiated using a 15% window centered over the 140-keV photopeak. The region of interest (ROI) was drawn on the muscles around the osteotomy site on the perfusion images. The same size ROI was also drawn approximately in a similar location on the contralateral extremity. Counts were derived from the both ROIs to calculate the perfusion ratio (counts from the osteotomy site/counts from the normal extremity).

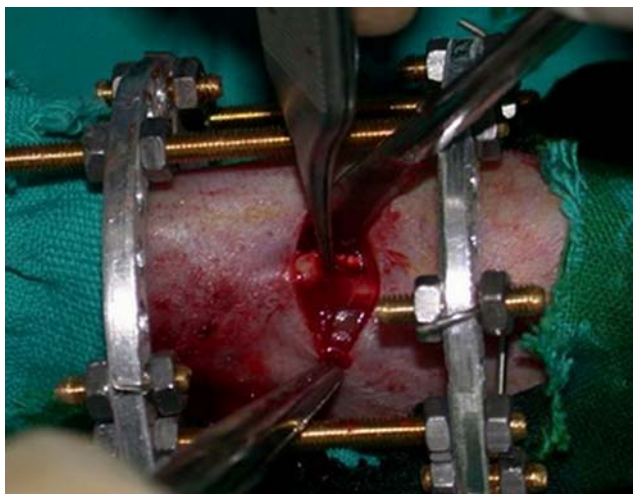

Fig. 1 Application of circular external fixator and the ostetomized tibia

The distraction (Day 7–17)

The distraction process was started 7 days after the osteotomy with the rate of 0.125 mm per 6 h (0.5 mm/day) in both groups, and continued for 10 days.

Hyperbaric oxygen therapy (Day 7–27)

The experimental group received 2.5 atmospheres of absolute (ATA) hyperbaric oxygenation for 2 h daily, beginning on the first day of distraction and lasting for 20 days in a monoplace pressure cabinet with 210 × 70 cm. dimensions (KRL, Turkey 2001). Oxygen was released into pressure cabinet as free flow.

Biochemistry (Day 7 and 27)

Blood samples were drawn from both groups on the 7th and 27th days to measure serum CPK, LDH and AST levels. Blood samples collected in containers (Vacutainer system, Becton Dickinson, NJ, USA) were centrifuged in GMMA Biochemistry Laboratory for 10 min at 4,500 cycle/min to separate serum and stored at −70°C before analysis. Serum CPK, LDH and AST levels were determined by Olympus AU 2700 (Mishima/Japan) auto analyzer using its own kits.

The sacrificing process (Day 27)

Inhalational anesthesia with N_2O/O_2 and Sevoflurane (Sevorane; Abbott, Queenborough, England) (1–2 vol%) at a tidal volume of 15–20 ml/kg and at a rate of 30–35 cycles/min was employed for the muscle biopsy. Tibialis posterior was totally excised for the preparation of specimens. Afterward, all rabbits in each group were killed with high-dose intraperitoneal sodium pentobarbital (Pental Sodium®, IE Ulagay, Istanbul, Turkey).

Histomorphometric evaluation with light microscopy

Two biopsy specimens with smallest dimensions of 0.5 × 0.5 × 0.3 cm were obtained for each case from the tibialis posterior muscle. One of the specimens was fixed with 10% formaldehyde for 24 h. After routine pathological processing 4-micron-thick slides were prepared. These slides were stained with Hematoxylin–Eosin (HE) and Gomori trichrome. The components of morphometric evaluation were as follows: a personal computer running Microsoft Windows NT 4.0 Service Pack 6a operating system (Redmond, Washington, USA), a light microscope with monitorized stage (Zeiss Axioscope, Göttingen, Germany), a frame grabber card (Matrox Meteor, Matrox Inc., Quebec, Canada) a digital camera attached to the microscope (Sony, ATV Horn 3 CCD Sony, Tokyo, Japan) and special image analysis software (Zeiss Vision KS 400 3.0 for Windows). For each subject, mean area of 50 randomly chosen muscle fiber was measured (Fig. 2).

The second specimen was frozen immediately in liquid nitrogen and sliced into 8–10 μm sections by frozen slicing device. Specimens were sectioned perpendicular to the axis of myofibrils. These sections were stained with NADH and trichrome and assessed under light microscope.

Histomorphometric evaluation with electron microscopy

Electron microscopic analysis was performed on 1-cm length fibers, which were dissected from musculotendinous junction of the tibialis posterior in all animals. These fibers were preserved in 2.5% glutaraldehyde solution and were rinsed in Sorensen buffer after two days of fixation period. Postfixation was done with 1% OsO_2 solution. Specimens were dehydrated with increasing concentrations of alcohol. Fiber pieces were blocked into plastic blocks (Araldit) at 60°C. Blocks were sliced into 40-nm-thick slices with an ultramicrotome (Leica, Germany). Sections transferred into grid surface were stained by uranyl acetate and lead citrate. Sections were examined and photographed by Carl Zeiss EM-900 electron microscopy at 4,500× magnification. The number of myofibrils was counted in the 4 × 4 cm area at the photographs.

Statistical analysis

Data were analyzed by SPSS for Windows V.10.0 software. Statistical significance was determined as $P < 0.05$.

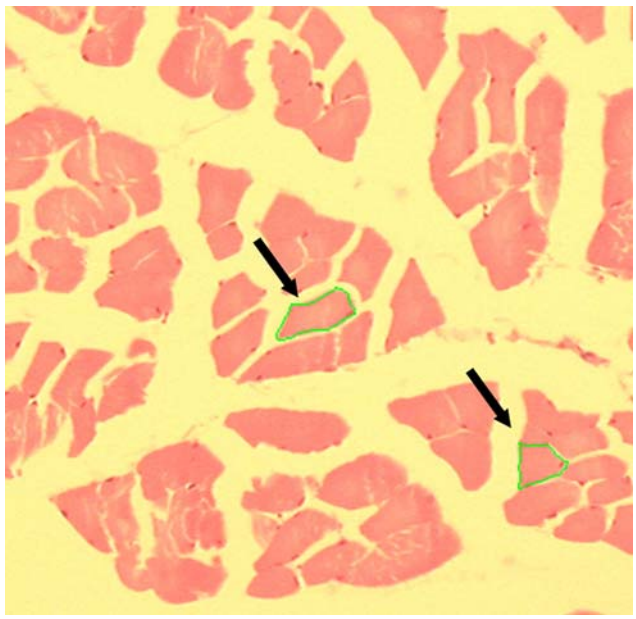

Fig. 2 Histomorphometric measurement on H&E stain with light microscopy. *Arrows* show the randomly selected fibers for measurement (original magnification ×20)

Comparisons were made with Wilcoxon test for dependent and with Mann–Whitney *U* test for independent groups.

Results

One of the rabbits in the control group died because of early post-operative complication on the fourth day. One of the rabbits in the experimental group died on the seventh day during HBO therapy. We have collected the data from the remaining 18 rabbits, which completed the experiment without any complication.

Muscle perfusion scintigraphy

Muscle perfusion ratio of both the control and the experimental groups before the distraction was similar statistically ($P = 0.258$). Muscle perfusion ratio of the control group demonstrated statistically significant decrease after the distraction ($P = 0.008$). However, muscle perfusion ratio of the experimental group did not change after the distraction ($P = 0.678$). Muscle perfusion ratio of the control group was significantly lower compared to experimental group at the final scintigraphic study (Table 1; Fig. 3).

Biochemistry

Serum CPK, LDH and AST levels were statistically similar within the control group before and after the distraction. However, serum LDH level was statistically decreased within the experimental group while serum CPK and AST levels did not change. Serum CPK, AST and LDH levels were statistically similar between groups before and after the distraction. CPK, LDH and AST levels and their *P* values are summerized in Table 2.

Table 1 Muscle perfusion scintigraphy results of the groups

	Control group	Experimental group	*P* value
	Distracted/ normal ratio	Distracted/ normal ratio	
Before distraction (5th day)	1.05 ± 0.25	0.90 ± 0.15	0.258
After distraction (27th day)	0.69 ± 0.05	1.02 ± 0.25	0.000
P value	0.008	0.678	

P values at the last column demonstrate the statistical difference between groups calculated with Mann–Whitney *U* test. *P* values at the last row demonstrate the statistical difference of repeated measurements of the same group calculated with Wilcoxon signed ranks test

Morphometric evaluation and light microscopy

In morphometric evaluation of the muscle with light microscopy on H&E stain, the mean area of the muscle fibers was measured as $398.66 \pm 9.16\ \mu^2$ in the experimental group and $349.44 \pm 5.76\ \mu^2$ in the control group. The difference between groups was statistically significant ($P = 0.000$). NADH-stained specimens showed similar characteristics in each group. However, trichrome-stained specimens showed increased endomysial–perimysial–epimysial fibrosis in the control group (Fig. 4). There was no necrosis in each group in each stained specimens.

Electron microscopic evaluation results

Electron microscopy examination revealed intracytoplasmic myofibrillary enlargement in the control group. In the experimental group, 26 myofibrils (20–32) were counted in a unit area of $16\ cm^2$ on photographs by morphometric analysis, whereas, in the control group 50 myofibrils (35–65) were counted in $16\ cm^2$ unit area by morphometric analysis. The difference between groups was statistically significant ($P = 0.000$).

Organization of myofibrils was less distorted in the experimental group and myofibrillary intervals were enlarged in the control group. Degenerative changes in the nuclear cytoplasm and intermyofibrillary sarcoplasmic retinacular swelling were also observed in the control group (Fig. 5).

Discussion

Distraction osteogenesis is an effective technique used in extremity lengthening and regeneration of new bone. However, it is limited by the surrounding skeletal muscle and neuromuscular structures rather than the bone itself. The development of contractures and muscle weakness has been interpreted as to be a result of limited adoptive response of skeletal muscle to distraction process [23]. In this study, we investigated whether HBO therapy could reverse these detrimental effects of distraction on the skeletal muscle.

Relevant literature contains conflicting knowledge about the adaptation of skeletal muscle to the distraction process. Calandriello et al. [5] proposed that during distraction muscle fibrils are torn and heal with fibrosis. Lee et al. [17] agreed with this hypothesis, upon histological findings of necrosis and fibrosis in their experimental study. Simpson et al., while evaluating the muscle response to different lengthening regimens in the rabbit experimental lengthening model, found muscle fiber disorganization, perimyseal and endomyseal fibrosis and necrosis at 1-mm/day distraction rates [25]. In this current study, we have also found

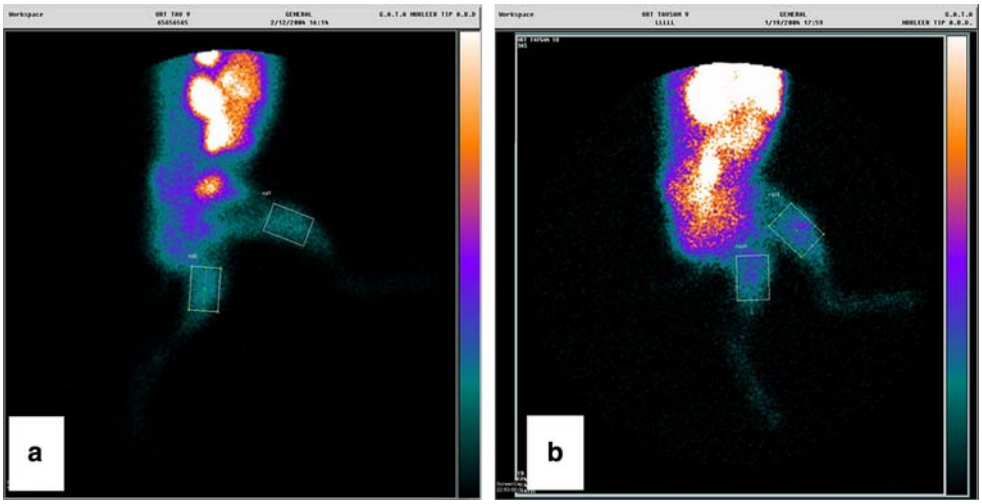

Fig. 3 Muscle perfusion scintigraphy after the HBO therapy. **a** Control group, **b** experimental group. Note the increased activity in the experimental group

Table 2 Results and statistical significance of serum CPK, LDH and AST levels

	Control group			Experimental group			*P* value
	CPK[a]	LDH[b]	AST[c]	CPK	LDH	AST	
Before distraction	1043.33 ± 333.04	756.88 ± 821.18	46.77 ± 21.7	1402.22 ± 900.20	1388.22 ± 718,11	74.44 ± 70.16	$P = 0.863^a$ $P = 0.063^b$ $P = 0.730^c$
After distraction	1832.66 ± 1026.01	331.11 ± 196.74	42.66 ± 13.9	2295.66 ± 1195.97	645.77 ± 333.49	57.77 ± 48.20	$P = 0.340^a$ $P = 0.077^b$ $P = 0.796^c$
P value	0.86	0.139	0.678	0.173	0.011	0.441	

P values at the last column demonstrate the statistical difference between groups calculated with Mann–Whitney *U* test. *P* values at the last row demonstrate the statistical difference of repeated measurements of the same group calculated with Wilcoxon signed ranks test

[a] *P* stands for CPK

[b] *P* stands for LDH

[c] *P* stands for AST

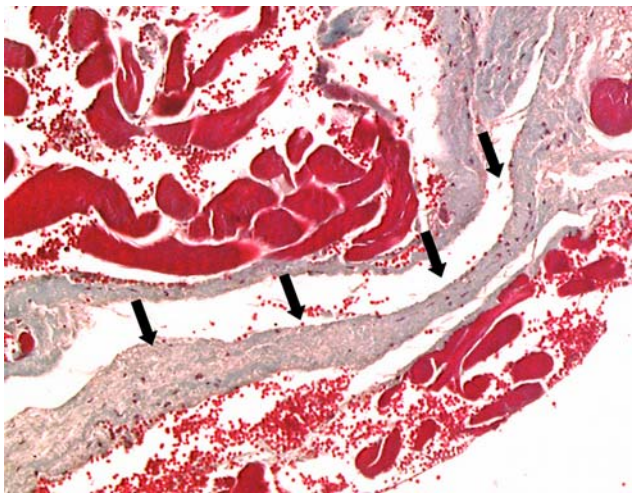

Fig. 4 Trichrome-stained sections of the control group. *Arrows* show the increased fibrosis (original magnification ×20)

increased endomysial–perimysial–epimysial fibrosis in the control group. Electron microscopic evaluations demonstrated disorganization of myofibrils, enlarged myofibrillary intervals and degenerative changes in nuclear cytoplasm and intermyofibrillary sarcoplasmic retinacular swelling in control group.

The connective tissue framework of muscle consists of fibrillar collagen, which provides both myofiber-to-myofiber connections and a three-dimensional support structure with important elastic properties [4, 26]. Thus, even small changes in the concentration of this element would be expected to have a substantial effect on muscle function. It would appear that during prolonged stretch the connective tissue elements remodel less readily than the contractile component. This may lead to damage to the perimysial and endomysial network with subsequent fibrosis and reduction in contractile function [15].

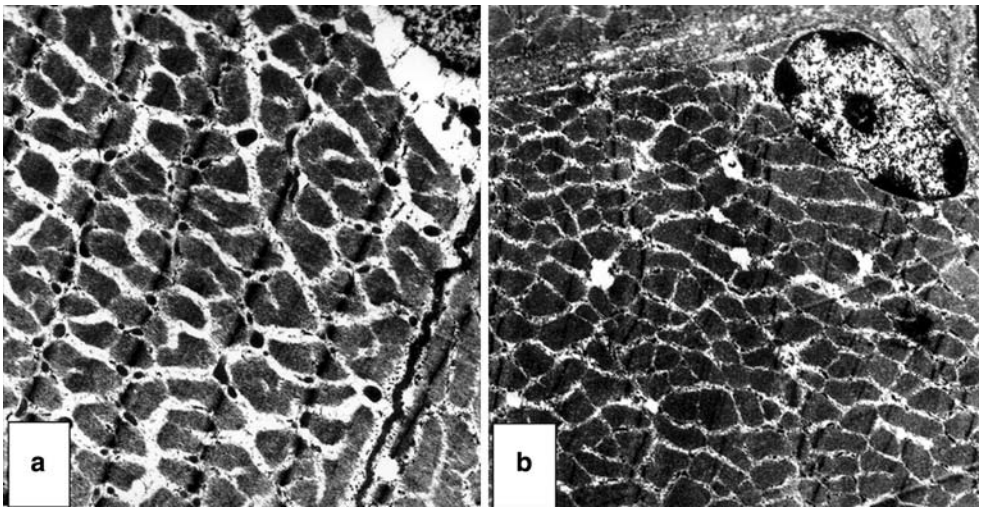

Fig. 5 Electron microscopic appearance of the muscle. **a** Control group, **b** experimental group (original magnification ×4,500). Note the enlarged myofibrillary intervals and distorted organization in control group

Yamazaki et al. [33] investigated the changes in the muscle fiber type during distraction on rabbits. He reported that Type 1 fiber diameters increased; type 2A fiber diameters were unchanged; and type 2B fiber diameters decreased. He suggested that qualitative changes in muscles might explain the weakness after distraction. On the other hand, Fink et al. [9], measured the electromyographic action potentials on the distracted muscles and found longer duration of potentials and lower amplitudes. Muscle weakness may be a result of neuromuscular disfunctioning rather than the qualitative changes in muscles.

Besides those reports on the detrimental effects of distraction on the muscle, contrary reports are also found in the literature. Tsumijura et al. [28] suggested that regeneration of skeletal muscle might be achieved by inducing the activation and proliferation of satellite cells, which eventually fuse with preexisting muscle fibers or fuse to form new muscle fibers. Ilızarov et al. [13, 14] suggested muscular elongation with myofibrilogenesis without tears upon distraction. Schumacher et al. [24] reported increased muscle volume and mitosis during distraction. Tuz et al. [29] observed central localization and increase in the number and sizes of the nuclei of the muscle fibers which indicated regenerative and reactive tissue responses. Day et al. [6] demonstrated that limb lengthening promotes muscle growth by triggering myoblast proliferation and fusion into the lengthened muscle using bromodeoxyuridine, a thymidine analogue that is incorporated during cell division, and desmin, a muscle-specific marker.

One of the handicaps of our study is the lack of histopathological evaluation of undistracted muscles in each group. Morphometric evaluation revealed increased myofiber enlargement in the experimental group compared to the control group. However, it is not possible to interpret this influence related to distraction. It may also be a result of HBO therapy or combination of both.

Current research on the effects of HBO therapy during distraction ostegenesis mostly focuses on the bone formation, maturation and the quality of newly formed bone. To the best of our knowledge, there is no experimental study investigating the effects of HBO therapy on skeletal muscle during distraction process. Administration of HBO during distraction osteogenesis resulted with the increase in bone mineral density of the distracted segment [8, 31]. Furthermore, It has been shown that HBO therapy increases the osteoblastic activity and angiogenesis in the distracted segment [19]. There have been reports of clinical improvement following the application of HBO therapy to individuals with established non-union treated with aggressive debridement and internal bone transport through distraction osteogenesis [3].

Hyperbaric oxygen therapy utilizes the inhalation of 100% O_2 at pressures greater than 1 atmosphere absolute (ATA). Adjunctive HBO therapy has been used as an orthopaedic treatment for several decades for orthopaedic infections, wound healing, delayed union and non-union of fractures, acute traumatic ischemia of the extremities, compromised grafts, and burn injuries [32].

However, there are few studies concerning the effects of HBO therapy on skeletal muscle tissue upon various experimental muscular injury models. It has been shown that HBO therapy reduces the interstitial hemorrhage, neutrophil infiltration and cellular necrosis and gives better results when administered in the ischemia-reperfusion injury to the muscles [21, 30]. HBO treatment lessens the metabolic, ischemic derangements and improves recovery in post-ischemic muscle and preserves the contractile capacity of the muscles after myotoxic injury induced with bupivacaine hydrochloride.

Moreover, it protects the muscles by modulating the antioxidant enzyme activity [11, 12]. In a recent study, it was shown that HBO therapy induced basic fibroblast growth factor and hepatocyte growth factor expression, and enhanced blood perfusion and muscle regeneration in mouse ischemic hind limbs [2]. Results of our study agree with these previous studies. Decreased muscular perfusion during distraction simulates ischemic injury. Experimental group animals showed significantly higher counts on muscular perfusion scintigraphy which can be interpreted as increased angiogenesis.

AST, LDH and CPK are intra-cytoplasmic enzymes found in the skeletal muscle and increased levels of these enzymes may be predicted as indirect signs of muscle injury [10, 18]. We were expecting to find increased serum enzyme levels. However, we could not see any significant change in these enzymes before and after the distraction process. Furthermore, HBO therapy had no effect on them. AST, LDH and CPK are found in various tissues other than the skeletal muscle such as liver, kidney and heart. We have distracted only one group of muscles in one leg. Serum AST, LDH and CPK levels that we measured project the total body and not the distracted muscle group alone. Therefore, we conclude that muscle enzyme release cannot be used to predict the muscle function impairment caused by distraction in limb lengthening.

Hyperbaric oxygen therapy may be utilized as an adjunctive treatment during distraction osteogenesis to overcome the detrimental effects of distraction to the skeletal muscle. Further clinical studies are needed to clear the conflicting information about this issue.

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