Hyperbaric oxygen therapy improves medial collateral ligament healing in a rabbit model

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Abstract

Purpose: Hyperbaric oxygen (HBO) therapy has been shown to enhance bone, muscle, skin, and wound healing, particularly in conditions of ischemia and low oxygen tension. Preliminary data show that HBO therapy may be beneficial in the treatment of muscle–tendon and ligament injury. However, the effects of HBO therapy on ligament healing have not been thoroughly investigated. The purpose of this study was to assess the effects of HBO on the healing of the medial collateral ligament (MCL) by using biomechanical and histological analyses in a rabbit model.

Methods: We investigated the effects of intermittent HBO therapy on ligamentous healing in rabbits. Sixty-four male rabbits were divided into eight groups consisting of eight animals each. The experimental group (32 rabbits) was subjected to HBO at 2.5 atmospheres absolute for 2 hours per day, whereas the control group (32 rabbits) did not undergo HBO therapy. All animals underwent a standardized surgical severance of the right MCL, whereas the left MCL was not surgically lacerated. Rabbits from each group were euthanized at 2, 4, 8, and 12 weeks postoperatively for biomechanical testing (six rabbits) and histological analysis (two rabbits).

Results: By using the intact left MCL as a control, the mean percentages of failure load calculated at 2, 4, 8, and 12 weeks in the HBO group were 20.6%, 49.1%, 63.9%, and 76.3%, respectively, whereas in the non-HBO group, the mean percentages of failure load at 2, 4, 8, and 12 weeks were 13.5%, 25.3%, 36.5%, and 57.3%, respectively. For all time intervals, the mean percent failure load of the severed MCLs from the HBO group was significantly higher than that from the non-HBO group. The mean percentages of regenerative collagen fibers in the HBO group at 2, 4, 8, and 12 weeks were 53.2%, 75%, 87.8%, and 95.1%, respectively, and 32.4%, 55.7%, 72.4%, and 82%, respectively, in the non-HBO group. For all time intervals, the HBO group showed a significantly higher amount of regenerative collagen fibers than the non-HBO group.

Conclusion: The present study results suggest that the healing of the MCL is enhanced by intermittent HBO therapy. HBO therapy may be useful in shortening the healing time of the injured ligaments.

Keywords:
hyperbaric oxygen therapy
ligament healing
medial collateral ligament

Introduction

Hyperbaric oxygen (HBO) therapy has been shown to enhance bone, muscle, skin, and wound healing, particularly in conditions of ischemia and low oxygen tension. Ligament healing is a slow but highly organized process involving hemorrhage, inflammation, proliferation, and remodeling. Preliminary data show that HBO therapy may be beneficial in the treatment of muscle–tendon and ligament injuries. This has positive implications for sports medicine, given the frequency at which such injuries occur and the wide variety of treatment options available. In the United States and Europe, some athletes with partial or complete periarticular ligament disruptions have been treated with HBO therapy; anecdotal reports have indicated a faster return to competition after using this therapy. However, there are no existing controlled trials or clinical research that delineate and define the benefits of HBO therapy in ligament healing. Furthermore, the effects of HBO therapy on ligament healing have not been thoroughly investigated.

In the present study, the medial collateral ligament (MCL) was selected as a model for ligament healing, which can theoretically be applied to other ligament injuries. Previous laboratory and clinical studies have demonstrated that even with various treatment methods, such as suture repair or early motion, normal ligament
properties are difficult to restore during MCL healing after an injury.\textsuperscript{10,17,19,20} HBO is known to stimulate fibroblast proliferation and remodeling in healing wounds. Because wounds and ligaments heal through similar cellular events, HBO, in addition to these proteins, is believed to play a major regulatory role in ligament healing.\textsuperscript{21,22}

The purpose of this study was to assess the effects of HBO on the healing of the MCL by using biomechanical and histological analyses in a rabbit model. We hypothesize that HBO therapy is a useful method for shortening the healing time of injured ligaments.

2. Materials and methods

2.1. Animal experimental design

The Institutional Animal Care and Use Committee of the Taiwan Department of Health and Human Services approved the methods described herein. All the animals studied were cared for in accordance with the regulations of the National Institute of Health of the Republic of China (Taiwan), under the supervision of a licensed veterinarian.

Sixty-four skeletally mature male New Zealand white rabbits (12–17 months of age), weighing 3.5 ± 0.6 kg [mean ± standard deviation (SD)], were randomly divided into two groups: the experimental group (n = 32) underwent intermittent 2.5-atmosphere absolute (ATA) HBO therapy, and the control group (n = 32) did not undergo HBO therapy, but subjects were kept in the HBO chamber. Under sterile conditions and general anesthesia using ketamine hydrochloride (Ketalar; Parke-Davis, Taipei, Taiwan, ROC) and Rompun (Bayer, Leverkusen, Germany) intravenous injection, each animal underwent a standardized complete severance of the right MCL.\textsuperscript{5} The MCLs of both hind limbs were exposed through longitudinal medial incisions in the skin and fascia. The MCL of the right leg was completely severed by passing a 3-0 braided steel suture beneath it at the level of the suprameniscal recess. The MCL in the left leg of each animal was simply exposed. Skin was approximated with 3-0 interrupted nylon sutures. All animals were intravenously injected with Keflin prophylactic antibiotics. After surgery, the animals were permitted unrestricted activity in cages and monitored daily for food and water intake as well as wounding and ambulation. Each test group of animals was housed in a hyperbaric research chamber (Model 17-48-100; Reimers System Inc, Springfield, VA, USA) for 2 hours per day. Beginning on the day of surgery, when the animals were in the chamber, the experimental group was exposed to 2.5 ATA of pure oxygen for 2 hours a day for 5 days. The control group was exposed to 1 ATA of normal air using the same regimen.

The control and experimental groups were then separated into four subgroups of eight rabbits each for study. These subgroups were euthanized at 2, 4, 8, or 12 weeks for biomechanical and histological studies.

2.2. Biomechanical test

After the animals were euthanized, both hind limbs of each animal were disarticulated at the hip, wrapped in saline-soaked gauze, and stored at −20 °C. The night before biomechanical testing, the specimens were thawed at 4 °C.\textsuperscript{23} For the first stage of biomechanical testing, the skin, subcutaneous tissue, and muscles were removed; care was taken not to damage the ligaments or joint capsules. Specimens were kept moist with 0.9% saline solution during testing. All soft tissues were stripped from the femur and tibia, leaving only the periarticular soft tissue around the knee. A unique surgical-grade drill (3-mm diameter) was used to drill holes perpendicular to the sagittal plane on both distal femur and proximal tibia sides; this allowed the insertion of two rods (3-mm diameter). To obtain the optimum relative position of specimens before testing, each rod was placed on a custom designed symmetrical grip with two hoops that allowed both perpendicular (linear movement and rotation) and horizontal (linear movement) adjustments. The remaining soft tissue around the knee, including the intra-articular ligaments, was sharply divided, leaving only the MCL intact. To provide consistent comparisons between specimens, all specimens were maintained in a condition of 0° flexion of the knee joint and the MCL parallel to the axis of tension by adjusting the grip to the optimum position before tensile testing. After the specimens were clamped in place, an axial tensile force was applied at a crosshead rate of 7.2 mm/min using a material-testing machine (Bionix 858; MTS Company, Minneapolis, MN, USA). The relationship between force and displacement was recorded simultaneously at increments of 0.05 mm using MTS Teststar II software (MTS System Corporation, Minneapolis, MN, USA). The experimental setup and testing configuration are shown in Fig. 1. To assess the effects of HBO therapy on the healing rate of ligaments at various time periods, the magnitude of force at failure for each individual specimen was selected for comparison. By using each rabbit’s other intact limb as a control, the percentage of failure force of the ruptured MCLs of each rabbit was calculated. Statistical analysis was done using an independent two-tailed test for discrete variables. A significant difference was reported when p < 0.05.

Fig. 1. The experimental setup and testing configuration. A unique surgical-grade drill (3-mm diameter) was used to drill the holes perpendicular to the sagittal plane on both distal femur and proximal tibia sides; this allowed the insertion of two rods (3-mm diameter). To obtain the optimum relative position of specimens before testing, each rod was placed on a custom designed symmetrical grip with two hoops that allowed both perpendicular (linear movement and rotation) and horizontal (linear movement) adjustments of the specimen.
2.3. Histological analysis

Two sacrificed specimens from each group underwent histological analysis; entire strips of MCLs of both hind limbs were excised from these specimens. The strips included areas of old ligament at both ends, connected by scar tissue in the middle in addition to comparable areas of controls. After the specimens were fixed in 10% buffered formalin and embedded in paraffin, they were stained using standard Masson’s trichrome stain24 and hematoxylin and eosin for subsequent examination. In trichrome staining, collagen fibers were identified as blue color stains and muscle fibers were identified as red color stains. Trichrome staining was used to monitor the amount of regenerating collagen fibers at the injured sites treated with different doses of HBO; the results among the different groups were used for comparison. Blue-stained areas were considered to be regenerating collagen fibers. The observer performing the counting was blinded to the source of the samples. Five random fields were selected for each sample, and the total areas of regenerating collagen fibers were calculated. The intensity of the blue staining was quantified using a computer-assisted image analysis system. Each image was captured by a digital camera (Olympus C-5060; Olympus, Tokyo, Japan). The intensity of illumination and the magnification of the image were kept constant. Areas containing the repairing tissue and normal adjacent ligaments of interest were identified; positive-staining regions were quantified using computer-assisted image analysis software (Image-Pro Plus version 5.1; Media Cybernetics, Inc., MD, USA). The results were expressed as the ratio of blue-stained areas of the regenerative collagen fibers in each random field. The mean ratio for sections of untreated defects was compared with the mean ratio of sections from defects treated with HBO using Student’s t test.

3. Results

3.1. Biomechanical test

Surgery was successful in all 64 rabbits; no infections were observed. Fig. 2 illustrates the corresponding diagram of force versus displacement; the load magnitude increased linearly with increasing displacement before failure. However, force magnitude declined significantly once maximal force was reached. After the failure of the MCL ligament, the fluctuation phenomenon of force value was observed—this was considered to be the result of the damping effect of the tibia mounted on the MTS load cell. In the HBO group, the mean percentages of failure force at 2, 4, 8, and 12 weeks were 20.6% (SD = 4.8), 49.1% (SD = 11.0), 63.9% (SD = 9.5), and 76.3% (SD = 10.2), respectively, and 13.5% (SD = 4.3), 25.3% (SD = 6.6), 36.5% (SD = 8.9), and 57.3% (SD = 10.9), respectively, in the non-HBO group. At all time intervals, the HBO group exhibited a significantly higher failure force than the non-HBO group (Fig. 3; two-tailed t test; p values were <0.05, <0.01, <0.01, and <0.05 at 2, 4, 8, and 12 weeks, respectively).

3.2. Histological analysis

Representative sections (Fig. 4) from the HBO and non-HBO groups at different intervals revealed that the healing processes were identical in both groups. At 2 weeks, the defects were filled by a vascular inflammatory tissue. By 4 weeks, the reparative zone in the midsubstance areas of healing MCLs was occupied by active fibroblasts. At 8 weeks, there was an increase in the number and size of fibroblasts in addition to some evidence of longitudinal (along the long axis of the ligament) alignment of their nuclei. At 12 weeks, continued remodeling, with improving realignment, had occurred. Although the phases of healing in the severed MCLs from both groups were identical, the amount of fibroblasts and the extent of the reparative zone were greater in the HBO group. The mean percentages of regenerative collagen fibers in the HBO group at 2, 4, 8, and 12 weeks were 53.2% (SD = 6.8), 75% (SD = 7.2), 87.8% (SD = 11), and 95.1% (SD = 10.5), respectively, and 32.4% (SD = 8.3), 55.7% (SD = 8.5), 72.4% (SD = 9.9), and 82% (SD = 8.5), respectively, in the non-HBO group. The HBO group showed a significantly greater amount of regenerative collagen fibers compared with the non-HBO group at all time intervals (p values were <0.05, <0.01, <0.01, and <0.01, at 2, 4, 8, and 12 weeks, respectively). The histological findings corroborated the biomechanical results.

4. Discussion

HBO therapy has been shown to enhance the rate of healing in various problematic wounds, including diabetic foot ulcers, osteoradionecrosis, necrotizing soft tissue infections, refractory osteomyelitis, and ischemic tissue flaps.2,6,25–31 Kivisaari and Niinikoski26 showed that HBO enhanced the rate of wound healing in ischemic wounds in rats. Hammarlund and Sundberg26 reported

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**Fig. 2.** The corresponding diagram of force versus displacement; load magnitude increased linearly with increasing displacement before failure.

![Fig. 2](image)

**Fig. 3.** The time course of the mean percent failure load in the HBO and non-HBO groups. The percent failure load equals the mean failure load of the right severed MCL divided by the mean failure load of the left intact MCL of the same rabbit. The data points of the HBO group at 2, 4, 8, and 12 weeks (mean ± standard deviation) were 20.6% ± 4.7%, 49.2% ± 11.0%, 64.0% ± 9.5%, and 76.3% ± 10.2%, respectively, whereas the data points of the non-HBO group were 13.5% ± 4.3%, 25.3% ± 6.6%, 36.5% ± 8.9%, and 57.3% ± 10.9%, respectively. HBO = hyperbaric oxygen; MCL = medial collateral ligament.
a significant enhancement of healing in chronic leg ulcers with the use of HBO therapy in a randomized study of 16 patients. Hunt et al.\(^2\) found that the greatest benefits of HBO were achieved in situations in which nutritive flow and oxygen supply to the repair tissue were markedly compromised—in a local injury or infection, where the regional blood is intact, for instance. HBO has also been shown to accelerate normal fracture healing and skin healing in animal models.\(^1,3,7,9\) Various mechanisms have been proposed that may explain the positive effects of HBO on injured tissues, including increased fibroblast proliferation, collagen deposition,\(^4,6\) stimulation of vascular proliferation at the site of injury,\(^1,2,6\) and decreased leukocyte adhesion to injured endothelium with decreased elaboration of inflammatory cytokines.\(^4\)

Ligament healing is a slow but highly organized process involving hemorrhage, inflammation, proliferation, and remodeling.\(^10\) There are very few reports concerning the effects of HBO therapy on ligament healing; thus, its role is still controversial. In a model of MCL healing, Horn et al.\(^3,2\) found that a greater force was required to cause the failure of the ligament in rats that received HBO therapy than the rats that had not, but only at 4 weeks after surgery; no additional significant increase in stiffness or force was observed at 6 or 8 weeks. However, the rat MCL heals so rapidly that, as early as 14 days after injury, the site of failure shifts from the site of ligament division to the tibial insertion. The strong remodeling ability of the rat MCL may have reduced the positive effects of HBO to a nonsignificant level between the HBO and non-HBO groups.

Fig. 4. Representative sections of midsubstance areas of healing MCLs at different intervals, taken from the non-HBO and HBO groups. Panels A, B, C, and D show the severed MCL in non-HBO group at 2-week, 4-week, 8-week and 12-week after injury, respectively. Panels E, F, G, and H show the severed MCLs in HBO group at 2-week, 4-week, 8-week and 12-week after injury, respectively. Both groups showed progressive changes in cell number, size (metabolic activity), distribution, and orientation are noted as stages of inflammation, proliferation, and remodeling take place. However, collagen fibers (blue color stain) in the HBO group had denser blue color (higher density) and better alignment than those in the non-HBO group at 2, 4, 8, and 12 weeks (Trichrome stain, ×200). HBO = hyperbaric oxygen; MCL = medial collateral ligament.