

ORIGINAL ARTICLE

THE EFFECT OF HYPERBARIC OXYGEN THERAPY ON ACUTE WOUND HEALING IN RABBITS: AN EXPERIMENTAL STUDY AND HISTOPATHOLOGICAL ANALYSIS

Jakub Tlapák ^{1,2}✉, Petr Chmátal ¹, Jaroslav Pejchal ², Boris Oniščenko ^{1,3}, Věra Radochová ², Petr Došel ¹, Jiří Páral ², Petr Lochman ²

¹ The Institute of Aviation Medicine, Prague, Czech Republic

² Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic

³ Third Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

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Summary

Background: The goal of our research is to show the effects and impacts of hyperbaric oxygen therapy (HBOT) on acute model wounds in animal subjects.

Methods: Three experimental groups were created using injured rabbits (N=36)—randomly divided into three groups (N=12 per group). One group was treated only with standard wound care management. Two groups were additionally treated with HBOT either once or twice a day. The wounds were surgical, uninfected, and in healthy animal test subjects. We compared the immunohistochemical and histological parameters in 4-, 7- and 10-day intervals.

Results: The detection of epidermal leaf parameters, the number of microabscesses, the Histopathological Superficial Epithelium Healing Score, Connective Tissue Healing Score, Histopathological Acute Inflammation Score and Total Histopathological Wound Healing Score all showed significant changes between time intervals within the individual groups.

Conclusion: The results did not show that HBOT had a significant effect on the healing process of uncomplicated acute wounds.

Key words: Hyperbaric oxygen; Wound healing; Animal models; Adjunctive treatment

Introduction

Hyperbaric oxygen therapy (HBOT) uses a combination of inhalation of 100% oxygen and pressure acting on the body, exceeding 1 atmosphere absolute (ATA). Both mechanisms increase the level of oxygen in the tissue, with a many times increase in the content of dissolved oxygen in the blood plasma and full hemoglobin saturation. The combination of these many effects of HBOT contribute to a favorable course of wound healing. Recent human and animal model works have shown that HBOT influences the healing process—It positively affects angiogenesis (1),

fibroblast proliferation, collagen synthesis (2), epithelialization (3), wound edema reduction (4), the redistribution of flow to the hypoxic area, the correction of tissue hypoxia (5) and also has an antibacterial effect (6).

Methods and procedures accelerating the healing process are, in the long term, in the interest of all teams dealing with traumatology and health care management, especially in terms of economizing treatment costs. The studies included in the latest systematic review by Smet, et al. (7) show a positive outcome on wound healing when using HBOT. The economic impact of the use of HBOT in this indication is shown by the results of a systematic review by Santema, et al. (8). In this systematic review is demonstrated little direct evidence of the cost-effectiveness for HBOT despite its wide application. Review articles indicate a lack of available strong evidence for both the economic outcome and effect of HBOT on acute wounds. The application of hyperbaric oxygenation as an adjuvant therapy appears to be beneficial not only in the civilian field, but also in the military, regarding complex battle wounds (9).

This randomized controlled experimental study was conducted to evaluate the effectiveness of HBOT in the treatment of acute open uncomplicated wounds on an animal model using histological and immunohistochemical analyses.

Methods and Materials

This experimental study of histopathological changes in animal wound models treated with hyperbaric oxygen was analyzed using histological and immunohistochemical techniques. Thirty-six adult female New Zealand White rabbits (initial weight between 2.4 – 2.9 kg) were randomly divided into three parallel-groups to compare the use of standard wound management (wound and incision sites were cleansed, disinfected and used sterile dressings) and HBOT in two different regimens. Four wounds were evaluated. Sampling was performed at 4- (right distal wound), 7- (left distal wound) and 10-day (right proximal wound) time intervals. The left proximal wound (the fourth wound) was left without a biopsy to compare the average healing time between the groups. Surgery day was defined as day 0 (wound induction). The experiment was terminated after the complete healing of the left proximal wound. The study was conducted at the Institute of Aviation Medicine, Prague.

Drugs

The introduction of the subjects to general anesthesia was through a combination of an intramuscular application of ketamine (50 mg/kg) and xylazine (3 mg/kg), and supplemented with 100% oxygen (flow rate 3 L/minute) via a face mask. General anesthesia was administered via an inhalation anesthetic with isoflurane. This drug application was used for both the wound procedure and biopsy.

Animal and tissue preparation

Four wounds, measuring 2 x 2 cm in the area of the dorsal muscles that penetrated the level of the muscular fascia, were created. The wound position was chosen to achieve as much tissue tension as possible. The proximal wound was 6 cm from the blade-bone on both sides of the spine and 1.5 cm from the midline of the spine; distal wounds were located 11 cm distally from the blade-bone on both sides of the spine, at the same distance from the median line as the proximal wound. In order to maintain the greatest possible unification of the wounds, the wounds were formed according to a template with an internal dimension of 2 x 2 cm of sterilizable material. Sampling was again performed by a unified method using a template with internal dimensions of 3.5 x 3.5 cm. The methodology of wound procedure and the technical implementation of HBOT had already been used and applied in another experiment at our Institution (10).

Bandages were monitored three times a day. Every second day, wounds were cleansed and disinfected (with a Betadine® solution) and covered with a new sterile dressing.

Hyperbaric oxygen treatment

A hyperbaric chamber with a volume of 10 cubic meters was slightly modified to comply with our experiment. Subjects were placed into steel, semi-hermetic, monoplace boxes filled with a continuous flow of oxygen to provide

Figure 1. Semi-hermetic monoplace boxes placed in the hyperbaric oxygen chamber and their connection to the oxygen system.



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sufficient fresh gas exchange (*Figure 1*). The suitability of the materials used was based on the recommendations of the American Society for Testing and Materials International and WHA International. All of the boxes had been tested for their resistance to pressure. Welfare monitoring of the animal subjects was done through a transparent top plate on these boxes by a continuous camera system. A tympanoscopy (Otoscope OX1-USB, Germany) was performed on sampling days when each animal was anesthetized. HBOT was applied using two different regimens—Two hours once a day and two hours twice a day, both under 2.5 atmospheres absolute (ATA) of pressure and a fixed oxygen flow. Slow compression and decompression rates were chosen, with each phase lasting for fifteen minutes. Length of exposure, including compression and decompression, was 120 minutes without a break. The length of isocompression was 90 minutes. A 4 L/minute flow rate of oxygen was input into each box. The therapy started two days after the wound induction. The animals were exposed for eight consecutive days.

Histology analysis

After the processing of bioptic material (fixation, standard processing, sectioned at 5 μ m for stains), the samples were evaluated under the Olympus BX51 (Olympus, Tokyo, Japan) and Olympus IX71 microscopes, equipped with MMI software (Olympus) for computer image analysis (ImagePro 5.1.; Media Cybernetics, Bethesda, MD, USA) by a certified independent histologist blinded to the experimental groups. A microscope magnification of 400x was used. The histopathological evaluation included epidermal leaf parameters (length and surface, mitotic activity, and the distance of epidermal leaves), the number of microabscesses, the Histopathological Superficial Epithelium Healing Score, Connective Tissue Healing Score, Histopathological Acute Inflammation Score (HAIS) and Total Histopathological Wound Healing Score between the groups. The stains used to evaluate the samples were hematoxylin-eosin (H-E), Masson's Trichrome, Van Gieson's stain, and naphthol AS-D chloroacetate esterase-positive cell detection.

The Histopathological Superficial Epithelium Healing Score parameters (with H-E stain) included the extent of epithelialization and differentiation. Epithelialization was scored from 0-4, with 0 = in front of the cut edge; 1 = on the edge of the cut; 2 = in the wound < 50% of extent; 3 = in the wound > 50% of extent; 4 = complete epithelial coverage. Differentiation was scored from 0-2, with 0 = none or only spinous; 1 = granular differentiation-keratinization; 2 = adnexa creation. Their total scores were 0-6; a higher number indicating a better degree of wound healing.

The length, surface, mitotic activity, and distance of the epidermal leaves (H-E stain; ImagePro 5.1. analysis) were evaluated. To measure length, surface and distance of the epidermal leaves, whole tissue microphotographs were acquire 400x magnification using Olympus IX71 microscopes equipped with MMI software. The epidermal leaf length were defined as the shortest distance between the apex of the newly formed epidermal leaf and the nearest adnexa. The epidermal leaf surface represents the surface between the apex of the newly formed epidermal leaf and the nearest adnexa. Distance of the epidermal leaves represents the shortest straight distance between the two apexes of the epidermal leaves. Mitotic activity was assessed oculometrically as the number of mitotic figures in the epidermal leaf per 100 basal cells starting from the apex. Connective Tissue Healing Score was defined by five parameters, the criteria being shown in Table 1.

Table 1. Connective Tissue Healing Scoring criteria; scale modified from Sultana et al. (2009), (11)

Scoring criterion used stain	Scoring scale				
1 – amount of granulation tissue <i>Hematoxylin-eosin stain</i>	0 – profound,	1 – moderate,	2 – scanty,	3 – minimal,	4 - absent
2 – amount of early collagen <i>Masson's Trichrome stain</i>	0 – absent,	1 – minimal,	2 – scanty,	3 – moderate,	4 – profound
3 – amount of mature collagen <i>Masson's Trichrome stain</i>	0 – absent,	1 – minimal,	2 – scanty,	3 – moderate,	4 – profound
4 – collagen fiber orientation <i>Masson's Trichrome + Van Gieson's stain</i>	0 – without orientation,	1 – vertical,	2 – mixed,	3 – horizontal	
5 – pattern of collagen <i>Masson's Trichrome + Van Gieson's stain</i>	0 – amorphous,	1 – reticular,	2 – mixed,	3 - fascicle	

Total healing score of each member was calculated by adding the score of individual criteria and with higher scores indicating better wound healing. Healing status with values from 0 to 18 points.

HAIS indicates the amount of naphthol AS-D chloroacetate esterase-positive cells per field of view, which was defined as the microscopic region in close proximity to the fibrin or epithelial layer. For each sample, ten fields of view were randomly scored according to a semi-quantitative scale—from 0-4, with 0 = high (> 200); 1 = medium (50 – 200); 2 = low (20 – 49); 3 = minimal (10 – 19); 4 = level uninjured skin (< 10). A higher number indicates a higher degree of wound healing and a lower level of inflammatory response. The number of microabscesses was evaluated in the samples for the detection of naphthol AS-D chloroacetate esterase-positive cells.

The Total Histopathological Wound Healing Score equals the Histopathological Superficial Epithelium Healing Score (total score) plus the Connective Tissue Healing Score (total score) plus HAIS.

Immunohistochemistry

The immunohistochemical detection of CD34+ endothelial cells was made by using the manual peroxidase technique in the histological laboratory of the Department of Radiobiology (Faculty of Military Health Sciences, Hradec Kralove, Czech Republic) according to the previously published protocol (11). The samples were incubated with mouse anti-CD34 monoclonal antibodies (MA1-10202, ThermoFisher Scientific, Waltham, MA, USA) and rat anti-CD34 antibodies (GTX28158, GeneTex, Irvine, CA, USA) in a phosphate buffer (pH 7.2, Sigma-Aldrich). The primary mouse antibody was followed by a secondary biotinylated donkey anti-mouse antibody (715-065-151, Jackson ImmunoResearch Laboratories, Baltimore, PA, USA) using streptavidin peroxidase (DAKO Corporation, Carpinteria, CA, USA) during incubation. In the case of the primary rat antibody, a secondary goat anti-rat antibody (ab214882-15ml; Abcam, Cambridge, England) was utilized using bound polymerized peroxidase. During this step

of incubation, streptavidin-peroxidase was omitted and 3,3'-Diaminobenzidine tetrahydrochloride chromogen (Sigma-Aldrich) was added to visualize bound in situ antibodies. Negative (no primary antibodies) and positive controls (human spleen samples) were included to support the validity of staining.

Statistical analysis

To determine the significance of the data difference, the data obtained were analyzed using IBM SPSS Statistics version 24 (IBM Corporation, Armonk, NY, USA). Data were evaluated using a nonparametric Kruskal-Wallis test, to determine whether there was a significant difference between all groups, with a post hoc Mann-Whitney test, to determine the p-value between the groups. The level of statistical significance (p-value) was set at ≤ 0.05 . Results are presented as means $\pm 2 \times$ Standard Error of the Mean (SEM).

Results

The physical examinations were physiological in all the rabbits. The animal tolerance of the pressure changes did not show any abnormalities. We did not record any incidents or suspicion of discomfort during the experiment. No signs of barotrauma were observed during tympanoscopic examinations. The open wounds healed without the use of antimicrobial drugs and no signs of infection were observed. The collective weight gain of the rabbits was 18%. The average healing time of the left proximal open wound (the fourth wound in which no biopsy was taken) was 18 days in the control group and 16 days in both treatment groups. The macroscopic view of the wounds in the time intervals between the experimental groups is compared in the *Figure 2*.

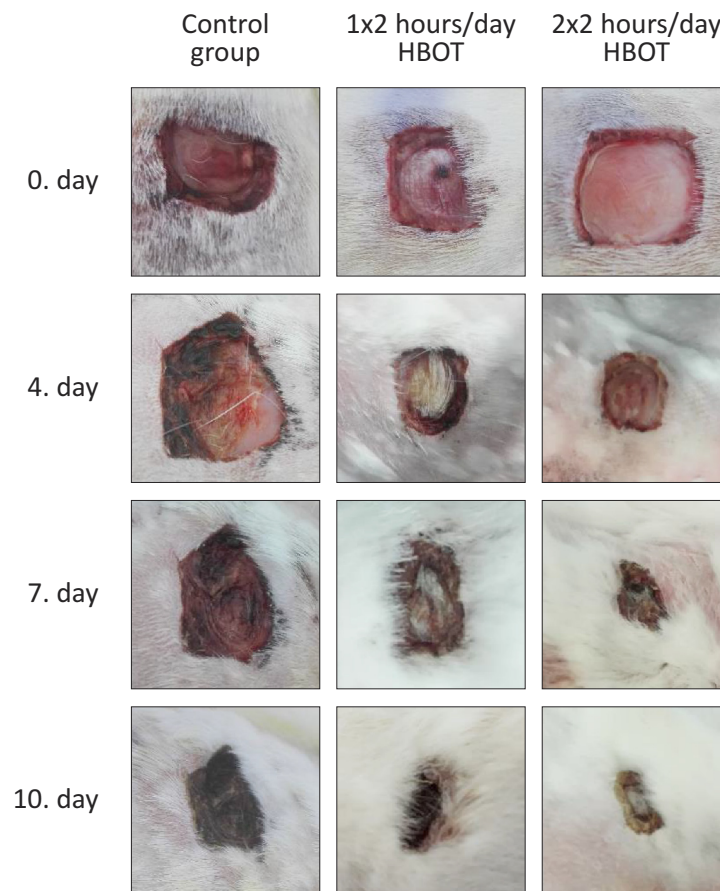
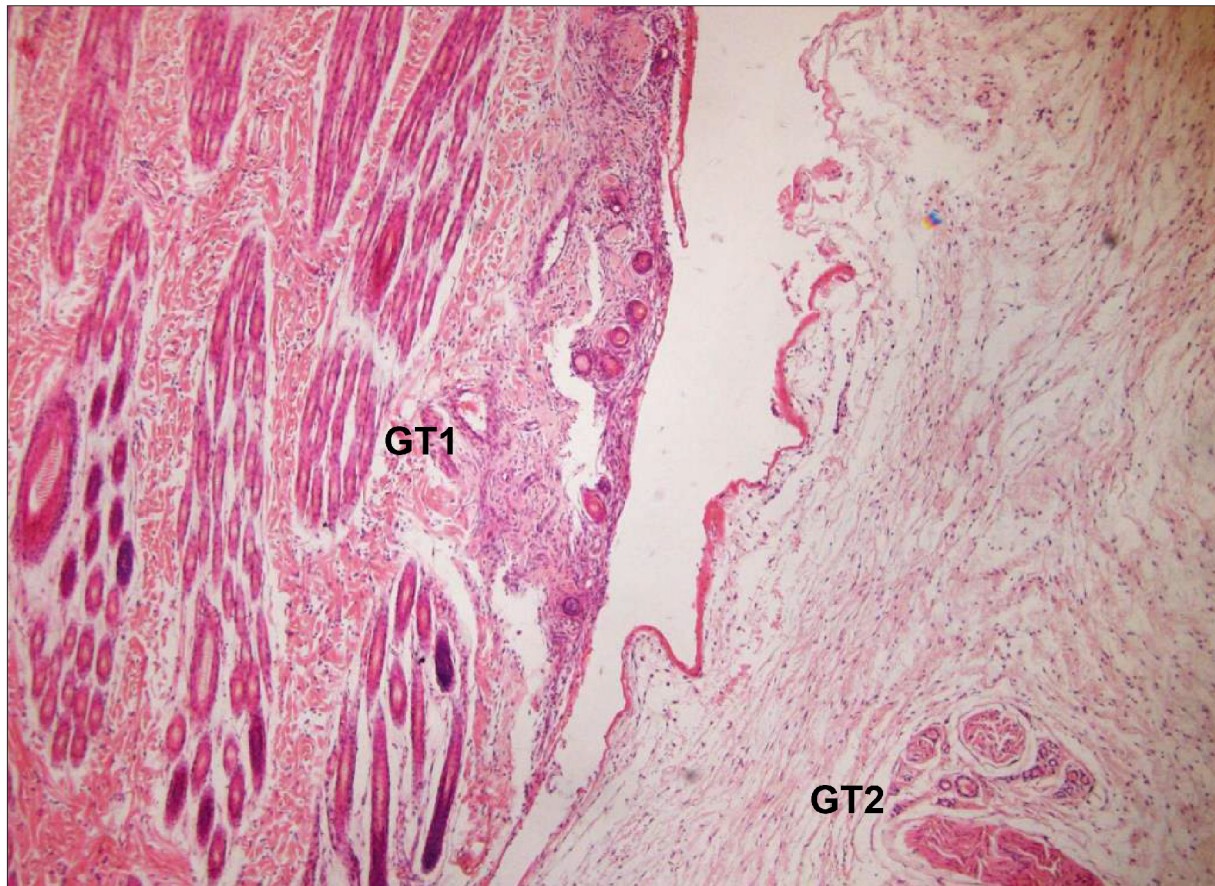


Figure 2. The wound healing progression of all groups in a rabbit model. Images taken during macroscopic size evaluation among different treated groups at days 0, 4, 7, and 10 after wounding.

A total of 108 samples were taken (12 samples from each time interval for each group) for histopathological analysis. Histopathological Superficial Epithelium Healing Scores covering the extent of epithelialization and differentiation were not significantly affected by HBOT. There were no statistically significant differences between the groups in the 4-, 7-, or 10-day time intervals of epidermal leaf parameters. Changes in epidermal leaf length and surface were observed only at time intervals of seven and ten days within groups. Epidermal leaf distance could only be assessed at 10-day intervals. In previous intervals, the total amount of ligament was low, which could affect the parameter. Concerning mitotic activity, no differences between the groups were found.

Figure 3. Granulation tissue evaluation. Granulation tissue formation (GT1) is visible at the cut edge of the sample. Another deposit of granulation tissue is the islet forming from the connective tissue around the nerve fiber (GT2).



GT, granulation tissue; hematoxylin-eosin stain; 400x magnification; 4-day interval; control group

The HAIS and Connective Tissue Healing Scores did not show a significant difference between the groups. Differences were noted when comparing parameters between time intervals within groups. In the case of the amount of granulation tissue (*Figure 3.*), differences were found only between the 7- and 10-day intervals in the control group and in the HBOT group 2×2 hours/day. The amount of early collagen showed changes in all groups between day intervals. The increase in mature collagen was observed in the control group between the 7- and 10-day intervals. Collagen fiber orientation showed a significant increase toward a more mature grade in the control and HBOT 1×2 hours/day group between the 4- and 7-day intervals, and in all groups between the 7- and 10-day intervals. In the collagen fiber pattern, there was a change in all groups only when comparing the 4- and 7-day intervals. Significant differences in the Connective Tissue Healing total score were noted between all three time intervals within all groups. The data of the mentioned histopathological parameters above are summarized in Table 2. Significant differences could only be observed between time intervals within groups.

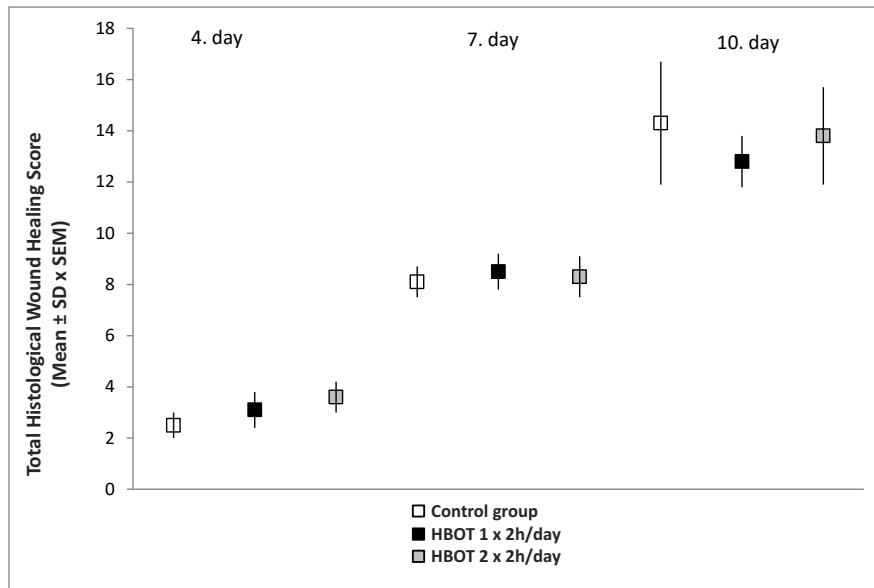
Table 2. Histopathological parameter scores from biopsies of uncomplicated open wounds evaluated on days 4, 7 and 10.

Day	Superficial Epithelium Healing Score			HAIS	Epidermal Leaf				Number of MA	Connective Tissue Healing Score					
	E	D	SE total score		length (μm)	surface (μm²) x 1000	distance (μm)	mitotic activity		granulation tissue	early collagen	mature collagen	collagen fiber orientation	collagen fiber pattern	total score
control group															
4	0.6 ± 0.3	0.0 ± 0.0	0.6 ± 0.3	1.3 ± 0.4	500 ± 100	20 ± 5	N	0.5 ± 0.3	0.3 ± 0.3	0.0 ± 0.0	0.3 ± 0.3	0.0 ± 0.0	0.3 ± 0.3	0.0 ± 0.0	0.7 ± 0.6
7	1.2 ± 0.2*	1.0 ± 0.0*	2.2 ± 0.2*	2.3 ± 0.4*	1800 ± 300*	163 ± 31*	N	0.5 ± 0.3	0.1 ± 0.2	0.0 ± 0.0	1.5 ± 0.3*	0.0 ± 0.0	1.1 ± 0.2*	1.0 ± 0.0*	3.6 ± 0.4*
10	2.2 ± 0.3*	1.4 ± 0.3	3.6 ± 0.5*	2.8 ± 0.5	3300 ± 1000*	281 ± 97*	3200 ± 1900	0.6 ± 0.3	0.1 ± 0.2	1.1 ± 0.8*	3.3 ± 0.5*	0.8 ± 0.3*	1.9 ± 0.5*	1.0 ± 0.0	8.0 ± 1.8*
HBOT 1 x 2 hours/day group															
4	0.9 ± 0.2	0.1 ± 0.2	1.0 ± 0.3	1.4 ± 0.3	500 ± 100	23 ± 6	N	0.4 ± 0.2	0.1 ± 0.2	0.0 ± 0.0	0.4 ± 0.3	0.0 ± 0.0	0.4 ± 0.3	0.0 ± 0.0	0.7 ± 0.6
7	1.5 ± 0.3*	0.9 ± 0.2*	2.5 ± 0.4*	2.5 ± 0.3*	1600 ± 500*	134 ± 45*	N	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 0.3*	0.0 ± 0.0	1.1 ± 0.2*	1.0 ± 0.0*	3.5 ± 0.4*
10	2.2 ± 0.2*	1.5 ± 0.3*	3.6 ± 0.3*	2.6 ± 0.6	2400 ± 600*	220 ± 51*	2400 ± 600	0.4 ± 0.2	0.1 ± 0.2	0.4 ± 0.3	2.9 ± 0.5*	0.5 ± 0.3	1.8 ± 0.4*	1.0 ± 0.0	6.5 ± 1.1*
HBOT 2 x 2 hours/day group															
4	0.9 ± 0.2	0.1 ± 0.2	1.0 ± 0.2	1.4 ± 0.3	500 ± 200	33 ± 14	N	0.4 ± 0.2	0.3 ± 0.4	0.0 ± 0.0	0.6 ± 0.3	0.0 ± 0.0	0.6 ± 0.3	0.0 ± 0.0	1.2 ± 0.6
7	1.3 ± 0.3	1.0 ± 0.0*	2.3 ± 0.3*	2.0 ± 0.3*	1900 ± 500*	184 ± 45*	N	0.3 ± 0.2	0.2 ± 0.2	0.1 ± 0.2	1.8 ± 0.4*	0.2 ± 0.2	1.1 ± 0.2	1.0 ± 0.0*	4.1 ± 0.7*
10	2.3 ± 0.3*	1.4 ± 0.3	3.7 ± 0.4*	3.0 ± 0.4*	2900 ± 900	276 ± 97	2300 ± 900	0.3 ± 0.2	0.0 ± 0.0	0.8 ± 0.4*	3.0 ± 0.5*	0.7 ± 0.4	1.8 ± 0.4*	1.0 ± 0.0	7.2 ± 1.6*

E, epithelialization; D, differentiation; SE, superficial epithelium; HAIS, histopathological acute inflammation score; MA, microabscesses; HBOT, hyperbaric oxygen therapy; N, not evaluated. Data are mean ± SD x SEM. * significant difference from the previous time point (p-value ≤ 0.05).

No significant differences in the Total Histopathological Wound Healing Score were found between the groups in the 4-, 7-, and 10-day intervals after wound induction (mean ± SD x SEM, $p \leq 0.05$). Results of the control group 2.5 ± 0.5 , 8.1 ± 0.6 , 14.3 ± 2.4 (4-, 7-, 10-day; $n=12$), the HBOT 1 x 2 hours/day group 3.1 ± 0.7 , 8.5 ± 0.7 , 12.8 ± 1.0 (4-, 7-, 10-day; $n=12$) and the HBOT 2 x 2 hours/day group 3.6 ± 0.6 , 8.3 ± 0.8 , 13.8 ± 1.9 (4-, 7-, 10-day; $n=12$). Summarized in the *Figure 4*.

Figure 4. Graph of the Total Histopathological Wound Healing Score for individual groups and time intervals.



HBOT, hyperbaric oxygen therapy; control group ($n=12$) versus HBOT 1 x 2 hours per day ($n=12$) versus 2 x 2 hours per day ($n=12$); 4-, 7-, 10. sampling day; mean ± SD x SEM, $p \leq 0.05$

Because both the mouse and rat antibodies showed a false negativity to the immunohistochemical detection of CD34+ endothelial cells, the evaluation of the number of CD34 positive structures could not be performed.

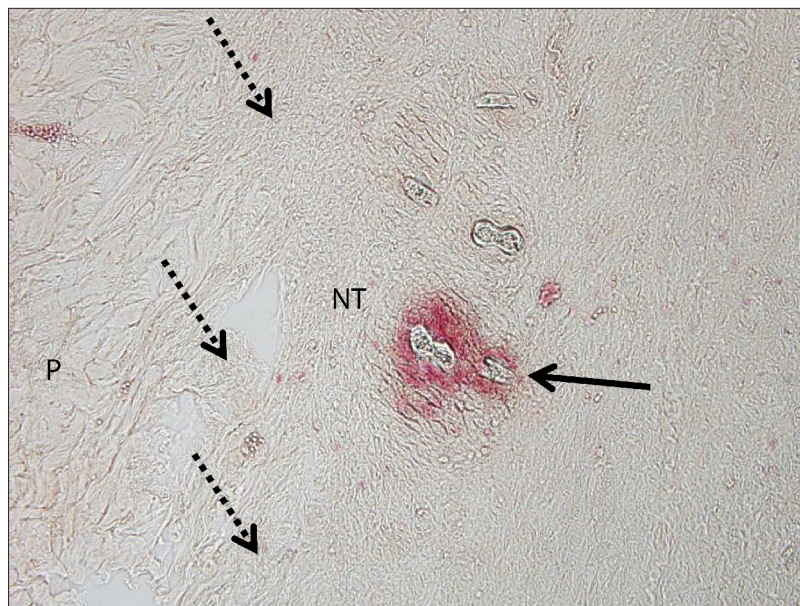
Discussion

This experimental study prospectively evaluates the effects of HBOT on uncomplicated wound healing processes in animal models. It complements the lack of evidence for acute wounds using this therapy. Despite the fact that pig models have been shown to have higher correlation to human healing, rabbits are frequently used as animal models to study wound healing process because of their cost, ease of handling and technical feasibility (12).

The treatment pressure used is based primarily on the general standard of treatment in the Czech Republic. 2.5 ATA is used not only for wound healing, but also for some otorhinolaryngological diseases (13) and other indications. However, for some indications, a different protocol may be considered—for example, lower overpressure for indications related to brain damage (14) or higher for anaerobic infections (15). A conservative approach was chosen, where 2.5 ATA therapy is commonly used in wound healing (16). HBOT can cause oxygen-induced seizures. Mammals may be more sensitive to oxygen-induced seizures than humans (17). Moreover, there are species differences in susceptibility to oxygen toxicity. Pulmonary oxygen toxicity is dependent on the concentration and duration of exposure to high oxygen concentrations (18). These are other arguments for a conservative therapy. The reason for comparing more intensive therapy—twice a day—with once daily HBOT is due to the conflicting results of previous studies. In another hyperbaric oxygen study dealing with lower limb trauma, an increased number of daily sessions at the beginning of acute wound healing treatment produced better results (19). On the other hand, other studies on skin graft survival in rats did not confirm this benefit (20). In our study, there was no significant difference between the individual HBOT regimens.

If the rate of healing according to histopathological evaluations with published models is compared, it appears to be slower than it was with punch biopsies on the backs of rabbits in the range of 6×10 mm (21). On the other hand, it corresponds to the healing rate of larger wounds (22). These comparisons are only approximate; the more accurate comparison corresponds to the results of the study in dogs with the same size wounds (23). Within the groups, it is

Figure 5. Microabscess (solid arrow; red color - stain) forming around the remain of two hairs at the borderline (dotted arrows) of the original tissue and the newly formed connective tissue.



NT, newly formed connective tissue; P, original tissue; naphthol AS-D chloroacetate esterase-positive cells detection; 200x magnification; 7-day interval; control group.

possible to observe a relatively high variance of the values of the Total Histopathological Wound Healing Score. Although it is not possible to make positive conclusions from the results of the histopathological analysis, factors that could affect the rate of wound healing are identified. The first factor is the quality of the edge cut. Furthermore, the variance of HAIS data (especially in the 10-day interval) could, on the one hand, simply reflect slower wound healing, and on the other hand, indicate the presence of infection in the wound. Macroscopically, the presence of infection was not observed. Microabscesses were rarely present in the wounds (*Figure 5*). There was a cluster of neutrophilic granulocytes that formed exclusively around the microscopic remnants of hair. The amount was very low within the groups and individual samples (individual cases) and the size of the microabscesses was small and did not affect the new formation of the surrounding connective tissue. There is also a question of whether their presence in a furry animal with a superficial wound can be completely eliminated. In the immunohistochemical analysis, both mouse and rat antibodies designed to detect CD34 positive structures in a rabbit model showed false negativity, therefore it would be appropriate to test more species of animal antibodies.

Wound contraction is one of the most significant limitations of using animals to model human wounds. The limitations of the present experimental study were the number of subjects and the quality of cutting the edge of individual samples. The results of the experiment can help increase the level of evidence, which is pointed out by survey analyses and the consensus of hyperbaric medicine (24). Further large scale studies will be required to confirm our findings.

Conclusion

Histopathological analysis did not detect an improvement in the healing process with HBOT both compared to the control group and between treatment regimens. Although there is literature evidence suggesting the effectiveness of HBOT, further studies are needed to evaluate the benefit of applying HBOT in acute wound care to treatment recommendations.

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Conflict of Interest

The authors declare that they have no conflicts of interest regarding the publication of this article.

Adherence to ethical standards

This article does not contain any studies involving human participants performed by any of the authors.

The study was approved by the Expert Committee on the Welfare of Experimental Animals at the Faculty of Military Health Sciences, University of Defence. The experiments were performed in accordance with the guidelines of EU Directive 2010/63/EU for animal experiments and with the conditions of the National Decree on the Breeding and Use of Experimental Animals. All workers who manipulated animals are holders of Certificates of Professional Competence to Design Experiments and Experimental Trials under the Animal Welfare Law Against Cruelty. The veterinary specialist was part of the research team.

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