Lasers Med Sci
DOI 10.1007/s10103-008-0629-0

ORIGINAL ARTICLE

The histological and clinical effects of 630 nanometer and 860 nanometer low-level laser on rabbits' ear punch holes

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Received: 7 May 2008 / Accepted: 23 October 2008

Abstract Low-level laser therapy (LLLT) studies on the musculoskeletal and cartilage tissues of rabbits have reported conflicting results. We aimed to investigate the effects of 630 nm and 860 nm low-level laser on injured rabbit cartilage. After punching 5 mm holes in both ears of ten rabbits, we grouped the rabbits randomly. The punched holes of the laser-treated group were irradiated with 630 nm and 860 nm diode laser on days 3–5 and then every other day until day 20. In both laser and control groups, the hole diameters were measured weekly. Histological evaluation was carried out on day 30. The inter-group difference in hole diameters was not significant. Mann–Whitney U tests showed significant inter-group differences in histological variables related to chondrocyte production and organization, growth rate, granulation tissue and pseudocarcinomatosis. LLLT improved cartilage formation and reduced inflammation and formation of granulation tissue. More accurate results on its healing effects warrant studies with larger sample sizes.

Keywords Low-level laser therapy · Cartilage · Diode laser · Healing · Histology

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Introduction

The use of low-level laser in medical science has gained popularity over the past 30 years [1]. Unlike other uses of laser, which usually depend on photothermal effects, the use of low-level laser depends on its photochemical and photobiological effects that do not increase the temperature in the exposed tissue [1, 2]. This type of laser has been successfully implemented in different fields, such as physiotherapy, veterinary medicine, dentistry, and acupuncture [2].

Recently, the healing effects of low-level laser on musculoskeletal tissue, with special attention to the healing process of the cartilage, have been investigated. Cartilage is a specialized form of connective tissue, which consists of chondrocytes sparsely distributed in a firm gel-like extracellular matrix [3], and is surrounded by the perichondrium, which is composed of two distinct layers. The layer immediately adjacent to the cartilage, the chondrogenic or cellular layer, is composed of chondroblasts and a network of small blood vessels. The outer layer of the perichondrium is made of irregularly arranged collagen fibers and fibroblasts [4]. Although the cartilage has limited capabilities for repair, the ability of the chondrogenic layer of the perichondrium to produce cartilage persists into adult life, but it is dormant until a need arises for new cartilage [3].

Studies on the use of low-level laser on rabbit musculoskeletal tissue and cartilage appear to have conflicting

Published online: 03 December 2008

findings [5], with reports of favorable [6, 7] and unfavorable [8] results. Findings from studies on the microcirculation are also conflicting [9, 10].

In light of the above, we designed a laboratory study to investigate the clinical and histological effects of exposure to 630 nm and 860 nm low-level diode laser on injured cartilage in rabbits' ears.

Materials and methods

In this study we used ten white rabbits of the same age, weighing between 2.5 kg and 3.0 kg. After disinfecting both ears of each rabbit with povidone iodine, we anesthetized the ears by local injection of 10% lidocaine and made 5.0 mm holes in the ears with sterile punches. After preparing the samples, we randomly assigned them to two groups of five rabbits each, which were treated equally in terms of nutrition and environment. Both groups were also injected with 200,000 units/kg of penicillin every 12 h during the first 5 days. The control group was only observed and was given no further treatment. The study group was subjected to the following low-level laser therapy (LLLT):

- Red (630 nm) laser with 30 mW power was radiated with a manual K30 probe over a 1.0 cm^2 surface area for 1 min in a direct and continuous fashion until the entire wound had been treated.
- Infrared (860 nm) laser with 100 mW power was radiated with a manual H100 probe over a 1.0 cm^2 surface area for 1 min in a direct and continuous fashion until the entire wound had been treated.

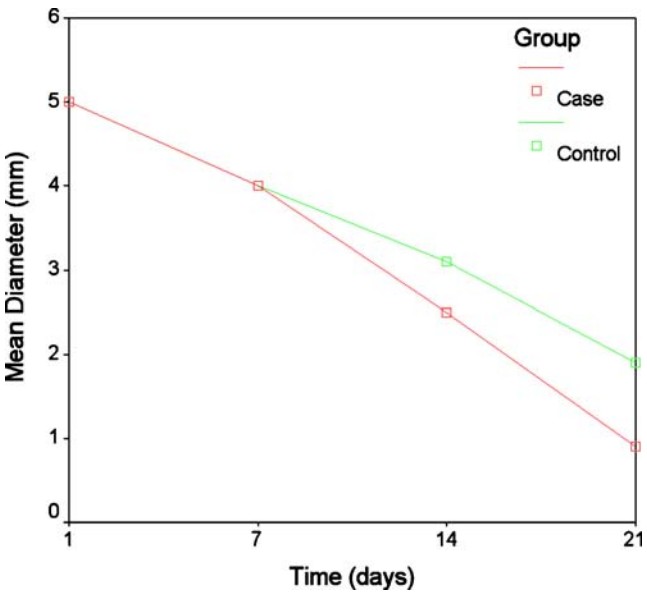

Fig. 1 Changes in mean punch hole diameters in the case and control groups with time

Table 1 Hole diameters (in centimeters) in the ears of the ten rabbits on days 1, 7, 14, and 21

Animal		Day 1	Day 7	Day 14	Day 21
Cases	Rabbit 1	5	5	4	3
		5	5	4	3
	Rabbit 2	5	4	2	0
		5	4	2	0
	Rabbit 3	5	4	2	0
		5	4	3	0
	Rabbit 4	5	4	3	2
		5	3	2	1
	Rabbit 5	5	4	2	0
		5	3	1	0
Controls	Rabbit 6	5	4	3	0
		5	4	2	0
	Rabbit 7	5	4	4	4
		5	4	4	3
	Rabbit 8	5	4	4	3
		5	4	3	2
	Rabbit 9	5	4	3	2
		5	4	3	2
	Rabbit 10	5	4	3	2
		5	4	2	1

In both cases laser was applied with the same apparatus (AZOR-2000, Russia), and the energy density was set at 14.6 J/cm^2. Laser irradiation was given once daily on days 3 to 5 after the punch holes had been created and then every other day for 2 weeks until day 20. To perform clinical evaluations, we measured the punch hole diameters with a ruler and recorded the readings on days 1, 7, 14, and 21. All measurements were taken by a single observer unaware of the grouping and laser treatments.

To perform histological and pathological evaluations, we cut and collected wound edge samples on day 30 after disinfecting and anesthetizing the wound. These samples were then placed in identical code-labeled dishes of 10% formalin. A veterinary pathologist, unaware of the dish codes, studied histological variables including sagittal growth, transverse growth, chondrocyte production and organization, growth rate, collagen synthesis, granulation tissue, hypercellularity, blood vessels, vessel diameter, vessel maturation, leukocyte invasion, pseudocarcinomatosis, focal bleeding, cartilage necrosis, and dermal growth. Semiquantitative variables were graded as zero for none (no change), 1+ for mild changes, 2+ for moderate changes, and 3+ for severe changes.

SPSS version 11.5 software was used for statistical analyses. We used the test of repeated measures to compare the changes in the punch hole diameters between the two groups and the Mann–Whitney U test to analyze semiquantitative variables.

Table 2 Comparison of mean rank of semiquantitative variables in five cases and five controls according to the Mann–Whitney U test

Variable	Case	Control
Chondrocyte production*	8.00	3.00
Chondrocyte organization*	8.00	3.00
Growth rate*	8.00	3.00
Granulation tissue*	3.00	8.00
Pseudocarcinomatosis*	3.00	8.00
Transverse growth	7.00	4.00
Sagittal growth	7.80	3.20
Collagen synthesis	6.60	4.40
Hypercellularity	4.30	6.70
Blood vessels	6.00	5.00
Vessel diameter	6.80	4.20
Vessel maturation	6.80	4.20
Penetration	4.50	6.50
Leukocyte invasion	5.00	6.00
Hyperplastic epidermis	4.60	6.40
Focal bleeding	5.40	5.60
Cartilage necrosis	5.50	5.50
Dermal growth	6.20	4.80

*$P<0.01$

Results

In terms of the punch hole size, the test of repeated measures showed a significant decrease with time in both groups ($P<0.0001$). The reduction in the punch hole size was greater in the laser-treated group after the first week than in the control group, but this difference was not statistically significantly different. Tests within each group also revealed that the second, third, and fourth measurements were statistically different from the first one ($P<0.001$). Table 1 shows the descriptive results of the punch hole diameters in the laser-treated group and the control group at all four measurement sessions. Figure 1 demonstrates these changes in a graph. At the fourth measurement session, the mean punch hole diameter in the laser-treated group was 1.0 mm smaller than that in the control group—an intergroup difference that was not statistically significant.

Results of semiquantitative and microscopic evaluations analyzed with the Mann–Whitney nonparametric U test are presented in Table 2. Overall, comparisons showed that chondrocyte production, and also their organization and growth rate, was significantly greater in the laser-treated group ($P<0.01$), while there was more granulation tissue and pseudocarcinomatosis in the control group ($P<0.01$) (Table 2).

In the treatment group, the chondroblasts were found in the regenerative part of the cartilage. The cells were oval, with a spherical nucleus. In this region the cartilage appeared to be immature and was slightly basophilic. Chondrocytes near the surface were small, with elliptic lacunae with often single and sometimes two cells, forming one or two member cell nests. Deep within the cartilage, the cells were larger and more polyhedral, with a dense network of elastic fibers. Also, relatively regular cartilage columns were observed. Another observation was an increased number of myoblasts and their fusion, producing myotube giant cells and enlarged fibers. In the control group cartilage columns were irregular. There was also considerable fibrous scar tissue, containing many fibrous-type cells, macrophages, and many foci of granulation tissue with newly formed capillaries that filled the holes. Myotube giant cell formation was rarely observed in the control group.

There were no significant differences between the two groups in terms of other histologic variables such as transverse growth, sagittal growth, collagen synthesis, hypercellularity, blood vessels, vessel diameter, vessel maturation, penetration, leukocyte invasion, hyperplastic epidermis, focal bleeding, cartilage necrosis, or dermal growth (Table 2).

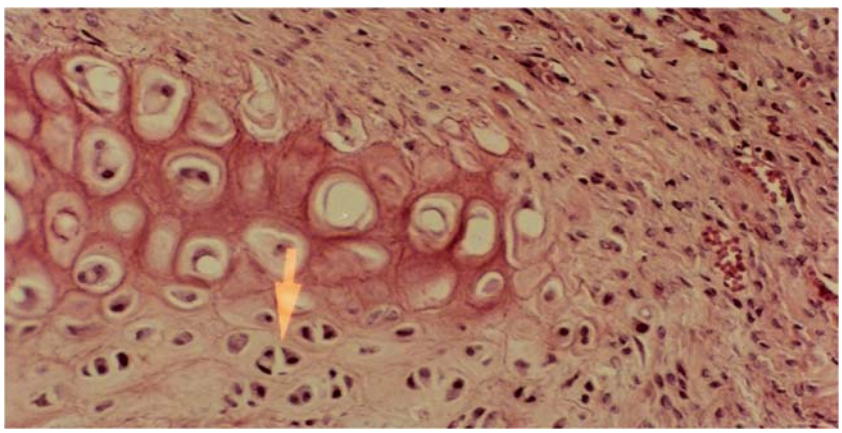

Fig. 2 Growth of cartilage, and mitosis (arrow), in mesenchymal perichondrial cells (appositional growth) in the laser-irradiated group

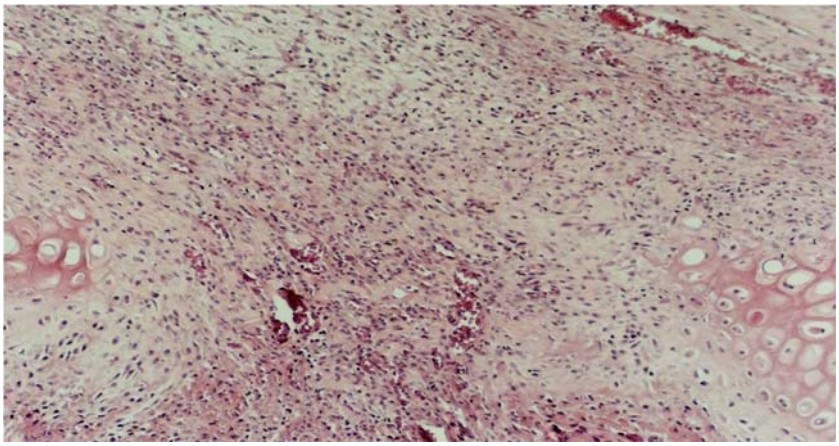

Fig. 3 Closing of perforation (reduced size of punched hole), longitudinal growth of cartilage, and regular pattern of connective tissue in the laser-irradiated group

Discussion

Low-level lasers have been used in different medical fields in the past 30 years. During these years, their biological stimulative effect on cell division and differentiation has been investigated [11, 12].

Studies on Hela cells treated with 685 nm, 630–694 nm, 752–774 nm, and 815–823 nm low-level laser beams showed stimulation of RNA and DNA synthesis [13–15].

In one study reduced edema and inflammatory cells in 72 rat skin wounds was reported as an effect of treatment with low-level laser [16]. Similarly, we found less inflammation in the wounds of the laser-treated group than in the control group. Jia and Guo reported that the exposure to helium–neon low-level laser of chondrocytic cells in laboratory animals led to direct stimulation of chondrocyte production and increased infiltration of these cells into the joints [17]. In another study Bayat et al. found that the depth of chondrocyte filopodia significantly increased after low-level irradiation with helium–neon laser in the immobilized articular cartilage of femora and knees of rabbits [18]. It has also been shown that LLLT on osteochondral defects in rabbits significantly enhanced the stiffness of repairing tissue [19].

In our study the first indication of cartilage regeneration within the holes was the division of fibroblast-like mesenchymal stem cells from the cellular layer of the inner layer of the perichondrium. These cells are transformed into chondroblast-like cells, which enlarge and divide into two separate daughter cells that form isogenous cell groups and secrete a typical cartilage matrix [3, 4]. These processes lead to the longitudinal and transverse growth of the cartilage. In mature cartilage the chondrocyte is a resting cell and functions in a well-regulated balanced system between the cell and matrix; therefore, repair of cartilage defects involves recruitment of cartilage chondrogenic cells to the injury site [3]. In the laser-treated group we observed more cartilage growth and mesenchymal perichondrial cell division than in the control group (Fig. 2). There was less granulation tissue in the laser-treated group but with better organization. Collagen fibers were better organized as well (Fig. 3). There were fewer vessels, but they were more mature. Schindl et al. have stated that LLLT can accelerate endothelial cell proliferation [20].

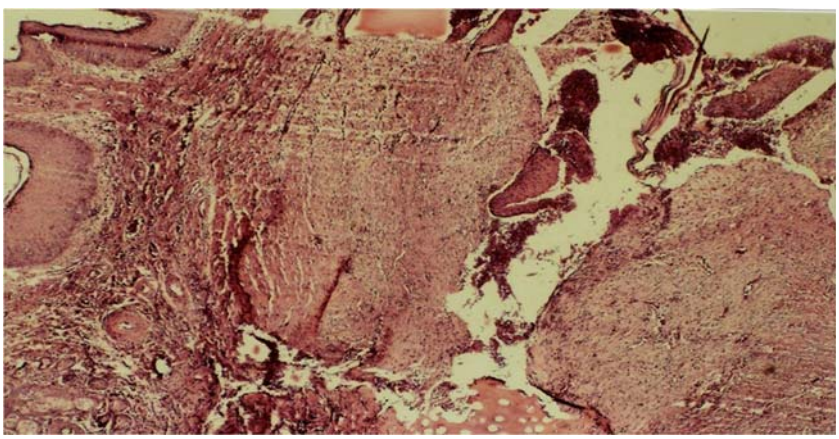

Fig. 4 Deficient cartilage growth and closing of perforation within the connective tissue in the control group

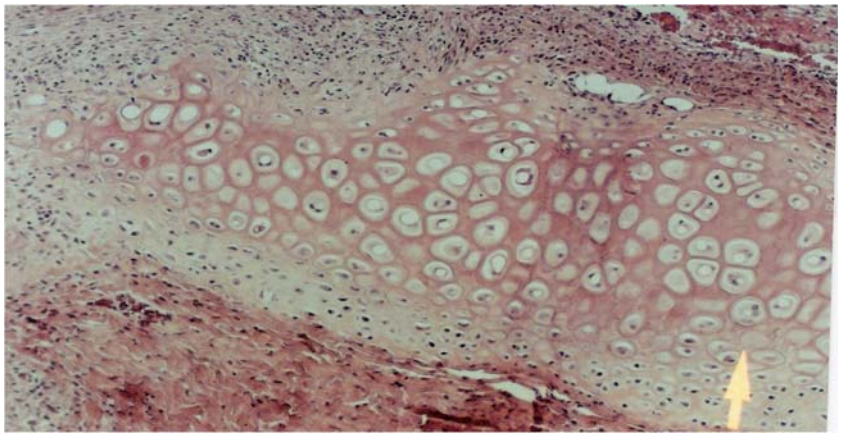

Fig. 5 The region of longitudinal cartilage growth (magnified in the same lamina) in the laser-irradiated group

In our control group we found a great amount of unorganized granulation tissue and a large number of inflammatory cells in the area where the cartilage had been cut. In addition, the gap between the two edges of the cartilage and the connective tissue under the skin was visible (Fig. 4). This indicated that low-level laser could increase the longitudinal growth of cartilage (Fig. 5), increase the number of fibroblasts and angioblasts in blood vessels and, thus, improve healing, with less granulation tissue formation and scarring.

The greater amounts of granulation tissue and severe epidermal hyperplasia in the control group may partly have been due to less growth of the cartilage and unorganized granulation tissue (Fig. 6), which, in turn, led to unacceptably high amounts of the growth factor, granulation tissue, and epidermal layers of the overlying skin. In addition to an association with rapid and organized growth, laser therapy was also associated with a lower risk of secondary infection. The higher numbers of inflammatory cells in the wounds of the control group, especially neutrophils and macrophages, were not only present because of secondary infection, but were also there to remove the debris of tissue degeneration and regeneration.

The wounds of the laser-treated group, in comparison with those of the control group, contained a larger number of polynuclear cells with large round cytoplasm. These cells were, in fact, myoblasts that were proliferating and could then have matured into myocytes. It therefore seems that LLLT can induce the proliferation of myoblasts, have a role in the formation of polynuclear cells and, eventually, improve muscle healing around the cartilage of rabbits' ears.

It must also be noted that LLLT may have a considerable effect on the granulation tissue and cartilage of the ear and nasal septum, which would be interesting to investigate. A cost-effective method with minimum complications, such as LLLT, can be very valuable for patients who undergo surgery of these areas.

In our study the lack of clinically significant differences in punch hole diameters between the two groups, despite significant histological differences, may be attributed to the small sample size of the study; accounting for a power of 0.3. Because of the importance of the issue where cartilage repair is concerned, such as cartilaginous wounds and defects, joint disorders, and also in ear and nose restorative and cosmetic surgery, it is suggested that studies with larger sample sizes be performed.

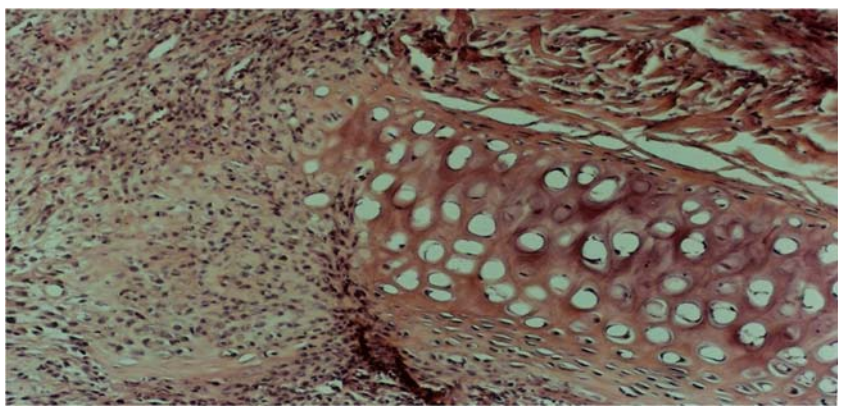

Fig. 6 Psudocarcinomatosis and lack of evidence of chondrocyte growth in the control group

Conclusion

Although LLLT had no statistically significant effect on the healing time of the punched hole, the histological effects led to increased growth of perichondrial cells, reduced granulation tissue with better organization, and increased longitudinal cartilage growth in the rabbits' ears.

Acknowledgments This project was funded by the ENT, Head and Neck Surgery Research Center of Iran University of Medical Sciences. The authors also wish to thank Dr. Shiva Mehravaran for her assistance in the preparation of the manuscript.

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