Lasers in Surgery and Medicine 41:487–491 (2009)

The Effects of Laser Irradiation of Cartilage on Chondrocyte Gene Expression and the Collagen Matrix

Paul K. Holden, MD,[1] Chao Li, BS,[2] Victor Da Costa, MD,[1] Chung-Ho Sun, PhD,[1,2] Susan V. Bryant, PhD,[3] David M. Gardiner, PhD,[3] and Brian J.F. Wong, MD, PhD[1,2,4]*

[1]*Department of Otolaryngology—Head and Neck Surgery, University of California, Irvine, California*
[2]*The Beckman Laser Institute, University of California, Irvine, California*
[3]*Department of Developmental and Cell Biology, University of California, Irvine, California*
[4]*Department of Biomedical Engineering, University of California, Irvine, California*

Objectives: Laser reshaping of cartilage is an emerging technology aimed at replacing conventional techniques for aesthetic and reconstructive surgery. Little is known about the mechanisms of wound healing following the photothermal heating during laser reshaping and, ultimately, how collagen remodels in the irradiated tissue. Healthy hyaline and elastic cartilage as found in the ear, nose, larynx, and trachea does not express collagen type I which is characteristic of fibro-cartilage and scar tissue. The aim of the study was to determine if collagen I and II gene expression occurs within laser irradiated rabbit septal cartilage.

Methods: Nasal septum harvested from freshly euthanized New Zealand White rabbits were irradiated with an Nd:YAG laser. After 2 weeks in culture, the laser spot and surrounding non-irradiated regions were imaged using immunofluorescence staining and evaluated using reverse transcription polymerase chain reaction (RT-PCR) to determine the presence of collagen I and II, and ascertain collagen I and II gene expression, respectively.

Results: All laser irradiated specimens showed a cessation in collagen II gene expression within the center of the laser spot. Collagen II was expressed in the surrounding region encircling the laser spot and within the non-irradiated periphery in all specimens. Immunohistochemistry identified only type II collagen. Neither collagen I gene expression nor immunoreactivity were identified in any specimens regardless or irradiation parameters.

Conclusions: Laser irradiation of rabbit septal cartilage using dosimetry parameters similar to those used in laser reshaping does not result in the detection of either collagen I gene expression or immunoreactivity. Only collagen type II was noted after laser exposure in vitro following cell culture, which suggests that the cellular response to laser irradiation is distinct from that observed in conventional wound healing. Laser irradiation of cartilage can leave an intact collagen matrix which likely allows chondrocyte recovery on an intact scaffold. Lasers Surg. Med. 41:487–491, 2009. © 2009 Wiley-Liss, Inc.

Key words: laser cartilage reshaping; collagen matrix; nasal septum; gene expression

INTRODUCTION

Laser cartilage reshaping (LCR) was first introduced in the early 1990s as a minimally invasive approach to alter cartilage structure and replace conventional cut and suture methods to reshape cartilage tissue [1]. Since then, LCR has been used successfully in various animal [2–4], and human studies [5]. Complementary studies have analyzed the effects of laser radiation on cartilage at both the tissue and cellular level. Flow cytometry has been used to develop a first-order rate process model for cell viability after thermal injury [6]. Wong et al. [7] showed that irradiated chondrocytes showed signs of regeneration using Brd-U to select for cells in the S-phase of the cell cycle. Mordon et al. [8] used PCNA staining to show chondrocyte proliferation after in vivo cartilage reshaping and identified the clonal expansion of chondrocytes in the region of photothermal heating. Karam et al. studied the long-term effect of laser irradiation on nasal cartilages in vivo and determined that tissue may undergo metaplasia and form calcified tissue as well. These and other studies have demonstrated that with appropriate dosimetry, the impact of laser irradiation on chondrocyte metabolism may induce a variety of cellular and tissue responses including chondrocyte proliferation. In septal LCR, the ideal cellular outcome is that wound healing and tissue remodeling in the heated region of tissue would result in the formation of new hyaline cartilage. Hyaline cartilage contains primarily collagen type II in contrast to soft tissues which consist primarily of collagen type I. Collagen type I is found only in fibrocartilage (i.e., at the annulus fibrosis of the intervertebral disk) and in

Contract grant sponsor: National Institutes of Health; Contract grant numbers: D00170, DC005572, RR-01192; Contract grant sponsor: The American Society for Lasers in Surgery and Medicine; Contract grant sponsor: Air Force Office of Scientific Research; Contract grant number: FA9550-04-1-0101.
*Correspondence to: Brian J.F. Wong, MD, PhD, The Beckman Laser Institute and Medical Clinic, University of California, Irvine, 1002 Health Sciences Road, Irvine, CA 92612.
E-mail: bjwong@uci.edu
Accepted 5 June 2009
Published online 28 July 2009 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/lsm.20795

pathologic conditions such as degenerative joint disease at articular surfaces in load bearing joints. Some modest degree of chondrocyte death and matrix injuries occurs in LCR as this is the trade-off cost of shape change. It is unknown what type of cartilage tissue forms after LCR and if the reparative response involves general wound healing mechanisms and the subsequent formation of scar tissue or something more sublime such as the formation of pure hyaline cartilage. Because of the profound mechanical differences between fibrocartilage and hyaline cartilage, it is important to determine whether fibrocartilage forms following LCR.

There is a delicate trigger balancing the expression of collagen type I, collagen type II, and proteoglycans during the classic model of cartilage wound healing [10]. Most of this work has focused on articular cartilage damaged by either chronic mechanical or inflammatory injuries. In the joints, injured cartilage is infiltrated by fibroblasts and capillaries causing the formation of fibrocartilage concomitantly with expression of collagen type I and the production of an accordingly different extracellular matrix. Studies by Hsu et al. [11] demonstrated that the presence of collagen type II in bioengineered cartilage matrix can prevent the migration of fibroblasts and capillaries.

In this study, we examine the cartilage matrix in rabbit nasal septal tissue after laser irradiation and subsequent tissue culture and determine whether collagen I and II immunoreactivity can be identified and whether collagen type I and II genes are expressed. The exclusive expression of only collagen II is critical in maintaining chondrocyte phenotype, and the absence of collagen I would suggest that observed repaired processes are unique and not related to generalized humoral wound healing processes.

MATERIALS AND METHODS

Tissue Specimens

This work was performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at University of California, Irvine. For reference, a schematic of the specimen allocation is shown in Figure 1. Rabbit nasal septal cartilage was extracted from New Zealand White rabbit crania aged 8–12 weeks obtained from local packing houses as previously described. The septal cartilage was prepared for laser irradiation by carefully removing the perichondrium using tissue paper followed by trimming the specimen to size with a razor blade (2 cm × 1 cm). A total of 12 specimens were examined using antibodies: three samples for each of the following four groups: native (no laser irradiation or antibody exposure), negative control (laser irradiation with no antibody exposure), collagen type I antibody with laser irradiation, and collagen type II antibody with laser irradiation.

Laser Irradiation

Specimens were irradiated with light from an Nd:YAG laser (4 mm laser spot diameter, 6 W, 6 seconds irradiation time, $\lambda = 1.32\,\mu$m, 50 HZ PRR, New Star Lasers, Roseville, CA). Specimens were supported by a bed of thin gauge stainless steel taxidermy pins as shown on Figure 2 as previously described [12]. Suspension of the specimen on points of taxidermy pins minimizes contact with conductive materials that could potentially serve as a secondary heat source or heat sink during laser irradiation. Dosimetry parameters were chosen based on previous work that established the relationship between tissue viability, mechanical properties and shape change, and thermophysical measurements [12]. Each specimen was irradiated on two separate spots separated by at least 1 cm from the center of each spot. The cartilage was then cut into 8 mm squares around each laser spot and prepared for tissue culture using a series of antibiotic and antifungal washes as previously described [7]. Twelve specimens were allocated for immunohistochemical evaluation while 12 were prepared for gene expression studies.

Tissue Culture

Irradiated specimens were then placed in tissue culture using sterile technique and incubated in Dulbecco's Modified Eagle's Media (DMEM) without phenol red (Sigma, St. Louis, MO) containing 10% fetal calf serum, gentamicin sulfate (Fisher Scientific, Waltham, MA),

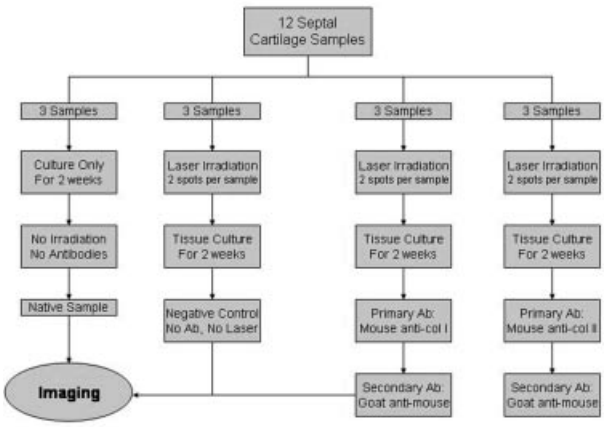

Fig. 1. Schematic of the study design.

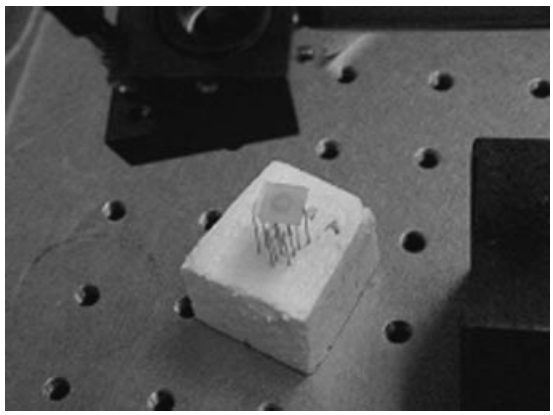

Fig. 2. Digital photograph of typical specimen placement on stainless steel taxidermy pins.

penicillin, streptomycin, and glutamine. Phenol red was not used in order to avoid red fluorescent artifact during microscopy. The samples were then incubated for 14 days at 37°C with 7.5% CO_2. Media was replaced every other day. The selection of the 14-day interval was determined in previous work using rabbit septal cartilage explants in culture [7], and is the time interval at which photothermally stimulated chondrocytes are first identified.

RT-PCR

After 10 days of cell culture, a portion of each set of samples was selected for reverse transcription polymerase chain reaction (RT-PCR) analysis. Three distinct regions were independently evaluated and are shown in Figure 3: (1) the center of the laser spot (CS); (2) the a ring of tissue encircling the margins of the laser spot (*); (3) and the distant unaffected periphery (UAP). The center of the spot is the region most intensely heated and likely devoid of viable chondrocytes using the present dosimetry parameter set [12]. The region just encircling the margins of the laser spot has been previously identified as the location where photothermal stimulated chondrocytes [7]. The distant unaffected periphery served as a control as this region was neither irradiated by the laser nor received any significant secondary heating due to heat conduction effects. One hundred milligrams of tissue was isolated from each of these three regions using dermal punch scalpels of increasing radii in order to obtain enough total RNA for PCR analysis; these samples were separately frozen in liquid nitrogen and then ground using a mortar and pestle. The resulting ground tissue was immediately placed into an RNA stabilizing solution and 2 μg of total RNA was extracted from each 100 mg sample using the RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA quality was then measured by spectrophotometer and acceptable samples (containing more than 2 μg of RNA that has a 260:280 ratio of 1.6 or higher) were treated with 4 Units of DNase I for 1 hour at 37°C. We aimed to isolate three different mRNAs: collagen type I, collagen type II and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which is a ubiquitous protein used here as our positive control. Retrotranscription was carried out using 2 μg RNA, 0.5 μl of 2 μg/μl Anchored oligo (dT) primers for collagen type 1(forward: 5′CAACATGGTGATCAAGGTGC 3′, reverse: 5′GCTGGTCAGCCCTGTAGAAG 3′), collagen type 2 (forward: 5′ AACACTGCCAACGTCCAGAT 3′, reverse: 5′ CTGCAGCACGGTATAGGTGA 3′), and RNA control GAPDH (forward: 5′ CTGACCTGCCGCCTGGAGAAA 3′, reverse: 5′ GGGTCTGGATGGAAACTGTG 3′), and Superscript™ II at 45°C for 90 minutes. PCR was performed on the resulting RT products using Taq polymerase and primers for collagen I and II for 30 cycles at 59°C. PCR products were analyzed using a 1.5% agarose gel and visualized with ethidium bromide.

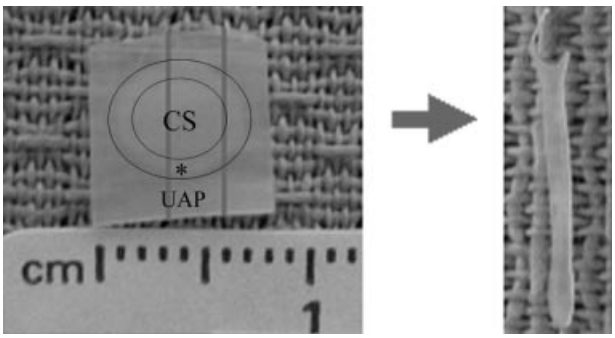

Fig. 3. Digital photograph demonstrating the three distinct areas affected by laser treatment on a cartilage specimen. CS, central laser spot;
*Surrounding ring and UAP, unaffected periphery; also shown is the cross-sectional view.

Immunohistochemistry

After 2 weeks in culture, samples allocated for immunohistochemical analysis were cut in cross section as seen in Figure 3 in order to allow en face imaging of the specimen. The cartilage was fixed for 2 hours in formalin. The 2-hour timeframe was chosen from pilot studies that showed that this was the minimum time for adequate fixation whilst still retaining immunogenicity. After washing with phosphate buffered saline (PBS), the samples were incubated for 15 min in pronase (Sigma). Samples were incubated with a proprietary signal enhancer from Molecular Probes (cat# I36933, Carlsbad, CA), before the addition of a blocking solution (5% goat serum Invitrogen #01-6201 diluted in sterile PBS). Primary antibody for collagen type I (mouse anti-collagen type I, Sigma #C 2456) was added to the collagen type I samples and the primary antibody for collagen type II (mouse anti-collagen type II, Developmental Studies Hybridoma Bank II-II6B3) was added to all samples except the negative control which received 250 μl blocking solution. After 60-minute of incubation at room temperature, the samples were washed, and then re-incubated with blocking solution. A secondary antibody (Alexa-Fluor 594 goat anti-mouse Molecular Probes #A21123) at a concentration of 1 μg/ml was added and allowed to incubate for another 60 minutes at room temperature. Samples were then washed with PBS and stored at 4 degrees until they were imaged.

Imaging

Rabbit septal cartilage specimens were imaged using a Zeiss-Meta 510 Multiphoton Microscope (MPM) (Jena, Germany). Alexa-Fluor 594 secondary antibodies were excited by a Helium Neon laser at 543 nm. Red emitted fluorescence was collected at 617 nm using a Plan-Apochromat 63× oil immersion objective lens.

RESULTS

After irradiation, all of our septal cartilage specimens had three visibly distinct areas of injury: the central laser spot (radius 2–2.5 mm), the surrounding ring (radius 3–4.5 mm), and the unaffected periphery which is consistent with prior work [7]. Analysis of cartilage mRNA and extra cellular matrix structure was based on these three zones.

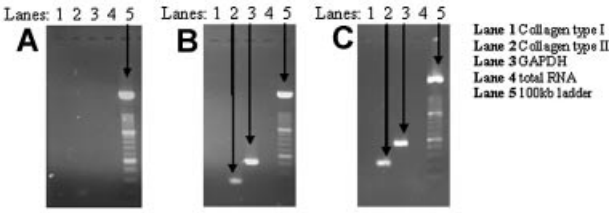

Fig. 4. Digital photograph of RT-PCR results for (**A**) the central laser spot, (**B**) the surrounding ring, and (**C**) The unaffected periphery.

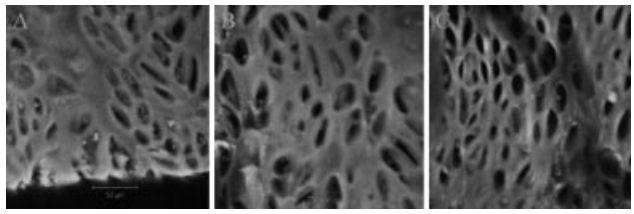

Fig. 6. Digital photomicrograph of immunofluorescence staining for Collagen type II for each portion of a treated specimen, including the (**A**) central laser spot, (**B**) surrounding ring, and (**C**) unaffected Periphery, 100×.

RT-PCR

Analysis of the irradiated cartilage shows complete absence of mRNA expression in the central laser spot (Fig. 4A) for all six specimens tested. There is a small, light band in lane 2 that is likely due to primer dimer formation. In the surrounding ring, we see the return of RNA expression with distinct bands for collagen type II and GAPDH, without mRNA expression seen for collagen type I (Fig. 4B). This was virtually identical in six of six specimens. The unaffected periphery specimens all showed the same expression as the surrounding ring (Fig. 4C). No bands were seen in the total RNA lane, as expected, since it did not contain PCR products.

Immunohistochemistry

We discovered that native cartilage (without laser irradiation or antibody exposure) was identical in appearance to the distal "unaffected" periphery which became a useful control to make more efficient use of our cartilage samples.

Using the mouse anti-collagen type I antibody, we were able to detect signal in the perichondrium only with no signal inside normal cartilage tissue (Fig. 5). There was no difference observed between the irradiated and non-irradiated tissue when using the collagen type I primary antibody for all 12 specimens, suggesting absence of collagen I which is further supported by the gene expression studies above.

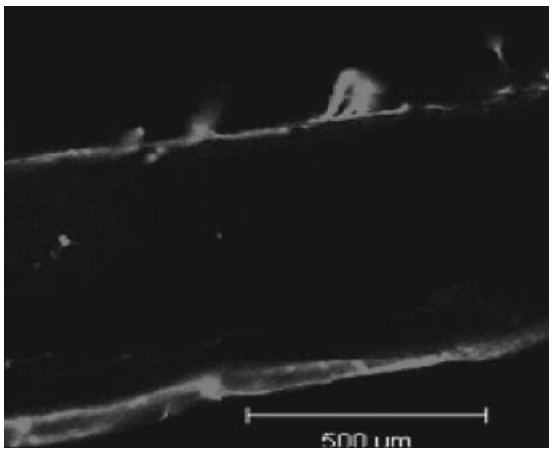

Fig. 5. Digital photomicrograph of immunofluorescence staining for Collagen type I for a treated specimen. This shows no activity (normal signal is present in the perichondrium), 40×.

Imaging using the collagen type II primary antibody yielded clear images depicting the outline of individual lacunae in the cartilage sample as seen in Figure 6 and normal matrix structure. The collagen type II found in the matrix remained immunogenic and appeared to be intact despite laser irradiation, as no major differences were observed between the three regions defined by the laser spot. This was virtually identical in six of six specimens.

Figure 6A is a representative image from the unaffected periphery. The lacunae have a uniform density with broad bands of collagen separating most of the lacunae. In comparison, Figure 6C (from within a central laser spot) also has uniform lacunae; however, the lacunae appear more densely arranged with thinner bands of collagen separating each from one another. Figure 6B represents the surrounding ring and shows an intermediate density of lacunae compared to Figure 6A,C. There is no evidence of increased collagen type II production in response to laser irradiation.

DISCUSSION

Laser cartilage reshaping relies upon the spatially selective heating of cartilage tissue. Heat is generated in regions of increased internal stress in mechanically deformed cartilage tissues with the aim of thermally accelerating the process of stress relaxation. The laser precisely controls the spatial and temporal evolution of heat. Shape change is achieved at the expense of chondrocyte injury within the region of light distribution, and limiting the extent off tissue damage is essential. Despite objective evidence of tissue injury demonstrated in previous studies using both classic and confocal microscopy methods [12,13], several in vivo studies have demonstrated that laser reshaping of cartilage in vivo actually may trigger a photothermally mediated reparative response in tissue as evidenced by the clonal expansion of chondrocytes [7]. Our previous ex vivo work has demonstrated that chondrocyte growth can be stimulated by laser heating in ex vivo cartilage grafts as well in an environment devoid of the cellular and humoral factors that choreograph wound healing in the intact organism. Questions remain regarding the nature of this wound healing process, and whether

the regenerating mesenchymal cells exhibit features of fibroblasts rather than chondrocytes. Unlike chondrocytes, fibroblasts produce collagen type I which is found is scar tissue and fibrocartilage, but not in the hyaline cartilages of the face. Question remains over the fate of laser reshaped cartilage and its matrix composition. This study attempted to identify whether collagen I was produced by regenerating cells using similar dosimetry parameters to earlier studies in our laboratory that identified regenerating chondrocytes using both flow cytometry and whole mount imaging techniques.

Previous work has determined that no viable chondrocytes exist in the center of the laser spot, which reaches a maximal temperature of 74°C when irradiated at 6 W for 6 seconds [12]. Interestingly, immunohistochemical staining for collagen II reactivity in this region persists despite total cell death. This can indicate that the laser parameters used in this study generated tissue temperatures adequate to kill the chondrocytes but insufficient to disrupt the collagen II protein in such a way that that antibody probe was no longer able to bind to its target epitope. At the margins of the laser spot, there is evidence to confirm that chondrocytes are still viable because of continued collagen II mRNA transcription. Expression of collagen I was not detected (both by immunohistochemistry and RT-PCR) which supports the hypothesis that there is no fibroblast activity after 2 weeks of explant culture. This study has shown that the regenerating cells at the margins of the laser spot produce collagen II mRNA but not collagen I mRNA, indicating that these cells maintain their hyaline cartilage phenotype in the wound healing process. However, immunohistochemistry revealed no evidence of increased collagen II production at the margins of the laser spot in response to laser irradiation. Although past studies have established the presence of an endogenous reparative response in terms of increased cell proliferation in this region, this study shows that the force of this reparative process is still incapable of generating a visible protein matrix response in vitro at 14 days post-laser irradiation.

Morphologically, there was noticeable thinning of the collagen bands between lacunae in the central laser spot (Fig. 6C). This could be from partial disruption of the collagen matrix or may be a dehydration artifact from laser heating.

Future studies could compare laser irradiation with other surgical tools such as monopolar or bipolar electrocautery and observe their effects on the histology of cartilage and its matrix. The importance of the presence of an intact collagen matrix for improved cartilage regeneration with minimal fibrocartilage formation may have important implications for the future of tissue engineering.

CONCLUSION

Laser irradiation of rabbit septal cartilage using dosimetry parameters similar to those used in laser reshaping does not result in the detection of either collagen I gene expression or immunoreactivity. Only collagen type II was noted after laser exposure in vitro following cell culture, which suggests that the cellular response to laser irradiation is distinct from that observed in conventional wound healing. Laser irradiation of cartilage can leave an intact collagen matrix which likely allows chondrocyte recovery on an intact scaffold.

ACKNOWLEDGMENTS

Tatiana Krasieva, Ph.D. provided technical support for confocal imaging. Dr. Jim Dennis gave important guidance on immunohistochemical technique and protocol guidance. Chao Li is now at Harvard Medical School. Victor Da Costa is now with the Division of Otolaryngology—Head and Neck Surgery at Duke University. Funding was provided by the Undergraduate Research Opportunities Program Grant of the University of California, Irvine, National Institutes of Health (D00170, DC005572, RR-01192), and The American Society for Lasers in Surgery and Medicine, and the Air Force Office of Scientific Research (FA9550-04-1-0101). Dr. Wong had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

REFERENCES

1. Helidonis E, Sobol E, Kavvalos G, et al. Laser shaping of composite cartilage grafts. Am J Otolaryngol 1993;14:410–412.
2. Chiu LL, Wong BJF, Chang JC. Minimizing superficial thermal injury using cryogen spray cooling during laser reshaping of composite cartilage grafts. http://www.laserexpress.net; 2003.
3. Jones N, Sviridov A, Sobol E, Omelchenko A, Lowe J. A prospective randomised study of laser reshaping of cartilage in vivo. Lasers Med Sci 2001;16:284–290.
4. Wang Z, Perrault DF, Jr., Pankratov MM, Shapshay SM. Endoscopic laser-assisted reshaping of collapsed tracheal cartilage: A laboratory study. Ann Otol Rhinol Laryngol 1996;105:176–181.
5. Ovchinnikov Y, Sobol E, Svistushkin V, Shekhter A, Bagratashvili V, Sviridov A. Laser septochondrocorrection. Arch Facial Plast Surg 2002;4:180–185.
6. Karamzadeh AM, Chang JC, Diaz S, Thomas EM, Wong BJ. Long-term in vivo stability of rabbit nasal septal cartilage following laser cartilage reshaping: A pilot investigation. Lasers Surg Med 2005;36:147–154.
7. Wong BJ, Pandhoh N, Truong MT, et al. Identification of chondrocyte proliferation following laser irradiation, thermal injury, and mechanical trauma. Lasers Surg Med 2005;37:89–96.
8. Mordon S, Wang T, Fleurisse L, Creusy C. Laser cartilage reshaping in an in vivo rabbit model using a 1.54 microm Er:Glass laser. Lasers Surg Med 2004;34:315–322.
9. Karam AM, Protsenko DE, Li C, Wright R, Liaw LH, Milner TE, Wong BJ. Longterm viability and mechanical behavior following laser cartilage reshaping. Arch Facial Plast Surg. 2006 Mar-Apr; 8(2):105–16.
10. Silver FH, Glasgold AI. Cartilage wound healing. An overview. Otolaryngol Clin North Am 1995;28:847–864.
11. Hsu SH, Chang SH, Yen HJ, Whu SW, Tsai CL, Chen DC. Evaluation of biodegradable polyesters modified by type II collagen and Arg-Gly-Asp as tissue engineering scaffolding materials for cartilage regeneration. Artif Organs 2006;30:42–55.
12. Li C, Protsenko DE, Zemek A, Chae YS, Wong B. Analysis of Nd:YAG laser-mediated thermal damage in rabbit nasal septal cartilage. Lasers Surg Med 2007;39:451–457.
13. Choi I-S, Chae Y-S, Zemek A, Protsenko DE, Wong BJF. Viability of human septal cartilage after 1.45 m diode laser irradiation. Las Surg Med 2008 Oct; 4D(8):562-9.