Published in final edited form as:

Lasers Surg Med. 2008 October ; 40(8): 550–561. doi:10.1002/lsm.20666.

Thermoforming of Tracheal Cartilage: Viability, Shape Change, and Mechanical Behavior

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Abstract

Background and Objectives—Trauma, emergent tracheostomy, and prolonged intubation are common causes of severe deformation and narrowing of the trachea. Laser technology may be used to reshape tracheal cartilage using minimally invasive methods. The objectives of this study were to determine: (1) the dependence of tracheal cartilage shape change on temperature and laser dosimetry using heated saline bath immersion and laser irradiation, respectively, (2) the effect of temperature on the mechanical behavior of cartilage, and (3) tissue viability as a function of laser dosimetry.

Materials and Methods—Ex vivo rabbit trachea cartilage specimens were bent and secured around a cylinder (6 mm), and then immersed in a saline bath (45 and 72°C) for 5–100 seconds. In separate experiments, tracheal specimens were irradiated with a diode laser ($\lambda = 1.45 \mu\text{m}$, 220–400 J/cm²). Mechanical analysis was then used to determine the elastic modulus in tension after irradiation. Fluorescent viability assays combined with laser scanning confocal microscopy (LSCM) were employed to image and identify thermal injury regions.

Results—Shape change transition zones, between 62 and 66°C in the saline heating bath and above power densities of 350 J/cm² (peak temperatures 65±10°C) for laser irradiation were identified. Above these zones, the elastic moduli were higher (8.2±4 MPa) than at lower temperatures (4.5±3 MPa). LSCM identified significant loss of viable chondrocytes within the laser-irradiation zones.

Conclusion—Our results indicate a change in mechanical properties occurs with laser irradiation and further demonstrates that significant thermal damage is concurrent with clinically relevant shape change in the elastic cartilage tissues of the rabbit trachea using the present laser and dosimetry parameters.

Keywords

tracheal cartilage; thermoforming; shape change; cell viability; mechanical property

INTRODUCTION

Tracheal stenosis is largely an acquired deformity that results from a combination of events including external trauma and infection as well as iatrogenic causes, such as prolonged endotracheal intubation and tracheotomy. Additionally, the increasing number of infants and children who survive previously fatal forms of respiratory failure also contribute to the rising rate of tracheal stenosis [1–5]. Current surgical treatments consist of serial endoscopic dilation, cartilage grafting, laser ablation, endotracheal stenting, tracheostomy, and segmental resection. These procedures are often associated with significant morbidity and mortality. Stenting is of particular interest, but in the airway, it is well-documented that prolonged stenting can aggravate ongoing tracheal inflammation and lead to worsening stenosis [6–8]. Although modern tracheostomy tubes have low-pressure, high-volume cuffs designed to decrease the tracheal injury, tracheal constriction still occurs and may go unrecognized in patients requiring prolonged mechanical ventilation [9–11].

The trachea is one of the most vital organs in the body, serving as the conduit for inspired oxygen and exhaled carbon dioxide. The tracheal wall is covered with respiratory epithelium circumferentially and has an underlying, semirigid cartilaginous support. Tracheal cartilage, like other hyaline cartilages, is a fiber-reinforced composite in which collagen fibers entrap a matrix of hydrated proteoglycans. There are few studies of the mechanical properties of airway cartilage, in contrast to what is known about articular cartilage biomechanics. Several authors have studied tracheal mechanics and have identified an age-related change in mechanical properties of tracheal cartilage in humans [12–15] and animals [16–19]. The correlation of collagen and proteoglycan organization has been established between changes in tensile modulus and the depth of human tracheal cartilage from the surface [20].

It is clear that there exists a need for the development of a minimally invasive procedure which can accomplish the task of permanently reshaping the trachea at success rates comparable to other conventional minimally invasive surgical approaches while minimizing the associated risks. Thermoforming is the process of altering the shape of cartilage which relies upon heat to accelerate the process of stress relaxation to achieve stable shape change. Its effectiveness has been demonstrated in cartilage reshaping in both in vivo and in vitro experiments using laser sources as a means to generate heat in tissue [21–25]. In the airway, endoscopic laser reshaping of fresh canine tracheal cartilage rings was first performed by Wang et al. [26] in both in vitro and in vivo experiments in canines using an Nd:YAG laser ($\lambda = 1.44 \mu\text{m}$) with fluences below the threshold for both carbonization and ablation.

Although the use of calcein AM and EthD-1 with CLM is a common method for cell viability determination, neither provides an absolute indication of cell metabolic activity relative to the condition of the matrix. In addition, some controversy exists regarding the application of calcein Am and EthD-1 accompanied by confocal laser microscopy (CLM) to

determine chondrocyte viability in cartilage explants [27–29], despite its use and presentation numerous peer-reviewed works [30–33].

Little is known about the extent of thermal damage and the changes in mechanical behavior in tracheal cartilage as a function of laser dosimetry, and this is important information required for further clinical evaluation. Nonetheless, it has been shown that cartilage irradiated below the ablation threshold can retain its new shape and maintain its ability to resist deformation. To further clarify the biophysical changes that occur during cartilage thermoforming in tracheal cartilage, we examined (1) the dependence of shape change on temperature and laser dosimetry using heated saline bath immersion and laser irradiation, respectively, and (2) the effect of temperature on mechanical behavior, and (3) tissue viability as a function of laser dosimetry. Our experiments were performed using transmucosal laser irradiation at relatively low power to reshape tracheal cartilage. To reduce the risk of full thickness tracheal injury and/or perforation, a 1,450 nm diode laser was selected due to its shallow optical penetration depth $\left(1/\sqrt{3\mu_a(\mu'_s+\mu_a)}\right)$ [34,35].

MATERIALS AND METHODS

Tissue Preparation

Rabbit tracheal segments were harvested from 50 New Zealand white rabbits (weight 5 lb) obtained from a local abattoir following euthanasia. Tracheae were obtained <24 hours postmortem from rabbits aged 10 weeks. Segments of trachea 4–5 cm in length were excised and immediately immersed in fresh 0.9% sodium chloride buffer solution and stored at 4°C before each experiment. Further micro dissection was performed immediately before either laser irradiation or mechanical experiments. Previous pilot studies in our laboratory observed the regional variations in biomechanics within the trachea. The distal trachea (closer to the lungs) has thinner rings and is more flexible while the proximal region (closer to the larynx) has thicker rings and is more rigid. In addition, elastic modulus is likely to vary with location around the trachea ring and across the cross section. To minimize experimental variation due to these differences, the specimens chosen for this study were taken only from the proximal (more rigid) region (Fig. 1) along the length of the trachea.

Each tracheal segment consisting of 5–6 tracheal rings was dissected from the larynx in a circumferential fashion, and any remaining tunica fibrosa or the trachealis muscle on the outer surface were removed by using scalpel and a dissection microscope. The mucosa and perichondrium were not removed from the surface of the tracheal lumen.

Saline Bath Immersion

Rabbit tracheal segments were deformed by securing each one to a small acrylic tube (outer diameter 3 mm) using dental rubber bands. Tracheal segment has a luminal (face airway) and abluminal surface (adjacent to the soft tissues of the neck). Tracheal segment was bent to reverse its natural convexity. The abluminal surface of the tracheal segment firmly affixed to the surface of an acrylic tube with use of rubber bands. This resulted in the tracheal segment being pressed against the acrylic tube. During bending, the lumen surface is stretched and the adventitial surface is compressed around a neutral axis where neither

compression nor stretching occurs (schematically illustrated in Fig. 2 as indicated by arrows) until internal stresses reached a state of equilibrium.

The neutral axis is defined as the line of demarcation across the cross section of trachea segment in bending which experiences neither tension nor compression stresses due to internal moment forces where the longitudinal stress is zero. However, the tracheal cartilage rings are not uniform and the cross-sectional area changes along the circumference of each ring slightly. In addition, the cross section of the tracheal segment is not a rectangle or other any other symmetric shape. Two complete tracheal rings (including the intermediate pars membranacea and its cartilaginous insertions) were mounted in the bending apparatus.

The entire assembly was placed into a room temperature (22°C) saline bath and allowed to equilibrate for 5 minutes. The assembly was then rapidly placed in to a temperature controlled saline bath at temperatures between 45–75°C and for immersion times varying between 5 and 300 seconds. To minimize thermal gradients within the bath, the saline bath was constantly stirred and a digital thermometer monitored the bath temperature. Once the designated time interval elapsed, the assembly was removed from the bath and immediately re-immersed in an ambient temperature isotonic sodium chloride (0.9%) solution bath for 10 minutes while secured around the acrylic tube to maintain the semicircular deformation. The distance between the ends of the unfurled specimen was measured after rehydration and removal from the device with a digital caliper to determine degree of shape change (Fig. 2). In control experiments, cartilage specimens were mechanically deformed and then submerged in a room temperature saline bath for duration of 5–300 seconds. Eighteen to 24 specimens were used for each temperature-time setting.

Laser Irradiation

The tracheal segments were each mechanically deformed by securing them to a small tube (outer diameter 3 mm) designed to maintain the specimen in a curved semicircular geometry (Fig. 2) using dental rubber bands. The lumen surface of each cartilage ring was stretched and the adventitial surface was compressed around a neutral axis where neither compression nor stretch occurs (schematically illustrated in Fig. 2 as indicated by arrows) until the internal stresses reached a state of equilibrium. Two complete tracheal rings (including the pars membranacea and its cartilaginous insertions) were mounted in the bending apparatus.

Light from a diode laser ($\lambda = 1,450 \mu\text{m}$, Candela Corp, Wayland, MA) was directed perpendicularly at the surface of the tracheal segments at the center of the segments using a side-firing 400 μm optical fiber (Laserscope, San Jose, CA) from a distance of 1 mm focus. The tip of the optic fiber contains a prism which refracts the laser beam at 90°. The trachea segment was irradiated and then the fiber was moved to next trachea ring (1 mm) parallel to the surface of the trachea segment and was irradiated (illustrated in Fig. 3) using a different power-irradiation time pair (80–200 W/cm², 2–3 seconds). Thus, irradiation along the tracheal specimen occurred in discrete steps. There was no overlap between successive laser spots. These dosimetry pairs were selected on the basis of preliminary studies, which narrowed the laser parameters and identified potential values of interest. Pilot studies in our laboratory examined a wide range of pulse duration and power pairs and examined their impact on shape change. Since there are a vast number of combinations, using trial and

errors approach we narrowed the possibilities down to the values used in this study based on observable shape change effects. A 10-second delay occurred between each irradiation to allow for thermal relaxation and thus minimize over-heating due to heat conduction.

In the longitudinal direction of trachea (proximal to distal), the side-firing fiber was advanced using a motorized mechanical stage (Physik Instrumente GmbH, Karlsruhe, Germany), while preserving a distance of 1 mm between the tip of the laser fiber and specimen surface (illustrated in Fig. 3). The laser spot size was measured using thermal paper (Kentak, Pittsfield, NH) to be 1.2 ± 0.2 mm. The surface area of cartilage specimens used in each laser irradiation experiments was approximately $3 \text{ mm} \times 13 \pm 2 \text{ mm}$, and the laser was programmed to continuously irradiate 3–5 spots in non-overlapping vertical sequences (illustrated in Fig. 3). In control experiments cartilage specimens were mechanically deformed and then submerged in an ambient temperature saline bath for a duration of 60 seconds. Four to six specimens were used for each dosimetry parameter pair.

Temperature Measurement

The temperature of each specimen's surface in the laser-irradiated region was monitored using a single element HgCd infrared thermal imaging system incorporating a $3\times$ telescopic lens (Inframetrics 600, emissivity set to 1.0, FLIR systems, Inc., North Billerica, MA) as previously described [36]. The focal distance was about 13–15 cm. The plane of the specimen surface was perpendicular to the angle of incidence for the laser beam within five degrees. The tip of laser fiber was placed at 1 mm from the tissue surface in the straight line between the plane of the specimen surface and the axis of the thermal camera (as previously described). The thermal images were calibrated using black body (BB701, OMEGA Engineering Ltd., Stamford, CT) radiation and processed with custom designed software programmed in MatLab ver 6 [36] to obtain time-dependent images of surface temperature.

Estimation of the Degree of Bending

The specimens were photographed with a digital camera immediately before and 10 minutes after thermal treatment. Change in curvature was determined from analysis of these images. The distance between the ends of each specimen was measured before reshaping and at 10 minutes after irradiation and submersion to estimate the degree of bending in order to visually compare the two thermoforming methods. The distance between the unfurled ends of each specimen was measured before each experiment and at 10 minutes after treatment to estimate the degree of shape change. The degree of change corresponding to each set of measurements was used for analysis. In order to compare the shape change between the various specimens, the degree of *bending or bending value* was estimated using this equation (illustrated in Fig. 4), where:

$$K = 1 - \frac{l^{Initial} - l^{Final}}{l^{Initial}} = 1 - \frac{h_1}{l^{Initial}} \quad (1)$$

h_1 is the length between the line, $l^{initial}$ which connect two ends of the tracheal ring and the tangent line, l^{final} which is parallel to the line, $l^{initial}$. $l^{initial}$ and l^{final} are defined from the tracheal rings before and after irradiation (or submersion), respectively. The shape of the tracheal rings which were not treated by thermal and mechanical loading (“concave”) is

defined as a positive bending value, otherwise, converse shape of the tracheal cartilage (“convex”) is defined as a negative bending value.

Biomechanical Analysis

Trachea segments heated in the saline bath were evaluated using a mechanical analysis platform (Electro-force 3200, Bose Corp., Eden Prairie, MN) (Fig. 5). Measurements of instantaneous Elastic modulus of the specimens subjected in tension were performed. After heat treatment, the specimens were maintained at 23°C for 5 minutes and allowed to equilibrate. A rectangular section approximately 12–15 mm long and 2 mm wide was dissected from the specimen. The average thickness of these sections was approximately 1.0 mm. The section was mounted vertically between the two grips in the apparatus. The grip surfaces were lined with sand paper to prevent slipping. To straighten the specimen in the holder a small initial tensile displacement resulting in a weak stress of ~0.01 MPa was applied to the specimen. The initial length (the distance between the two clips), width and thickness of the central gauge section were measured using a digital micrometer (accuracy $\pm 10 \mu\text{m}$) and the initial cross-sectional area was calculated.

Each section was stretched at a displacement rate of 10% strain per second up to a maximum 10% strain and held at this strain for 25 seconds. Cartilage stiffness is strain-magnitude and strain-rate dependent. In this study, 10% strain per second was chosen to obtain the instantaneous response of cartilage, and this rate was the maximum we could use without risking cartilage slipping from the clamps in our mechanical testing device. It was necessary to observe the behavior at the end of the loading cycle in order to confirm that the specimen remained adequately mounted and secured to the mechanical testing platform grips. Stress was defined as force of reaction to the tensile deformation divided by the initial cross sectional area of the specimen. Instantaneous Elastic modulus was determined by the linear least-square fit from the slope of the stress-strain curves obtained during stretching of the specimen. Four to five specimens were evaluated for each temperature-time setting.

Cell Viability Analysis

A cell viability assay system combined with fluorescent confocal microscopy was used to determine the amount of thermal damage generated in the laser irradiated specimens. An approximately 500 μm thick cross-section slice was cut lengthwise through the center of a laser target site from each tissue sample within 1 hour after laser irradiation. The tissue slice was then incubated in the cell viability assay solution and distribution of live and dead cells was visualized with a laser confocal microscope (Axiovert 135M; Zeiss, Göttingen, Germany). The two-color fluorescent live/dead dye solution was prepared using green fluorescent 0.6 μl Calcein AM dye and 13 μl red fluorescent ethidium homodimer-1 (Molecular Probes, Inc., Eugene, OR) in 1 ml of Hank balanced salt solution [37]. Images of strained specimens were analyzed using Photo-shop (ver 9.0, Adobe Systems, Inc., San Jose, CA) and the size of the thermal damage region (“dead region”) was determined by inspection of the image [36]. The extent of the thermal damage zone was defined as the location where the population of dead chondrocytes was above 85%.

The width of the damage was defined as a diameter of the dead region on the irradiated surface of the trachea and the depth was defined as the maximum axial distance between the irradiated surface and the thermal damage boundary. Three to five specimens were used for each dosimetry parameter pair.

Statistical Analysis

Statistical analysis was performed on bending value, instantaneous Elastic modulus, and thermal damage width and depth using a one-way general linear model analysis of variance (ANOVA) for means of pretreatment and post-treatment differences within thermal treatment groups, laser heating and saline bath submersion. When significant effects of thermal treatment were detected by ANOVA, a paired t test was performed to determine which treatments exhibited statistically significant results ($P < 0.05$).

Measurements of the bending dimensions, Elastic modulus, and thermal damage on each specimen were repeated at least three times to establish reproducibility and the results are expressed as mean+SEM.

RESULTS

Correlation Between Thermal Treatment and Shape Change

Saline Bath Immersion—Figure 6a–e demonstrates the bending value as a function of immersion time and saline bath temperature. The bending value of control samples in room temperature saline (23°C) was 1.0 ± 0.05 (Fig. 6a). There were no significant differences in the bending value of tracheal segment in the 23°C bath temperature between any time point and the control baseline data ($P > 0.05$), whereas the change in the bending value in the 45°C, 55°C, 55°C, 65°C, and 75°C bath temperatures significantly decreased compared to that in the 23°C bath temperature between any immersion time and the control baseline data ($P < 0.05$).

The bending value gradually decreased with increase in immersion time for bath temperature between 45°C (Fig. 6b,c) and 65°C (Fig. 6d). Samples heated in the 75°C bath (Fig. 6e) demonstrate that the bending value reaches a maximum level of 0.1 ± 0.4 after 15 seconds of immersion and remains fairly constant for immersion times > 15 seconds. At 55°C, the shape change decreases with time, however, a change in the sign of the bending value (indicating a converse curvature) was not observed. At 65°C, a maximum bending value of -0.2 ± 0.3 was observed after 100 seconds of heating with a change in the sign of the bending value, which was permanent (Fig. 6d). Based on the quantitative assessment of the shape change versus saline bath temperature, the threshold condition for the change in sign of the bending value occurs within 65 and 75°C.

Laser Irradiation—Figure 7a,b shows the bending value as a function of radiant exposure and peak temperature respectively. The data on the two figures are paired: each data point in Figure 7a corresponds to a data point in the same sequential order taken on Figure 7b. There were no significant differences in the bending value of tracheal segment in the 220 J/cm² compared to that in the control tracheal segment ($P > 0.05$), whereas the change in the bending value between 220 and 395 J/cm² significantly decreased compared to that in the

control tracheal segment ($P < 0.05$). The change in the segment curvature below this radiant exposure level is consistent with a surface temperature lower than $55 \pm 3^\circ\text{C}$ (Fig. 7b). The bending value decreases as fluence with an increase from 220 to 395 J/cm^2 . In this radiant exposure range, surface temperature rises from 60 to 85°C .

Above 370 J/cm^2 , pyrolysis, ablation, and vaporization on the surface of the cartilage were observed. The surface temperatures of the tracheal cartilage in the laser irradiated regions estimated using the IR camera showed a temperature of $80 \pm 3^\circ\text{C}$ corresponding to 370 J/cm^2 of laser density. Based on this quantitative assessment of the shape change versus laser dosimetry, the threshold condition for converse shape change of a tracheal ring likely occurs between 320 and 370 J/cm^2 corresponding to $65\text{--}80^\circ\text{C}$.

Biomechanical Measurements—The instantaneous Elastic modulus of rabbit trachea in tension measured in control experiments was $7.5 \pm 5 \text{ MPa}$. Figure 8 shows the stress-strain curve for rabbit trachea segment at 22°C and Figure 9a–c shows relative change of Elastic modulus with immersion time for bath temperatures of 55, 60, and 65°C . The changes in Elastic modulus after saline bath immersion were calculated as a difference between average modulus of the saline bath heated specimens and control value for each immersion time divided by control value:

$$P = \frac{E^{\text{Final}} - E^{\text{Initial}}}{E^{\text{Initial}}} = \frac{\Delta E}{E^{\text{Initial}}} \quad (2)$$

where E^{Initial} is the control value and E^{Final} the average Elastic modulus after the heating. The average Elastic modulus after heating was defined from two adjacent samples taken from one trachea, which consists of 4–6 trachea rings. There was no significant difference in the stiffness below 55°C bath temperature between any immersion time and the control baseline data ($P > 0.05$), whereas there was significant difference in the stiffness in 55°C bath temperature over 60 seconds, in 60°C bath temperature over 30 seconds, in 65°C bath temperature over 15 seconds between any immersion time and the control baseline data ($P < 0.05$).

In Figure 9, it was observed that the elastic moduli of tracheal cartilage decrease after a 55°C saline bath immersion temperature and significant changes in elastic moduli for each immersion temperature were observed at 55°C for 60 seconds, 60°C for 30 seconds, and 65°C for 15 seconds. Likewise, it was observed that longer immersion times for each temperature do not change the elastic moduli and the bending values.

Chondrocyte Viability and Thermal Damage Analysis—Figure 10 shows the extent of thermal damage to tracheal cartilage identifying live (green fluorescence signal, lighter sections within the gray scale images) and dead (red fluorescence signal, darker regions in gray scale images) cells in a cross section slice of a laser irradiated sample. The depth and width of the zone of chondrocyte death in the laser irradiated tissue as a function of radiant exposure are shown on Figure 11. The depths and widths of chondrocyte death over 220 J/cm^2 treatment group were significant difference in the depth and width of chondrocyte death below 220 J/cm^2 treatment groups ($P < 0.05$).

Figure 11 demonstrates that the width of the thermal damage zone significantly increases with irradiance until the irradiance reaches 220 J/cm^2 . The thermal damage depth was $0.31 \pm 0.16 \text{ mm}$ at 240 J/cm^2 which was significantly greater than the $0.17 \pm 0.08 \text{ mm}$ measured at 150 J/cm^2 . The depth of the damage zone increased with irradiance until the damage through the entire thickness of the specimen (about $500 \mu\text{m}$) was observed at radiant exposure above 236 J/cm^2 . The thermal damage widths were $1.3 \pm 0.3 \text{ mm}$ at 240 J/cm^2 which was significantly greater than $0.72 \pm 0.2 \text{ mm}$ at 200 J/cm^2 . Superficial tissue ablation was observed on the irradiated tracheal specimen surface above 270 J/cm^2 .

DISCUSSION

Laser cartilage reshaping in the head, neck, and airway neck offers a potentially less-invasive alternative to conventional reconstructive and cosmetic surgical procedures. Targeted cartilage tissue can be irradiated through the skin or mucosa without incisions and suturing. Using a mid-infrared laser which penetrates approximately 1 mm into tissue, energy can be delivered in a spatially selective pattern to produce effective shape change at the expense of only limited thermal injury. In vivo, the Er:Glass laser ($1.54 \mu\text{m}$) was demonstrated to reshape auricular cartilage in live rabbits [23]. Wang et al. [26] applied Nd:YAG laser ($1.44 \mu\text{m}$) to reshape tracheal cartilage rings in canines.

Correlation Between Thermal Treatment and Shape Change

A lumped parameter analysis gives a rough estimate that at least 10 seconds is required for a 1.0 mm thin cartilage specimen immersed in a saline bath at 75°C to reach thermal equilibrium. This time is significantly shorter than immersion time of 20–30 seconds required to obtain a visible shape change in a water bath (Fig. 6). Therefore, we assume that the immersion heating method permits relatively rapid thermal equilibration of a thin trachea specimen, and hence the temperature profile inside the specimen can be approximated as a uniform distribution. When calculating the bending value, the reshaped cartilage specimens from saline bath immersion were represented as an ideal arc segment, which is a fairly practical way to gauge shape change using linear dimensions to represent curved shapes. However, in laser irradiation studies, the curvature of the reshaped cartilage specimen did not as accurately mimic the shape of an arc segment because the laser-induced shape was concentrated only in the targeted irradiation zones.

This study shows how shape change in tracheal cartilage correlates with both temperature and heating time. The heat deposited into a deformed cartilage sample induces alteration in collagen matrix components, which results in a partial or complete fixation of the induced deformation. Several hypothetical mechanisms were suggested for this process of stress relaxation [22,25]. In saline bath studies, the change in tracheal shape from concave up to convex occurred at 65°C after 100 seconds and 75°C after 15 seconds. This threshold lies between 65 and 75°C . The bending value, $K = 0.2 \pm 0.3$ was similar to that produced using laser irradiation at a fluence of 370 J/cm^2 which corresponds to a peak surface temperature of 80°C . However, at 370 J/cm^2 , pyrolysis, ablation, and vaporization on the surface of the cartilage were also observed. The observed shape identified using saline bath studies may be thought of as the initial stage of the transition zone where a large change in the biophysical

properties of tracheal cartilage occurs. This set of values (75°C and immersion time of 15 seconds or and 370 J/cm² using a laser) define a threshold where significant change in segment curvature occurs and there is a change in the sign of the bending value.

These findings are in agreement with previous detailed studies that identified that changes in internal stress during the laser irradiation of porcine and rabbit nasal septal cartilage occurs when the surface temperature reaches 60–70°C [38–40]. Recently Szántó et al. [41,42] demonstrated that a marked endothermic peak was observed at 61.8°C in DSC scans (Differential Scanning Calorimetry) of tracheal cartilage and the transition was identified as an irreversible denaturation; accordingly the rate of heating in a calorimeter is profoundly slower than either of the two methods used herein. The immersion temperature of 75°C and the surface temperature of 80°C corresponding to a fluence of 370 J/cm² may be a consequence of performing studies using multi-layered tracheal segments (pericondrium, mucosa, and smooth muscle) as well as by the non-uniform heating in depth produced by a laser. Again, we defined the transition conditions as when there is a change in the sign of the bending value (a converse shape change).

In the saline bath studies, we assumed that the immersion technique produces instantaneous step-wise temperature changes that occur uniformly throughout the entire specimen, however, non-uniform heating due to heat conduction at a finite rate through the thickness of these specimens occurs, though this is a relevant effect for short immersion times.

Correlation Between Mechanical Property and Temperature

Tracheal cartilage is anisotropic and inhomogeneous, which makes selection of specimen orientation for mechanical analysis critically important. To straighten the specimen in the holder a small initial tensile displacement resulting in a weak stress of ~0.01 MPa was applied to the specimen. When tracheal segment is loaded in tension, the inner layer of the tracheal cartilage is compressed and the outer layer is stretched around a neutral axis where neither compression nor stretch occurs and the specimen was initially non-zero-stress configuration as the reference state. In this study, we measured elastic modulus with preconditioned specimens. In our analysis, we measured the temperature-dependent intrinsic static mechanical properties in the elastic region of a material's stress-strain curve, rather than those at equilibrium. When step changes in displacement were applied, there was a time-dependent elongation of the specimens, and equilibrium was reached after 5–10 minutes. Equilibrium measurements may thus be subject to artifacts due to specimen dehydration, which occurs rapidly under ambient conditions. Rains et al. [14] has described that there is a significant inverse relationship between tensile stiffness and water content in human tracheal cartilage. Hence with tracheal segments, equilibrium tensile tests maybe unable to quantify adequately the mechanical contribution of the aggregating proteoglycans to cartilage behavior, thus biasing the results toward the measurement of the mechanical properties of collagen which are less heavily dependent upon hydration state.

When loaded in tension as in this study, the realignment of the collagen fibers imposes a compressive stress on the entrapped proteoglycans. During mounting of isolated curved tracheal cartilage rings in the mechanical testing apparatus, the abluminal surface is compressed while the luminal layer is stretched around a neutral axis where neither

compression nor stretch occurs. The stretching of a tracheal segment contributes to the change in residual stress and produces a nonzero initial internal stress in the specimen.

The mechanical properties of cartilage depend on temperature [43] and there are several hypotheses about the behavior of cartilage which is dependent of temperature [22,44]. The results of this study show a progressive decrease in the tensile stiffness of tracheal cartilage with temperature and duration of heating. A significant change in tensile stiffness occurs after 60 seconds of immersion at 55°C and 30 seconds at 60°C, 15 seconds at 65°C (Fig. 9a).

The temperature-time pair of 60 seconds at 55°C can be designated as the threshold of the transition zone where large changes in cartilage biophysical properties and permanent reshaping (such as the change in sign of the bending value) are observed. This threshold in tensile stiffness is in agreement with the threshold in the bending value, Figure 6c and with previous observations of changes in shape, internal stress and elastic modulus in porcine and rabbit septal cartilage at the temperatures of 60–70°C [40].

Correlation Between Tissue Viability and Shape Change

Cartilage is an avascular and undergoes a wound healing and tissue repair process via mechanisms different from soft tissues. Cartilage thermal injury can result in depopulation of chondrocytes and subsequent matrix changes, including depletion of proteoglycans, scar/fibrosis, calcification, ossification, and under some conditions even repopulation by chondrocytes, provided a stem cell source such as perichondrium is close to the injured region. In in vivo experiments, the thermal injury observed following several days or weeks after laser irradiation may be deeper and wider than thermal injury observed immediately after laser exposure due to additional chondrocyte death resulted from a continued necrosis or induction of apoptosis.

In this study, no viable populations of chondrocytes were identified in the region of laser energy deposition for any dosimetry settings that produce at least some measurable shape change. This suggests that shape change and thermal damage are concomitant and significant shape change and preservation of cell viability are mutually exclusive. Similar findings were reported by Karam et al. [24] in rabbit nasal septal cartilage. Hence, the effective shape change of tracheal cartilage is likely to be achieved using spatially selective heating, in which damaged tissue regions are small and superficial and surrounded by healthy tissue or stem cell-rich perichondrium.

The thickness of rabbit tracheal cartilage has been estimated to vary from 0.3 to 0.6 mm and the tracheal mucosal thickness on the luminal surface was $150 \pm 2.5 \mu\text{m}$. Avoiding full thickness tracheal wall injury using a laser operating at 1,450 nm is less likely due to two factors, namely: (1) the optical penetration depth of light at 1,450 nm in cartilage is less than these dimensions, and (2) irradiation was performed through the thick endotracheal mucosa. Short radiation time confines thermal injury and minimizes the impact of heat conduction thus resulting in the heating of only the superficial layers. Physical changes occur only in the most superficial regions of the tracheal cartilage. The deeper regions of the tracheal cartilage and the abluminal tracheal lining may be spared thermal injury using suitable dosimetry. The use of relatively small laser spot sizes ensures that reasonable wound healing would

occur due to the surrounding viable tissue. The change in the width and the depth of thermal injury, from 220 to 270 J/cm² corresponds to peak surface temperatures of 55–70°C. The significant change in the bending value in laser irradiance studies (Fig. 7) was observed when the full thickness injury to the specimen occurs.

Proteoglycan or collagen synthesis within the specimen is potentially a better indicator of whether tissue is metabolically normal [45]. Further research that would include determination of proteoglycan production by chondrocytes would be especially helpful, as it would provide insight into chondrocyte metabolism after thermal injury. Correlation of proteoglycan or collagen synthesis with each vital cell staining technique with CLM would provide more conclusive evidence with regard to which vital staining method best reflects the metabolic state of chondrocytes.

CONCLUSIONS

Laser thermoforming remains a promising technology with many potential clinical applications in head and neck surgery. This study was the first attempt to examine ex vivo heat-induced shape change and correlate this change with cell viability in rabbit tracheal cartilage. We identified the transition zones in rabbit tracheal cartilage for thermo-forming using both saline immersion and laser irradiation and found these corresponding values to be 62–68°C and 350 J/cm², respectively. Thermoforming using heated saline baths provides useful information on the temperature ranges where shape change occurs, though is not useful in clinical settings. With laser irradiation, the threshold dosimetry value of 350 J/cm² corresponds to a surface temperature of approximately 65°C; however there was a loss of viable chondrocytes within the laser-irradiation zone, which increased with dosimetry. Mechanically, above these transition zones, the elastic moduli were higher than at lower temperatures.

Our study underscores the 1,450 nm laser may be suitable for use in clinical LCR, and that the putative mechanism for shape change likely relies upon maintaining spatially selective heating and modification of the tracheal wall. While not perfect and accurate, the live/dead viability assay combined with fluorescent confocal microscopy used in this study do provide rough guidance on the degree of thermal injury; and further it is the most heavily studied and implemented damage model in thermal medicine. A better understanding of cell and tissue function may result from combining the use of viability stains with determination of other cell function such as proteoglycan, collagen, or cytokine production when evaluating chondrocyte. Future studies will focus on replicating the current work in tissue models more closely approximating the dimensions of the human trachea.

Acknowledgments

The authors thank Dr. Krasieva and Dr. Zhu for their insights and assistance with the laser scanning confocal microscopy expertise. The authors also thank Dr. Mukai for allowing access to fresh tracheal cartilage specimens. This study was supported by Candela Corporation, 2007 US Air Force Travel Grant, LAMMP (P41RR001192), NIH (D00170, DC005572, RR-01192) and Air Force Office of Scientific Research (FA 9550-04-1-0101).

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Fig. 1.
a: Rabbit trachea, **(b)** tracheal segment, and **(c)** dimension of the tracheal segment: ①, tracheal cartilage; ②, intermediate pars membranacea.

Fig. 2.

Schematic diagram for the deformed specimens (a) and saline bath immersion experiment (b, control specimens; c, test specimens).

Fig. 3. Schematic diagram for laser experiment (a), the position of the side-firing fiber tip (b), and the laser spot on the specimens (c).

Fig. 4. Schematic diagram for defining the bending value of the trachea ring (a) diagram, (b) equation.

Fig. 5. Mechanical analysis of trachea cartilage in tension. a: Schematic of mechanical test device (ELF 3200), (b) Displacement curve in tensile test.

Fig. 6. Bending values of trachea segment with various immersion temperatures and times. a: at 23°C, (b) at 45°C, (c) at 55°C, (d) at 65°C, and (e) at 75°C.

Fig. 7. Bending values (K) of trachea segment for various radiant exposures (a) and peak temperature (b).

Fig. 8.
Characteristic static stress-strain curve for rabbit tracheal cartilage in tension at 22°C.

Fig. 9.

Change in Young's modulus (P) for various immersion temperatures and times (a) 55°C, (b) 60°C, (c) 65°C.

Fig. 10.
Confocal LSM visualizations of damaged trachea by diode laser irradiation for various radiant exposures.

Fig. 11.

Damage in trachea tissue for various radiant exposures. **a:** Damaged diameter versus radiant exposure and **(b)** Depth of the damaged in tracheal cartilage versus radiant exposure.