

Ultrastructure of Collagen Thermally Denatured by Microsecond Domain Pulsed Carbon Dioxide Laser

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Background: Clinical improvement in photodamaged skin after carbon dioxide (CO₂) laser resurfacing is thought to result in part from thermal collagen shrinkage. The presence of such collagen has not been unequivocally demonstrated. To identify and characterize the morphological features of collagen after CO₂ laser exposure, we irradiated ex vivo human facial skin and bovine calcaneus tendon with microsecond domain pulsed CO₂ laser energy and examined specimens for histopathological and ultrastructural changes in collagen.

Observations: In dermis and tendon, 3 zones of collagen structure were apparent on electron microscopy. The first, most superficial zone demonstrated loss of collagen structure. The second zone consisted of admixed normal collagen fibers and thickened colla-

gen fibers. Zone 3 consisted of normal-appearing collagen fibers.

Conclusions: Ultrastructural examination of irradiated collagen revealed distinct morphological zones of denatured collagen fibers. Partially denatured fibers had an increased diameter consistent with lineal shrinkage. Zonal distinction was undetectable by light microscopy. Ultrastructurally, the zones of denatured collagen located above the normal fibers correlated with the zone of altered material seen on light microscopy. These findings suggest that collagen fiber shrinkage does occur after pulsed CO₂ laser irradiation and that this phenomenon contributed, at least in part, to the immediate tissue contraction observed clinically.

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SHORT (submillisecond) pulsed carbon dioxide (CO₂) lasers have assumed a major role in skin resurfacing. Carbon dioxide laser resurfacing accomplishes clinical and histopathological improvement in skin by removal (ablation and debridement) of photodamaged epidermal and dermal tissue, regeneration of the epidermis and dermis, and tissue contraction.¹ Clinicians and investigators have observed tissue contraction immediately after laser pulses. After CO₂ laser irradiation of ex vivo human skin, Gardner et al² found a positive linear correlation between laser pass number and unidimensional skin shrinkage. Similarly, Ross et al³ observed a positive correlation between contraction of the treatment area and both increasing fluence and pass number on irradiation of in vivo pigskin. They attributed tissue contraction to tissue desiccation and collagen shrinkage, both recognized results of tissue heating.^{2,3} Light microscopic (LM) examination after CO₂ laser resurfacing demonstrates a zone of residual thermal collagen damage characterized by colla-

gen tinctorial change (increased basophilia) and by a loss of birefringence on polarized LM. The area just subtending this zone of altered extracellular matrix has been assumed to be the locus of shrunken collagen.⁴

Collagen denaturation and shrinkage is a phenomenon that has been reported in various areas of research.⁵⁻⁹ The role of heat in collagen denaturation and shrinkage has been variably quantified. The threshold temperature at which native collagen fibers shrink varies among species, with increased temperature correlating with increased imino acid (proline and hydroxyproline) content and a more thermally stable helix.¹⁰ Suprathreshold heating then disrupts the semicrystalline fibril structure rapidly over a narrow temperature range. Numerous weak, interdependent intermolecular bonds confer structural stability and account for the rapid structural dissolution observed with small temperature increases. Beyond this narrow thermal range, progressive denaturation occurs until there is complete fiber dissolution.¹¹ Partial thermal disruption of collagen's tightly maintained triple- α he-

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MATERIALS AND METHODS

Fresh (<3 hours old) ex vivo facial human skin removed as redundant tissue during reconstruction provided a clinically relevant in vitro model for laser resurfacing and its effects on dermal type I collagen; fresh (<12 hours old) bovine calcaneus tendon served as a concentrated array of essentially pure type I collagen for this study.¹⁸ Fresh specimens were wrapped in saline-moistened gauze and stored at 4°C before irradiation. Room temperature specimens were individually exposed to 1 (tendon and skin) or 2 (skin only) passes each with 3 different CO₂ laser systems (UltraPulse 5000C, Coherent Medical, Palo Alto, Calif; TruPulse, Palomar Medical Technologies, Beverly, Mass; and SilkTouch, Sharplan, Allentown, NJ). The laser parameters that we used in this study are shown in the **Table**. Skin specimens were irradiated perpendicular to the skin surface and cylindrical tendon specimens, once sectioned longitudinally to reveal a flat internal surface, were irradiated perpendicular to the open face so created. Two specimens each were irradiated with 1 or 2 passes using 0% (TruPulse and SilkTouch) or 10% (UltraPulse) pulse overlap and with interpass debridement using a saline-dampened cotton-tipped applicator. Each area was approximately 1.0 cm². Specimens were fixed for LM in 10% buffered formalin, processed in paraffin, and stained with hematoxylin-eosin. Specimens were fixed for transmission electron microscopy (EM) in glutaraldehyde and prepared as previously described.¹⁹ Briefly, ultrathin (1-μm) sections were cut with a microtome (MT-7, RMC, Tucson, Ariz), placed on copper grids, and stained with uranyl acetate-lead citrate. The tissue was then examined with a transmission EM (EMU-4, RCA, Camden, NJ).

lical tertiary structure induces a phase transition necessitating contraction along the fiber's length (*shrinkage*), with a correlate increase in fiber diameter, much like the thickening seen by releasing tension on a taut rubber band. Early chemical studies reported that sheepskin collagen shrank in the 63°C to 67°C range,¹¹ intact rat-tail tendon in the 54°C to 59°C range,¹² human scleral collagen in the 61°C to 63°C range, and human corneal collagen in the 55°C to 59°C range.¹³

Tissue welding depends on heat-induced collagen changes. In a laser-welded rat carotid artery, loss of collagen periodicity and increased fibril caliber where fibrils interdigitate at the weld were cited as the structural basis for the laser-induced welding effect.¹⁴ Also, welded rat-tail tendon demonstrates gross tissue contraction.¹⁵ Tang et al¹⁶ examined collagen structure transitions created by diode laser (830-nm) welds of rat aorta using multiple 8-second laser pulses. Within the welds, collagen fibers demonstrated a variety of morphological changes. Adjacent collagen fibers might show normal ultrastructural features (diameter, birefringence, and periodicity), intermediate changes (increased diameter with normal periodicity but reduced birefringence), or complete denaturation (loss of recognizable fibrillar structure, periodicity, and birefrin-

Carbon Dioxide Laser Parameters

Laser System*	Fluence, J/cm ²	Pulse Duration, μs†	Pulse Energy, mJ‡	Power, W‡
UltraPulse 5000C	5.7	900	400	...
SilkTouch	5.4	900	...	18
TruPulse	5.5	100	400	...

*The UltraPulse 5000C is manufactured by Coherent Medical, Palo Alto, Calif; the SilkTouch, by Sharplan, Allentown, NJ; and the TruPulse, by Palomar Medical Technologies, Beverly, Mass.

†Pulse duration also refers to the dwell time of the scanned continuous wave beam used in the SilkTouch system.

‡Ellipses indicate not applicable.

gence). Moreover, all these changes could occur within single fibers, and when periodicity was observed, fibers always retained a 55-nm regular banding pattern. Tang and colleagues attributed the presence of adjacent normal and denatured fibers to differential thermal stability of different collagen types within the vessel wall and to protection by overlying elastic tissue.

Similar thermal alterations have been observed in cutaneous collagen, but with a more distinctly zonal pattern. Zweig et al¹⁷ examined the lateral thermal damage along erbium and CO₂ laser incisions in murine skin. Both lasers delivered approximately 25 J/cm² within 250-microsecond pulses using an effective beam diameter of 0.36 mm, overlapped by 50%. Two zones of thermally altered collagen were described. Within the "coagulation zone," collagen fibers were swollen and devoid of optical birefringence and periodicity. Within the "transition zone," both denatured and normal collagen fibers were intermixed. Birefringence of fibers within the zone was reduced and their periodicity "partly recognizable." These changes were correlated with those found in murine skin immersed for 160 seconds in a controlled temperature bath. Under these conditions, collagen fiber denaturation began at 57°C; the number and diameter of swollen fibers increased at 65°C (periodicity partly maintained); and fiber coagulation was nearly complete at 73°C. The width of the transition zone caused by the CO₂ laser was approximately double that produced by the erbium laser (375 ± 52 μm vs 198 ± 70 μm, respectively).

Common speculation asserts that the zone of shrunken collagen seen immediately after resurfacing human skin acts as a tightened scaffold over which new collagen is laid down in the delayed phase of wound healing. Some believe that this taut scaffold contributes to the final improvement seen months after laser resurfacing. In human laser skin resurfacing, collagen fiber shrinkage has been assumed, but not unequivocally demonstrated. In this study we sought to identify, verify, and characterize by ultrastructural analysis a zone of collagen fiber shrinkage after microsecond domain pulsed CO₂ laser exposure.

RESULTS

No gross tissue shrinkage was observed after first passes of CO₂ laser irradiation on skin and tendon specimens. With second passes of laser energy, immediate tissue



Figure 1. Light micrograph of a cross section of bovine calcaneus tendon after a single 100-microsecond pulse (5.5 J/cm^2) of 10 600-nm laser irradiation demonstrating a "festooning" or "scalloped" zone of residual thermal collagen damage (hematoxylin-eosin, original magnification $\times 200$).

shrinkage was evident. Light microscopy after laser resurfacing revealed superficial tissue ablation in the skin and tendon specimens. A layer of denatured collagen, characterized by tinctorial change and loss of birefringence on polarized LM, subtended each laser impact site. In tendon specimens cut in cross section, this layer assumed a scalloped or festooned configuration (**Figure 1**). In concert with this configuration, undulating variations in the thickness of altered collagen were observed in tendon specimens cut longitudinally, but presumably slightly tangentially to this cross-sectional pattern. The thickness of the layer of altered collagen measured 16 to 120 μm in the skin and 8 to 80 μm in the tendon specimens. While residual thermal collagen damage zone thickness correlated positively with pass number and pulse duration, the small number of specimens prohibited meaningful statistical analysis of this trend. Electron microscopic analysis in both skin and tendon revealed 3 distinct zones of collagen fiber morphological change; these zones were most clearly evident in the densely packed tendinous collagen (**Figure 2** through **Figure 4**). In the most superficial tissue, just subtending the laser impact, zone 1 consisted of homogenous, completely denatured collagen, without discrete fibrillar components. Just below this, zone 2 demonstrated a significant population of less structurally defined collagen fibers of increased diameter interspersed among normal collagen fibers. Where seen in longitudinal section, these zone 1 and 2 fibers of increased diameter also manifested a loss of normal collagen fiber banding periodicity (**Figure 4**). Finally, ultrastructurally normal collagen fibers were found in zone 3.

The relative thicknesses of zones 1 and 2 were measured on ultrastructural samples of tendon alone. Zone 1 thickness measured approximately 60 and 50 μm , and zone 2 approximately 9 and 15 μm , for single- or double-laser passes, respectively (all lasers). It must be noted that the indistinct zonal margins created in the transition between zones 1 and 2 made measurement difficult. Also, these measurements represent a sampling of a very small area within each specimen. Thus, these measurements should be viewed as estimates.

Measurements of collagen fiber diameter were carried out on tendon specimens, since these morphologi-

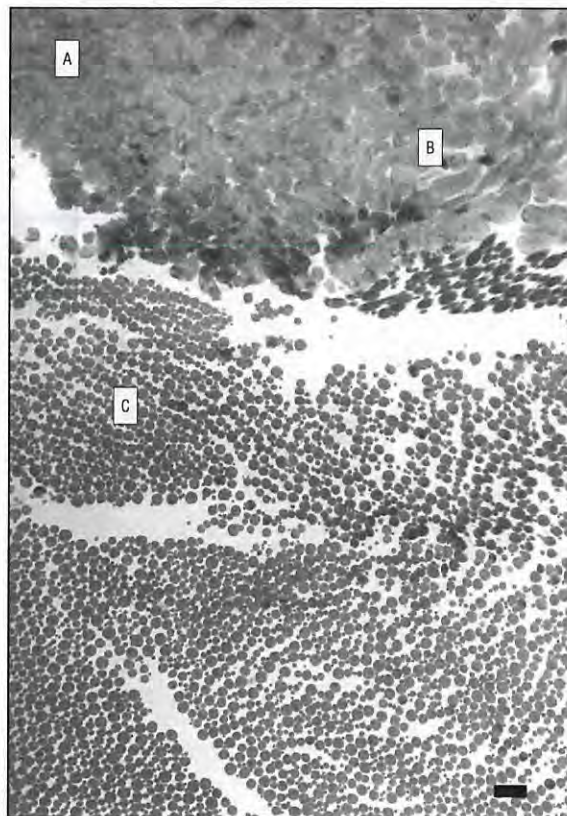


Figure 2. Transmission electron micrograph of bovine tendon collagen after carbon dioxide laser irradiation (TruPulse, Palomar Medical Technologies Beverly, Mass; 1 pass), demonstrating the zones of morphological change. Zone 1 (A) shows complete collagen fibril denaturation; zone 2 (B) shows discrete fibrils with increased diameter; and zone 3 (C) shows normal structure and diameter collagen fibrils (original magnification $\times 6500$; bar = 1 μm).

cal zones were largest and most distinct in these specimens. The total population of collagen fibers measured within zone 2 of the treated tendon had a mean $\pm$ SD fiber diameter of $0.24 \pm 0.12 \mu\text{m}$ ($n = 912$) compared with $0.14 \pm 0.07 \mu\text{m}$ ($n = 898$) of fibers from the untreated tendon. There was no significant difference in the mean fiber diameter in zone 2 between the 3 laser systems. Visual examination of the fiber structure in zone 2 of the treated tendon revealed 2 subpopulations. Structurally normal fibers, identical to those found in zone 3, had a fiber diameter of $0.15 \pm 0.06 \mu\text{m}$ ($n = 491$), and thickened fibers had a fiber diameter of $0.35 \pm 0.06 \mu\text{m}$ ($n = 421$) (**Figure 5**). Measurements of smaller numbers of fibers in skin specimens revealed similar-caliber fibers in similar subpopulations.

COMMENT

Previous studies¹ examining the LM sequelae of CO_2 laser resurfacing have recognized changes in the cutaneous extracellular matrix, composed predominantly of type I collagen.¹⁸ While our findings confirm similar changes present by LM, we determined that EM is required to discern the finer morphological alterations in collagen fibers immediately after resurfacing.

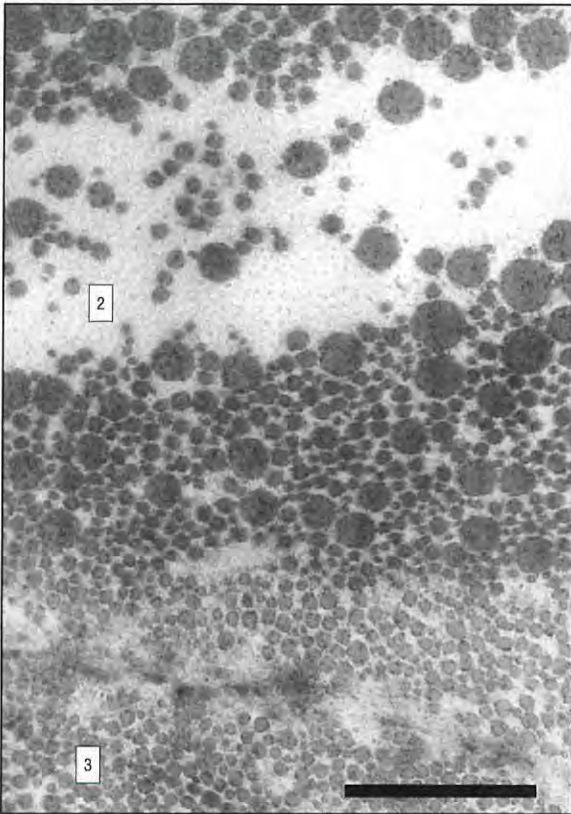


Figure 3. Transmission electron micrograph of bovine tendon collagen after carbon dioxide laser irradiation (UltraPulse 5000C, Coherent Medical, Palo Alto, Calif; 1 pass), looking more closely at zones 2 (2) and 3 (3) (original magnification $\times 40\,000$; bar = $1\,\mu\text{m}$).

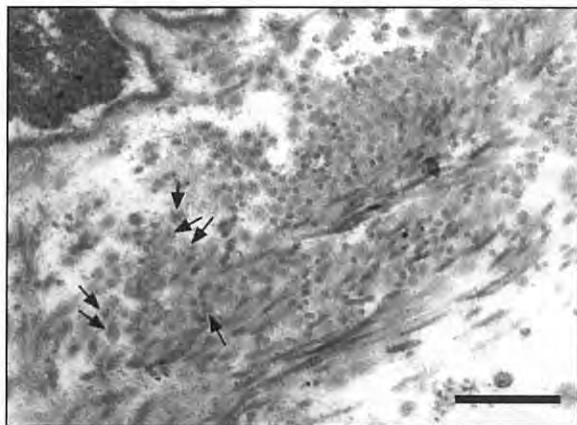


Figure 4. Transmission electron micrograph of ex vivo human skin after carbon dioxide laser irradiation (UltraPulse, 1 pass), demonstrating the zone 2 of collagen fibril features in the papillary dermis. Thermal denaturation and separation of basilar keratinocytes have occurred in what would have been zone 1 change. The arrows indicate enlarged zone 2 collagen fibrils. Loss of normal type I collagen banding periodicity is evident in denatured collagen fibers seen in longitudinal section (original magnification $\times 29\,000$; bar = $1\,\mu\text{m}$).

Ultrastructural examination of human dermal and bovine tendon collagen subjected to microsecond CO_2 laser pulses did reveal distinct zones of completely and partially denatured collagen fibers, similar to those de-

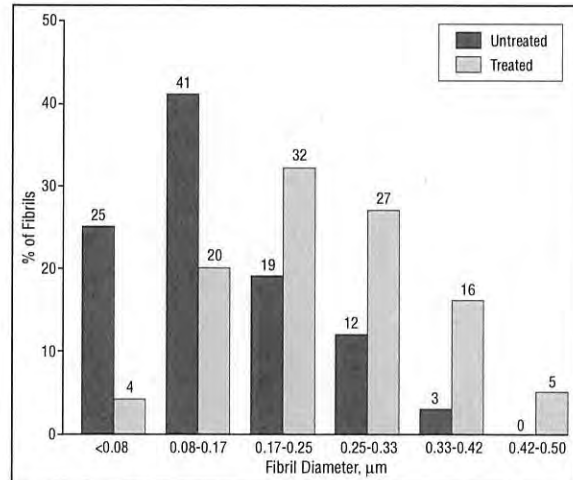


Figure 5. Collagen fibril diameter before and after treatment of bovine tendon with a high-energy carbon dioxide laser. The number at the top of each bar represents the number of fibrils counted.

scribed by Zweig et al.¹⁷ This gradation of morphological alterations in collagen fibers mirrors that seen in tissue welding^{14,15} and collagen fiber dissolution experiments.⁵⁻⁹ The increased cross-sectional diameter of partially denatured collagen fibers observed in zone 2 was consistent with lineal shrinkage of these fibers. Excessive heating, occurring closest to the laser energy impact, causes the complete fiber denaturation observed in zone 1. In zone 2, some fibers, or certain areas of fibers, absorbed sufficient heat energy to allow relaxation of hydrogen bonding and lineal shrinkage of the fibers (partial denaturation). These findings suggest that collagen fiber shrinkage did occur after pulsed CO_2 laser irradiation and that this phenomenon contributed, at least in part, to the immediate tissue contraction seen clinically. Since tendon contains a single collagen type, it is unlikely that the admixture of normal and denatured collagen fibers in zone 2 results from differences in imino acid content. It is perhaps more likely that there are differences in local heat diffusion through the tendon and relative cooling of 1 fiber by heat uptake in an adjacent fiber.

An intact tertiary structure is also necessary for diffraction of plane polarized light (birefringence). Loss of tertiary structural integrity of the triple helix is associated with loss of birefringence, owing to disruption of collagen fibril arrangement in larger collagen fibers, and with tinctorial change, owing to exposure of stain-binding sites within the unraveled collagen fiber.⁴ Because of the irregular nature of the transition boundary and the inability to simultaneously assess exactly the same tissue by LM and EM, it is difficult to judge whether zones 1 and 2, which are seen distinctly on EM, are both found within the zone of altered dermal matrix that is seen on LM. One can distinguish 10- to 15- μm objects on LM at high power, yet no authors have reported a readily distinguishable zone of intermediate damage. Since disrupted zone 2 fibers would stain differently and manifest altered birefringence, our inability to discern a layer of intermediate degree alteration argues that the zone 2

seen on EM lies within the residual thermal damage zone seen on LM.

Although 3 morphological zones of collagen have been characterized ultrastructurally after irradiation, whether the first homogenous zone or the second partially denatured zone or both contribute to the immediate gross tissue contraction seen clinically remains unproved in any dynamic model. Other processes likely contribute to immediate gross tissue contraction after laser resurfacing. Tissue water is the major chromophore for CO₂ laser irradiation. The differential role of tissue volume loss from tissue water vaporization in overall tissue contraction has not been determined. Ross et al³ observed wrinkles immediately after laser resurfacing that resolved over the ensuing 6-month period. They postulated that wound contraction and biological remodeling of the extracellular matrix may represent a third mechanism in creating overall clinical improvement. Smith et al²⁰ demonstrated the presence of smooth muscle actin-positive dermal stromal cells 14 days after CO₂ laser resurfacing. They postulated that these cells might provide for wound contraction later cemented in place by new collagen production. The role of shrunken collagen fibers in long-term clinical improvement after laser resurfacing has not been delineated with certainty. The

The role of shrunken collagen fibers in long-term clinical improvement after laser resurfacing has not been delineated

differential tensile strength of completely and partially denatured collagen fibers is unknown. Previous studies have shown that partially denatured collagen does maintain at least 30% of its tensile strength.⁵ Our findings show that zone 2 in skin consists of a mixture of partially denatured and normal collagen fibers. Together, these findings suggest that this layer may have enough tensile strength to account for the maintenance of tissue contraction that is seen clinically. The fate of these 2 layers over time has not been established. If the layers are sloughed within days, much as chemically denatured collagen sloughs after chemical peeling,²¹ it is difficult to imagine that they could be the source of persistent tissue contraction. Gross tissue contraction seen months after resurfacing may instead be attributable to extracellular matrix remodeling or myofibroblast activity.

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