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Cosmetic

Fat Liquefaction: Effect of Low-Level Laser Energy on Adipose Tissue

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Low-level laser energy has been increasingly used in the treatment of a broad range of conditions and has improved wound healing, reduced edema, and relieved pain of various etiologies. This study examined whether 635-nm low-level lasers had an effect on adipose tissue in vivo and the procedural implementation of lipoplasty/liposuction techniques. The experiment investigated the effect of 635-nm, 10-mW diode laser radiation with exclusive energy dispersing optics. Total energy values of 1.2 J/cm², 2.4 J/cm², and 3.6 J/cm² were applied on human adipose tissue taken from lipectomy samples of 12 healthy women. The tissue samples were irradiated for 0, 2, 4, and 6 minutes with and without tumescent solution and were studied using the protocols of transmission electron microscopy and scanning electron microscopy. Nonirradiated tissue samples were taken for reference. More than 180 images were recorded and professionally evaluated. All microscopic results showed that without laser exposure the normal adipose tissue appeared as a grape-shaped node. After 4 minutes of laser exposure, 80 percent of the fat was released from the adipose cells; at 6 minutes of laser exposure, 99 percent of the fat was released from the adipocyte. The released fat was collected in the interstitial space. Transmission electron microscopic images of the adipose tissue taken at ×60,000 showed a transitory pore and complete deflation of the adipocytes. The low-level laser energy affected the adipose cell by causing a transitory pore in the cell membrane to open, which permitted the fat content to go from inside to outside the cell. The cells in the interstitial space and the capillaries remained intact. Low-level laser–assisted lipoplasty has a significant impact on the procedural implementation of lipoplasty techniques. (*Plast. Reconstr. Surg.* 110: 912, 2002.)

The science of lipoplasty has advanced significantly since its 1921 inception, when Charles Dujarrier of France attempted to remove subcutaneous fat from a dancer's calves using a uterine curette.[1] Although Dujarrier's results were less than acceptable, he proved the viability of attempting to beautify the human body. Lipoplasty and its first rudimentary tools were improved on through the innovative thinking of professionals such as Babcock,[1] who initiated techniques to contour the breast and abdomen in 1939. Babcock was followed by Pitanguy in 1967,[2] Regnault and Daniel in 1975,[3] Illouz in 1980,[4] Jackson and Downie in 1978,[5] and Juri et al. in 1979,[6] all of whom contributed to the growing popularity of contour operations. The process was revolutionized in 1980, when Schrudde[7] introduced lipexeresis as a means of eliminating local adiposity. Fournier and Oteni[8] used uncut edge cannulas for contouring bodies through lipolysis. Fodor[9] described the superwet technique in 1986, and Klein's[10,11] development of the tumescent technique that allowed near bloodless liposuction using only local anesthesia increased the popularity of lipoplasty. In 1992 the internal ultrasound technique was developed by Zocchi,[12] and in 1998 the external ultrasound was developed by Silberg.[13] In 2000 Neira et al.[14] presented a new liposuction technique that demonstrated liquefaction of fat using a low-level laser device during a liposuction procedure. All of these techniques have improved the surgical procedure with varying degrees of contribution: some have reduced risk to the patient and others have expedited the process; yet all ultimately aim to decrease fat particles and thereby facilitate fat extraction. Each is a testimony of the develop-

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er's ability to use learned knowledge in a dynamic model to expand the science and application of lipoplasty.

Combining low-level laser energy with the established practice of lipoplasty to create a new technique[15] is a result of the same dynamic application of knowledge. Low-level lasers are often used in physical therapy and as antiinflammatory devices.[16] Low-level laser therapy is defined as treatment with a dose rate that causes no immediate detectable temperature rise of the treated tissue and no macroscopically visible changes in tissue structure.[16] Over the past decade, low-level lasers have been increasingly used in the treatment of a broad range of conditions and have improved wound healing, reduced edema, and relieved pain of various etiologies.[16,17] The dosage is a magnitude used to define the laser beam energy applied to the tissue. Normal units for the dosage are given in joules per squared centimeter, and the dosage is calculated as the laser power measured in milliwatts, multiplied by treatment time in seconds, and divided by area in squared centimeters of the laser spot directed toward the tissue.

Many studies have been conducted on the most efficient use and most effective application of laser energy,[18] the results of which depended on three factors: (1) coherent light verses noncoherent light, (2) wavelength, and (3) power.

Coherent Light

Based on quantam physics, Frohlich[19] predicted that the living matrix (i.e., the sets of protein dipoles) must produce coherent or laser-like oscillations if energy is supplied. The coherent radiation field of a laser and the biochemical energy form the surroundings that provide that energy. Frohlich deduced the existence of acousto-conformational transition, or coherent photons,[20,21] binding a Bose-Einstein condensate.[22] Such coherent vibrations recognize no boundaries at the surface of a molecule, cell, or organism: they are a collection of cooperative properties of the entire being. As such, they are likely to serve as signals that integrate processes such as growth, repair, defense, and the functioning of the organism as a whole. Research on electrically polarized molecular arrays of biological systems reveals that interactions repeated by the millions of molecules within a cell membrane give rise to huge coherent or Frohlich-like vibrations.[23–27] This singular response shows that the components of living matrices behave like coherent molecular antennas, radiating and receiving signals. In this way, coherent Frohlich excitations in cytoskeletal microtubules have been suggested to mediate information processing.[28–30]

Similar mechanisms could be evoked to explain the effects of low-level laser therapy. Nevertheless, the successful use of light-emitting diodes in low-level laser therapy[16,17,31] proves, apparently, that coherence is not an essential light property for the clinical effects of laser therapy. Coherence seems to be more important for light propagation and diffusion, producing speckle patterns from inhomogeneous tissues, which leads to local heating.[32]

Optimum Wavelength

Research supports 630 to 640 nm as the optimum[19–21, 33–35] wavelength because it facilitates biomodulation. Furthermore, this range of wavelength promotes the proliferation of fibroblasts and keratinocytes, increases skin circulation and microcirculation, and diminishes scar tissue. A 630- to 640-nm wavelength proved to be more effective on wound healing by a minimum of 6 to 14 percent when compared with other lasers.

Optimum Power

At high laser powers, the most important mechanism is heating. This occurs in all laser medical applications involving ablation, cutting, vaporization, and coagulation. All of these procedures involve tissue destruction and are roughly wavelength-dependent. Almost all high-power radiation beams are capable of producing tissue damage. The wavelength dependence appears in the absorption coefficient of the irradiated tissue, which defines the special application, the laser type, and length of the procedure. At low laser intensities, however, stronger wavelength dependence is present. The photochemical energy conversion generally involves the light absorption by special molecular light receptors. Also, the light absorption by nonspecialized molecules plays a significant role in medical applications because of the capacity of molecules to absorb light at certain energy levels and the possibility of energy transfer between molecules. An activated molecule can cause biochemical reactions in the surrounding tissue. In a complete chapter, Karu[36] established the most essential mechanisms of light tissue interaction. It is significant

that she wrote, "The photon receptors take part in a metabolic reaction in a cell that is not connected with a light response. After absorbing the light of the wavelength used for irradiation this molecule assumes an electronically excited state from which primary molecular processes can lead to a measurable biological effect in certain circumstances." In her comprehensive work, Karu[36] analyzed and discussed the most important findings concerning low-level laser therapy. In explaining the experimental results, she concluded "that one key event among the secondary reactions of cellular responses was the change in overall redox state of the irradiated cell," so "that the cellular response is weak or absent when the overall redox potential of a cell is optimal or near optimal for the particular growth conditions, and stronger when the redox potential of the target cell is initially shifted to a more reduced state."

It is known that power density and exposure time results show that laser power below 2.91 mW could enhance cell proliferation, whereas higher power had no effect. Stimulatory effects are most pronounced at irradiation times between 0.5 and 6 minutes. The Arndt-Schultz biological law states that weak stimuli excite physiologic activity, moderately strong stimuli empower it, strong stimuli retard it, and very strong stimuli inhibit physiologic activity. Laboratory analyses show that the 10-mW laser is more effective than a 100-mW laser for cell mitosis.

After assessing all known variables, we developed our hypothesis: The application of low-level laser energy—effectively administered according to established criteria addressing coherence, wavelength, and power—to proven lipoplasty/liposuction processes will result in a significantly safer, shorter, and relatively trauma-free procedure. Identifying this procedure as laser-assisted liposuction, our multidisciplinary team of experts set out to establish proof for our hypothesis using scientifically proved testing methods to evaluate the laser effects on the adipose cells.

Process/Protocol

First, in-vitro human adipocyte cultures were developed and then irradiated. After irradiation, it was shown that the adipose cell membrane lost its round shape and that the fat content left the cell through a transitory pore discovered in the cell membrane. Next, the irradiated adipose cells were recultured and shown to be able to recover their original cell membrane structure and remain alive or viable. After this, we took samples of adipose tissue from lipectomy; irradiated them for 0, 2, 4, and 6 minutes; and examined them under light microscopy. Although the first results of the optical studies were inconclusive because of the initial sample testing procedures, the clinical team decided to continue the case study because the preliminary clinical evidence achieved by the plastic surgeon (a co-author of this article)[14,15] was clearly impressive.

The clinical team sent samples for scanning electron and transmission electron microscopic study. Both microscopy protocols were performed on superficial and deep fat samples to establish the cellular effects correlated with the penetration depth of the laser beam after application of the tumescent technique. Samples without the tumescent technique but with exposure to laser for 0, 4, and 6 minutes were also taken. Results indicated that the tumescent technique facilitates laser beam penetration and intensity—fat liquefaction is thus improved.

Fat samples were processed as follows and analyzed by using both microscopy protocols:

1. adipose tissue taken from the abdominoplasty
2. application of tumescent technique and exposure to laser beam for 0 minutes
3. application of tumescent technique and exposure to laser beam for 4 minutes
4. application of tumescent technique and exposure to laser beam for 6 minutes
5. no application of tumescent technique and in vitro exposure of adipose tissue to laser beam for 4 and 6 minutes compared with samples without laser exposure (0 minutes).

Results indicated that the tumescent technique facilitates laser beam penetration and intensity and thus improves fat liquefaction.[14] The adipose cell membrane was also studied in detail with transmission electron microscopy to clarify the suspected pore.

Materials and Methods

Twelve healthy women who had undergone lipectomy were selected for random fat sampling. Their abdominal fat was analyzed after 0, 2, 4, and 6 minutes of external laser exposure. Follow-up observation was done 24 hours after surgery and continued for up to 12 months

after the procedure. The tumescent technique was applied, followed by external laser therapy using a low-level energy diode laser with a nominal wavelength at 635 nm and a maximal power of 10 mW diode. The laser light is line generated at a 60-degree angle with a maximum width of 3 mm. The length of the line generated is factored at an average of 23.7 mm per inch of generated line for each 25 mm of distance from the patient.

The laser light was passed in a sweeping motion about 6 inches above the targeted area. The adipose tissue was externally irradiated through the skin. Cellular effects were studied in samples after 2, 4, and 6 minutes of laser exposure time. Because the dosage is the measurement generally used to define the laser beam energy applied to the tissue, it is useful to reduce the above total applied energy values to these normal units that are given in joules per squared centimeter. In this case, dosage is calculated as the laser power measured in milliwatts, multiplied by treatment time in seconds and divided by area in squared centimeters of the laser spot directed toward the tissue. Considering the properties of the laser output optics and a normal laser at a target distance of 6 inches, the aforementioned energy values correspond to dosages of 1.2 J/cm^2, 2.4 J/cm^2, and 3.6 J/cm^2.

Superficial and deep fat samples of laser-treated tissue were taken from the infraumbilical area of all patients studied. Biopsies were taken with a scalpel (no. 11) from extracted abdominoplasty tissue and then introduced into a 0.1-cc glutaraldehyde phosphate 2.5% buffer at pH 7.2 and 4°C. Furthermore, fat samples extracted without the tumescent technique were also taken and irradiated following the aforementioned sequential procedure. These samples were then examined with scanning electron and transmission electron microscopy to study the laser beam effects on fat cells. The protocols used to study these samples are presented in the Appendix. Regarding the changes in the adipose tissue, there were no major observable differences between samples exposed to 2 and 4 minutes of laser radiation. The samples were to be standardized to those taken for 4 and 6 minutes of exposure time, in which different cell effects could be observed under each microscopy protocol.

RESULTS

Microscopic Findings

Application of tumescent technique without exposure to laser beam. Figure 1 shows a scanning electron microscopy photomicrograph of an adipose tissue sample without laser exposure. A tridimensional view of the adipocytes can be seen. The contours are regular and the traditional grape-cluster shape is evident (Fig. 1). This tissue received tumescent solution but was not exposed to the laser beam.

Application of tumescent technique and exposure to laser beam for 4 minutes. By 4 minutes of exposure, partial disruption of the adipose cell was observed, but several cells without disruption of the cellular membrane were preserved (Fig. 2, *above*). The adipose cells lost their round shape, and fat spread into the intercellular space, going from inside to outside of the cell (Fig. 2, *below*).

Application of tumescent technique and exposure to laser beam for 6 minutes. As shown in Figure 3, microscopic evidence was found that fat was completely removed from the cells and remained in the interstitial space. Some disruption of the connective tissue was also observed. Other structures, such as the capillaries and the remaining interstitial space, were preserved (Fig. 3).

No tumescent solution and in vitro exposure of adipose tissue to laser beam for 4 and 6 minutes compared with samples with tumescence and same laser exposure times. The findings of scanning

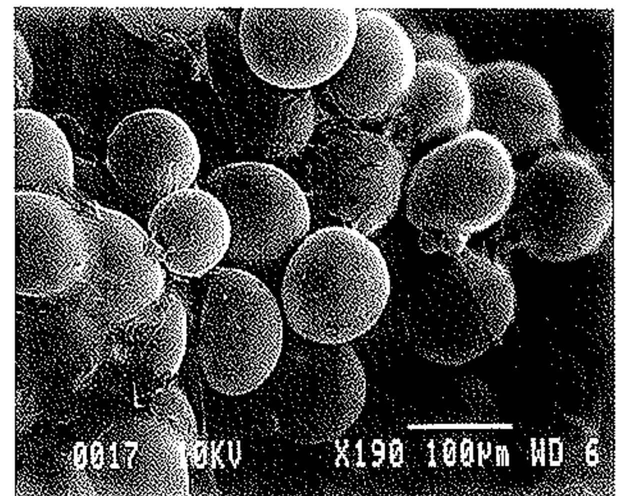

FIG. 1. Scanning electron microscopy photograph of normal adipocytes (×190). Note the round shape. The contours are regular with a grape-cluster shape. This specimen received tumescent solution.

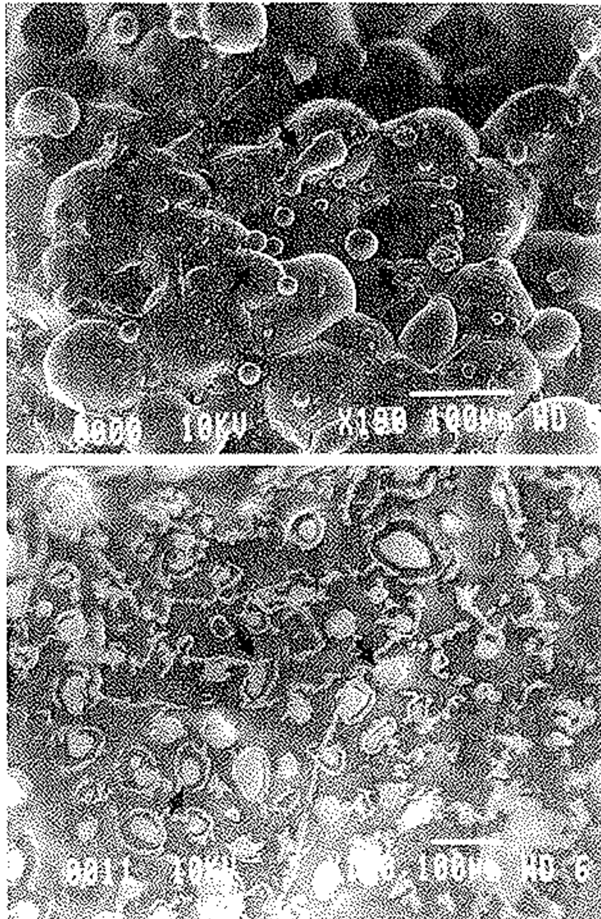

FIG. 2. Scanning electron microscopy photographs of adipocytes after 4-minute application of laser beam. (*Above*) Only a few adipocytes are liquefied, and there is preservation of some cell membranes, some of which have lost their original shape (×190). *Arrows* point out fat particles coming from inside to outside of the adipose cell. (*Below*) The adipocytes have lost their roundness, and some have a star or oval shape (×130).

electron and transmission electron microscopy, after 6 minutes laser exposure in samples taken without tumescent solution (Fig. 4), correspond to those observed in samples exposed to 4 minutes of laser irradiation of equal intensity (10 mW) taken with tumescent solution. Laser penetration through adipose tissue decreased when the tumescent solution was not used, suggesting that the application of the tumescent solution is an important enhancement factor (Fig. 4).

Comparison of adipose membrane of nonirradiated sample with cell membrane after 6 minutes of laser exposure. Figure 5 shows a ×40,000 magnification photomicrograph taken of the adipose membrane of a nonirradiated sample. The membrane remained intact when the laser was not applied. Figure 6 shows a cell membrane at ×60,000 magnification in a tissue sample with 6 minutes of laser exposure. It is possible to see that after irradiation, the membrane is temporarily disrupted, creating a transitory pore that allows the liquefied fat to come out of the cell and be released into the interstitial space.

In summary, without laser exposure, the adipose tissue remains intact and adipocytes maintain their round shape (Fig. 1). After 4 minutes of laser exposure, the membrane of the adipocyte is partially disrupted (Fig. 2, *above*), and 80 percent of the fat is liquefied. Fat particles build up, forming a "cell helmet." Adipocytes suffer partial disruption of their membranes, exposing fat bodies within the cell (Fig. 2, *below*). At 6 minutes of laser exposure, scanning electron microscopy shows almost total disruption of the adipose cell membrane and evacuation of fat (Fig. 3).

To our knowledge, until now, the use of low-level laser energy to open a transitory pore

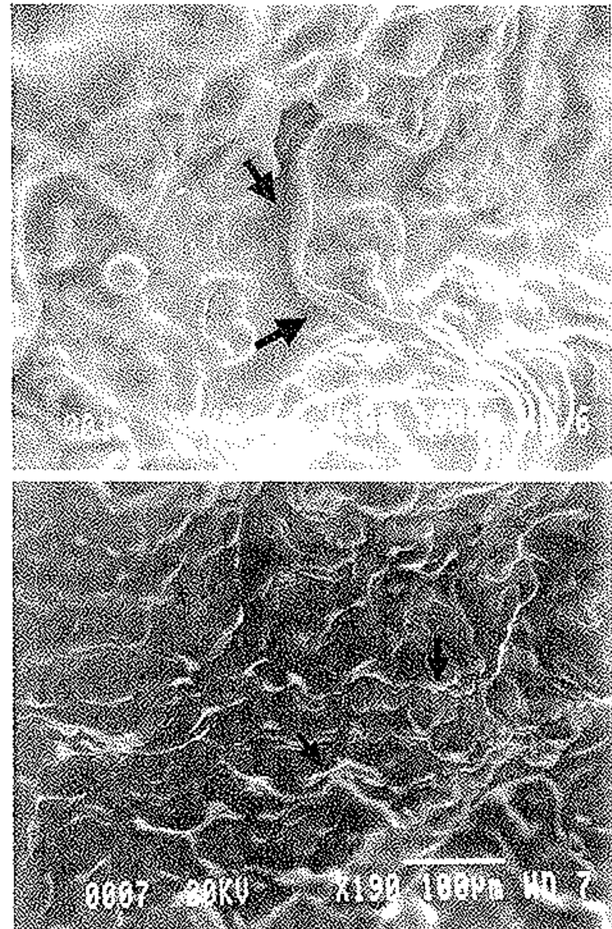

FIG. 3. Scanning electron microscopy photographs after 6 minutes of laser application (×180 and ×190). No round adipocytes are seen, only liquefied fat. *Arrows* point to fat coming out of the adipose cells.

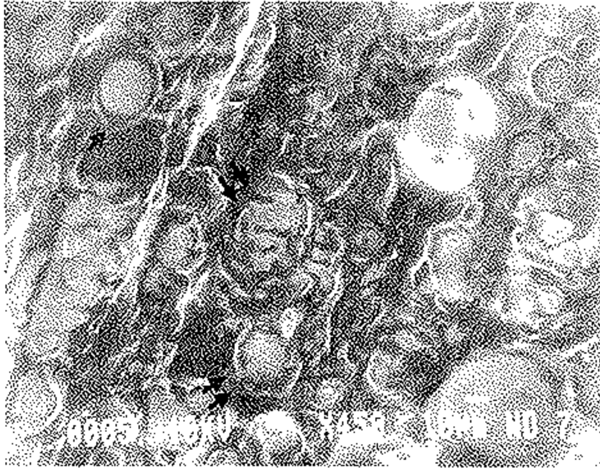

FIG. 4. Scanning electron microscopy photograph after application of the laser beam for 6 minutes without tumescence. Some adipocytes are intact, but others are disrupted (×450). *Single arrow* points to an intact cell, *double arrows* point to the disrupted cells.

in the adipose cell membrane has not been reported.[37] Therefore, the technique described in this article is a new application in the field of plastic surgery, and we have provided the science to support it. We have also demonstrated the effect of the laser beam on the adipose cell through the in vitro human adipose culture.

DISCUSSION

Liposuction techniques and co-adjuvants have been used for many years. Nevertheless, each time a new method or procedure is developed, there are expectations about its potential benefits for mankind. The scientific evidence provided in this article shows that the laser-assisted lipoplasty technique will serve as a valuable contribution to this specific field of medicine and will generate the same expectations as other techniques previously described by other authors. Among its benefits are the reduced risk and improved quality of life for patients.

Random samples taken from 12 patients and submitted to scanning electron and transmission electron microscopic studies demonstrated that the application of the tumescent technique is an important co-adjuvant to laser beam application because it facilitates beam penetration, and as a result, fat extraction. The consistently observed findings are discussed below.

The results of both microscopy protocols indicated that 6 minutes of laser beam exposure with application of the laser-assisted lipoplasty technique and without tumescent technique were comparable with the recorded results achieved from 4 minutes of laser beam exposure combined with the application of the laser-assisted lipoplasty technique and the tumescent technique. The tumescent technique, therefore, empowers the laser beam to affect the cell. Transitory pores were also observed in the cell membrane, with the subsequent spillage of fat into the interstitial space. In samples that underwent the tumescent technique but not laser exposure, a tridimensional scanning electron microscopy photograph showed that the adipocyte retained its original shape (Fig. 7). Several collagenic fibers can be observed in

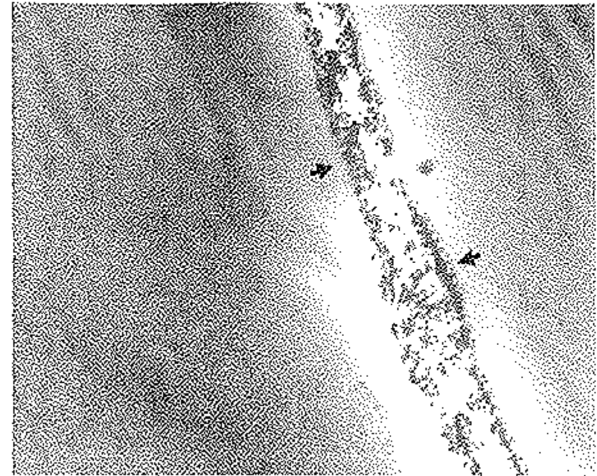

FIG. 5. Adipose membrane (×40,000). The membrane remains intact when the laser is not applied.

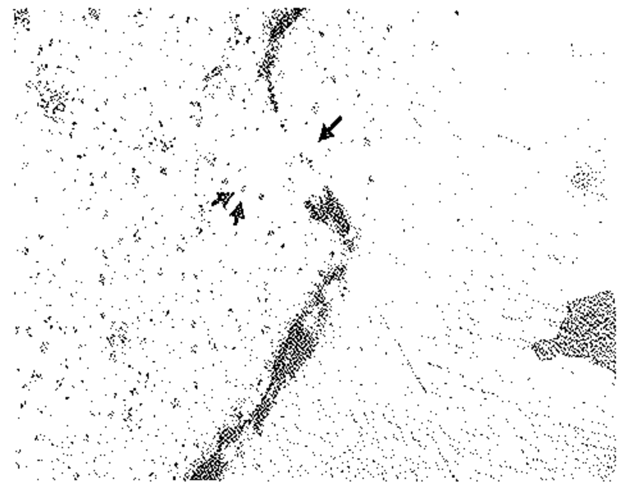

FIG. 6. Transmission electron microscopy photograph of cell membrane after 6 minutes of laser exposure (×60,000). The membrane is temporarily disrupted, creating a transitory pore (*single arrow*) that allows the liquefied fat to leave the cell and be released into the interstitial space. *Double arrow* points out the fat particles released from inside the cell.

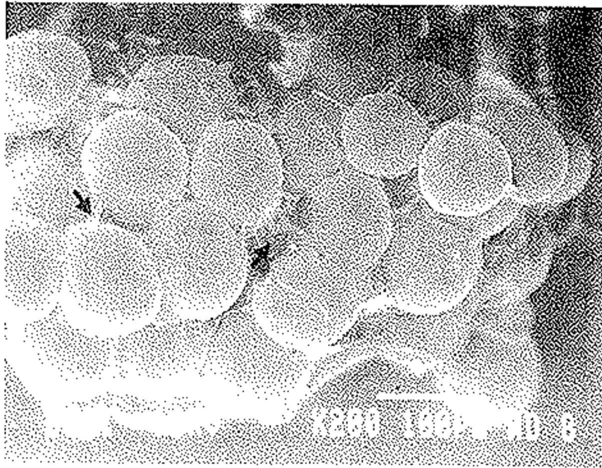

FIG. 7. Scanning electron microscopy photograph of adipocytes with no laser exposure (×200). Adipocytes are intact, and several collagenic fibers (*arrows*) can be seen surrounding the adipose tissue.

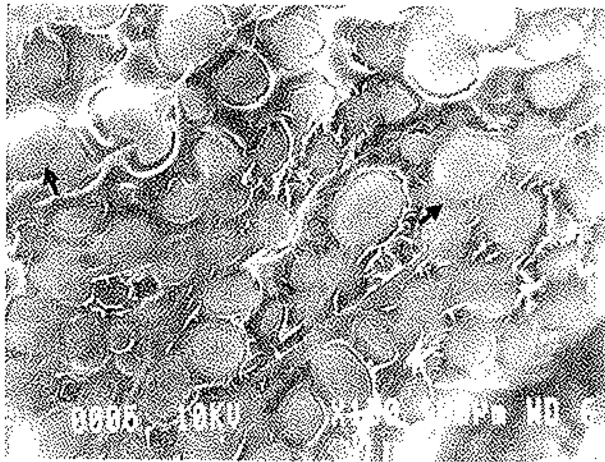

FIG. 8. Scanning electron microscopy photograph after 4 minutes of laser exposure (×190). No tumescence has been applied. Only a few adipocytes have been liquefied. *Arrows* point out intact adipose cells.

the interstice. At 4 minutes of laser exposure without the tumescent technique, liquefaction of only a few adipocytes occurred (Fig. 8). At 6 minutes of laser exposure without the tumescent technique, scanning electron microscopy showed liquefaction of a higher number of adipocytes but not all. When the traditional tumescent technique was combined with 4 minutes of laser exposure, scanning electron microscopy showed partial disruption of the adipocyte membrane with 80 percent of the fat extracted from the cell (Fig. 2).

By increasing the laser exposure to 6 minutes, scanning electron microscopy showed almost total disruption of the adipocyte membrane, which was empty and flexed with irregular contours (Fig. 3). In samples obtained from the traditional tumescent technique without laser exposure, transmission electron microscopy showed adipocytes completely saturated with homogeneous fat. Figure 9 shows four of those cells, which were of regular diameter, were close together, and had reduced intercellular space. When the laser-assisted lipoplasty technique was applied with 4 minutes of laser exposure, transmission electron microscopy showed partial loss of intracellular fat and increased intercellular space. Figure 10 illustrates this and shows three adipocytes. Deformed adipocytes that had lost their round shape were also observed. Capillaries remained completely intact after 4 and 6 minutes of laser exposure (Fig. 11). When the laser-assisted lipoplasty technique was applied with 6 minutes of laser exposure, transmission electron microscopy showed almost total disruption of the regular contours of the adipocyte. The intracellular fat was completely removed from the cell, and the adipocyte was deformed and did not maintain its original shape (Fig. 12). Figure 12 also shows the deformity, folding, and disruption of the adipocyte membrane with 6 minutes of laser exposure.

As an interpretive explanation of our study findings with regard to the biological performance of the adipose tissue, its interaction with laser light, and the environmental contributions of the tumescent solution, experimental studies show a 0.3 to 2.1 percent transmittance of red laser light in 2-cm-thick normal skin, depending on the laser wavelength.[38] Further,

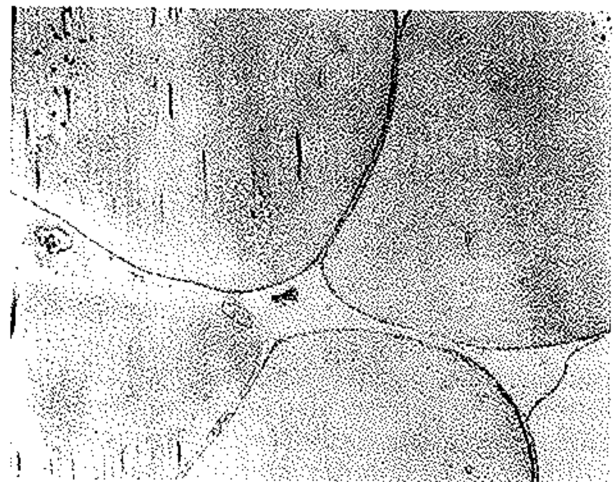

FIG. 9. Transmission electron microscopy photograph showing adipocytes completely saturated with fat and close to one another (×20,000).

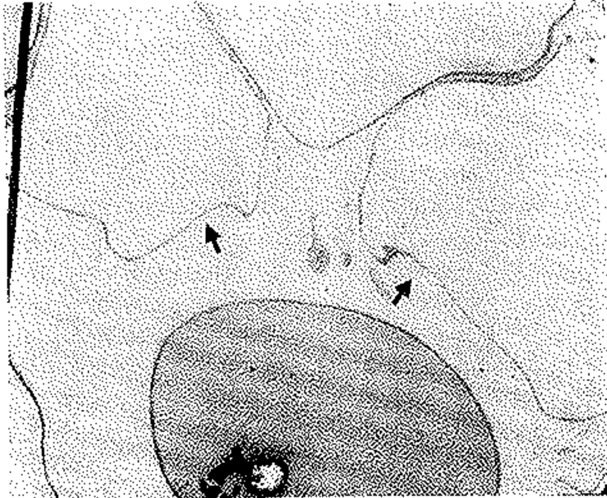

FIG. 10. Transmission electron microscopy photograph after 4 minutes of laser exposure (×20,000). There is partial loss of the intracellular fat, and the membrane has become flexed because it has lost part of its fat content.

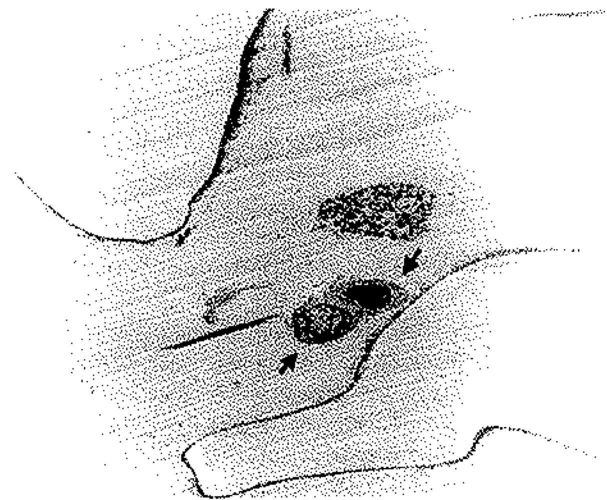

FIG. 11. Transmission electron microscopy photograph after 4 minutes of laser exposure (×20,000). The adipose membrane is flexed and deformed. The capillaries remain intact in the intercellular space (*arrows*).

it was found that the transmittance of granular tissue is 2.5 times higher than that of normal skin. Moreover, to find a method for increasing light transport deeply into target areas of tissue, the effects of a hyperosmotic agent on the scattering properties of rat and hamster skin were investigated,[39] and a transient change in the optical properties of in vitro rat skin was found. A 50 percent increase in transmittance and a decrease in diffusive reflection occurred within 5 to 10 minutes after introducing glycerol.[23] In our case, it is known that fat contains glycerol; therefore, laser transmittance through the adipocyte could be very effective.

FIG. 12. Transmission electron microscopy photograph after 6 minutes of laser exposure (×20,000). There is almost total disruption of the adipocyte membrane. The adipose cell has almost completely lost its fat content.

In addition, the tumescent solution has two mechanisms of action:

1. It is a polar solution that destabilizes the adipocyte membrane, thus facilitating the penetration of the laser beam. This was demonstrated by the findings in the samples subjected to both microscopy protocols.
2. The aqueous portion also serves as a co-adjuvant to laser action. These effects are co-adjuvants to the laser action, making the low-level energy laser a powerful tool in liposuction procedures.

The adipocyte membrane is activated by different cyclic adenosine monophosphate concentrations that stimulate, in turn, cytoplasmic lipase that triggers the conversion of triglycerides into fatty acids and glycerol, both elements that can easily pass through the cell membrane. The adrenaline, also found in the tumescent solution, stimulates the adenyl cyclase that, together with the effect of the laser beam on the internal and external media of the adipocyte, changes its molecular polarization. The exit and removal of fatty acids and glycerol into the extracellular space enhance this. The effectiveness of low-power laser light in producing changes in biological tissues and laser action on cells, even in low doses, has been reported recently.[40–42] Reproducible light-induced changes in the transmission spectrum of human venous blood under the action of low-intensity radiation from the helium-neon laser were found,[40] showing that laser light induced

the changes and highlighting the potential of the spectrometric studies. In addition, the influence of low-level laser irradiation on the degranulation process of the mast cells was studied in mesentery mast cells of the rat intestine,[41] showing that laser radiation (890 nm, in this case) stimulates degranulation of mesentery mast cells. This study also showed that the effect is dose-dependent, and maximal degranulation was registered after laser irradiation with power of 25 mW and an exposure time of 15 to 30 seconds. Finally, confocal microscopy was used for irradiation, and simultaneous observation of low-power laser effects in subcellular components and functions at the single-cell level was made.[41,42] Cultures of human fetal foreskin fibroblasts were prepared for in vivo microscopic evaluation. Cells were stimulated by the 647-nm line of the argon-krypton laser of the confocal microscope (0.1 mW/cm^2). Laser irradiation caused alkalization of the cytosolic pH and increased the potential of the mitochondrial membrane. Temporary global cytoplasmic calcium responses were also observed. The effects were localized to the irradiated microscopic fields, and no toxic effects were observed during experimentation.[27]

Conclusions

The low-level laser–assisted lipoplasty consists of the tumescent liposuction technique with the external application of a cold laser (635 nm and 10 mW intensity for a 6-minute period). This technique produces a transitory pore in the adipocyte membrane, preserving the interstice, particularly the capillaries. When adipose tissue is exposed to the laser beam for 4 minutes, 80 percent of the adipocytes' membranes are disrupted; this increased to almost 99 percent with 6 minutes of laser exposure, as demonstrated by both scanning electron and transmission electron microscopy.

The laser facilitates the releasing of fat and contributes to the disruption of the fat panicles, allowing the fat to go from inside to outside the cell and placing it in the interstitial space. With easier fat extraction, surgical trauma, ecchymoses, and hematomas are reduced to facilitate the patient's recovery.

The transitory pore formation induced by the laser occurs exclusively at the level of the adipocyte membrane. When tumescent solution was used as a co-adjuvant, almost 99 percent of the fat was released into the interstice, whereas the capillaries and the remaining interstice were preserved. The result of this development is a safer, more effective procedure with elimination of the need for pretunneling.[14,15,37]

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Appendix

Protocol to Identify Tissues by Scanning Electron Microscopy

1. Fix tissue in phosphate buffer with 2.5% 0.1 M glutaraldehyde at pH 7.2 for 24 hours.
2. Rinse buffer in 4.5% 0.1 M sucrose phosphate buffer for 15 minutes.
3. Dehydrate in alcohol at different concentrations, 30% to 100%, for 2 minutes per percentage of alcohol.
4. Dry until critical point is reached.
5. Place tissue on specimen holder previously prepared with colloidal graphite and attach with double adhesive tape.
6. Ionize with gold-palladium until a 10- to 18-monometer layer is formed.
7. Observe under a microscope (e.g., JEOL-820 [scanning] or JEOL-JEM 1010 [transmission]; JEOL USA, Inc., Peabody, Mass.).

Protocol to Observe Ultrafine Tissues by Transmission Electron Microscopy

1. Fix tissue in Milloning buffer with 2.5% glutaraldehyde at pH 7.
2. Rinse in 0.1 M phosphate buffer at pH 7.2 for 15 minutes.
3. Postfix in 1% osmium tetra-oxide in distilled water for 1 hour.
4. Rinse in 0.1 M phosphate buffer at pH 7.2 for 10 minutes.
5. Dehydrate in 25% to 100% alcohol for 15 minutes.
6. Add 70% uranyl acetate to the alcohol dur-

ing dehydration and leave tissues for 12 hours.

7. After last pass through 100% alcohol, pass three times through acetone for 15 minutes.
8. Infiltrate with 3:1 acetone + V plastic for 60 minutes.
9. Infiltrate with 2:2 acetone + V plastic for 60 minutes.
10. Infiltrate with 1:3 acetone + V plastic for 60 minutes.
11. Leave in pure plastic overnight.
12. Place in recently prepared plastic (Spurr).
13. Polymerize on stove at 60°C from 8 to 15 hours.
14. Make ultrafine 600-nm cuts with diamond-head scalpel in the ultramicrotome and collect in 1-hole grids covered with Fomvar (Structure Probe, Inc., West Chester, Pa.) membrane.

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