Title
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Journal
Lasers in Surgery and Medicine, 46(8)

ISSN
0196-8092

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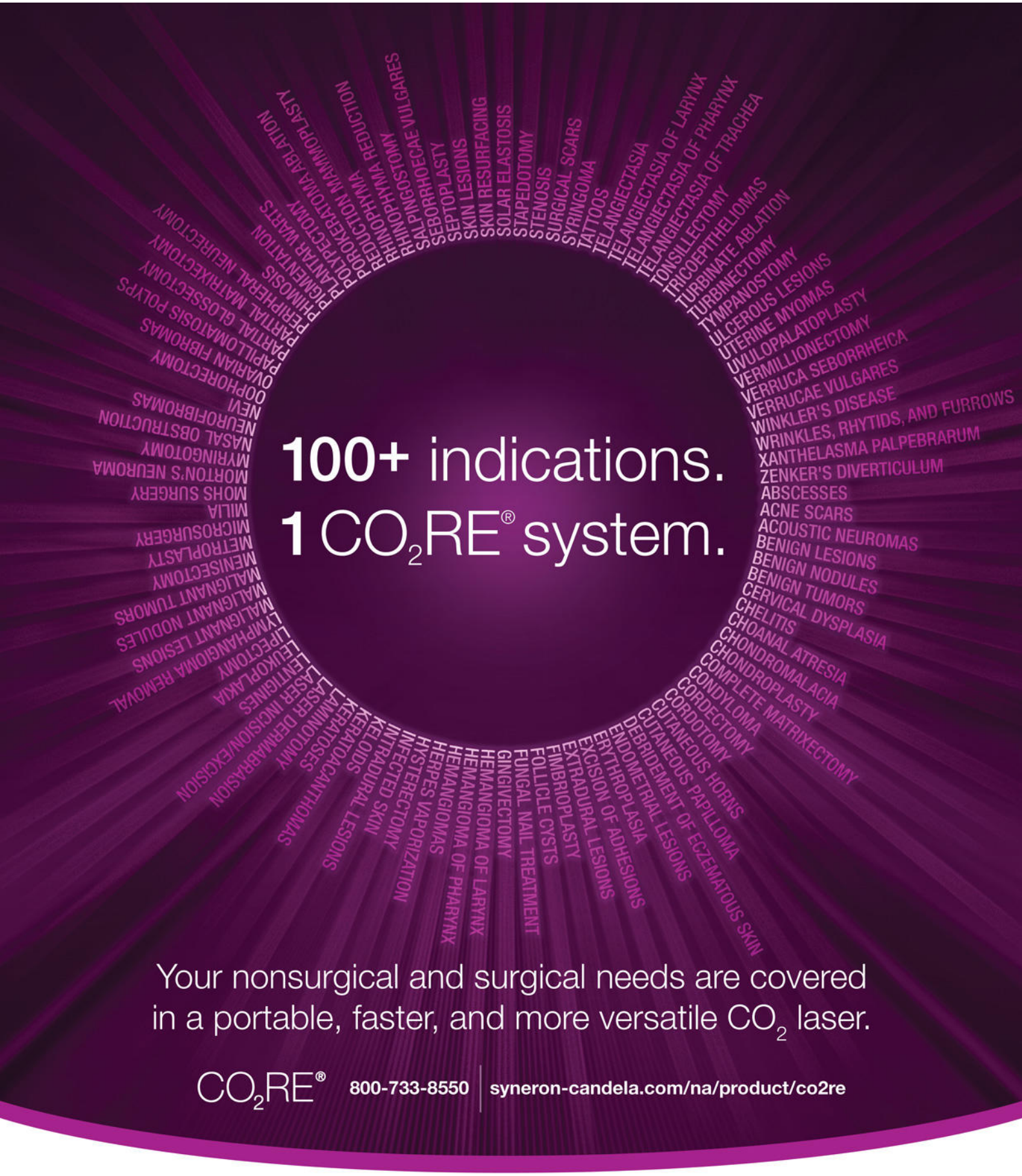
Publication Date
2014-10-01

DOI
10.1002/lsm.22278

Peer reviewed



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Effect of Laser Generated Shockwaves 1 on Ex-vivo Pigskin

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Introduction: Persistent bacterial infection prolongs hospitalizations, leading to increased healthcare costs. Treatment of these infections costs several billion dollars annually. Biofilm production is one mechanism by which bacteria become resistant. With the help of biofilms, bacteria withstand the host immune response and are much less susceptible to antibiotics. Currently, there is interest in the use of laser-generated shockwaves (LGS) to delaminate biofilm from infected wound surfaces; however, the safety of such an approach has not yet been established. Of particular concern are the thermal and mechanical effects of the shockwave treatment on the epidermis and the underlying collagen structure of the dermis. The present study is a preliminary investigation of the effect of LGS on freshly harvested *ex vivo* porcine skin tissue samples.

Materials and Methods: Tissue samples for investigation were harvested immediately post-mortem and treated with LGS within 30 minutes. Previous studies have shown that laser fluences between 100 and 500 mJ/pulse are capable of delaminating biofilms off a variety of surfaces, thus our preliminary investigation focused on this range of laser energy. For each sample, LGS were produced via laser irradiation of a thin layer (0.5 μm) of titanium sandwiched between a 50 and 100 μm thick layer of water glass and a 0.1 mm thick sheet of Mylar. The rapid thermal expansion of the irradiated titanium film generates a transient compressive wave that is coupled through a liquid layer to the surface of the *ex vivo* pigskin sample. Shocked samples were immediately fixed in formalin and prepared for histological analysis. A blinded pathologist evaluated and scored each section on the basis of its overall appearance (O) and presence of linear/slit-like spaces roughly parallel to the surface of the skin (S). The scores were given on a scale of 0–3.

Results: The present investigation revealed no visible difference between the tissue sections of the control sample and those that were subjected to laser-generated shockwaves. There was no relationship between the scores received by the samples and the energy with which they were shocked.

Conclusion: Preliminary investigation into the safety of the LGS treatment for biofilm delamination appears promising. Additional investigation will continue on *ex vivo* porcine samples, followed by an *in vivo* animal trial to better understand the physiological response to LGS treatment. Lasers Surg. Med. 46:620–627, 2014.

Key words: bacterial biofilms; Nd:YAG laser; laser-generated shockwaves; bacterial resistance

INTRODUCTION

Bacterial biofilm-infected wounds, arising from surgery or trauma, are a major burden on the US healthcare system. Treatment of these biofilm-based infections costs more than $1 billion annually [1–3], and it is estimated that biofilms are associated with 65% of nosocomial infections [4]. Bacterial biofilms are communal structures of microorganisms encased in an exopolymeric coat that attach on both natural and abiotic surfaces. Biofilms have been associated with a variety of persistent infections [5–7]. The sessile bacteria in these biofilms can withstand host immune responses, and are much less susceptible to antibiotics compared to their nonattached individual planktonic counterparts [8–11].

Treatment of Biofilm-Associated Chronic Wounds

Biofilms are a principal cause of wound chronicity [4,12,13]. Several strategies for treating biofilms associated with wounds are being developed [14]. Most, if not all methodologies involve disruption of the multicellular, communal structure of biofilms; if the aggregation of bacterial cells in biofilms can be broken down, the host defenses might be able to resolve the infection, and the efficacy of antibiotics might be restored [15]. Chemical therapies that could potentially help with this disruption include dissolving the matrix polymers of the biofilm using enzymes [16], or initiating chemical reactions that block

Contract grant sponsor: Telemedicine and Advanced Technology Research Center (TATRC).
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Accepted 8 July 2014
Published online 28 August 2014 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/lsm.22278

biofilm matrix synthesis [17]. Mechanical approaches to biofilm disruption are also being investigated. Here, the current gold standard treatment involves relatively painful wound debridement procedures. Pain notwithstanding, these procedures have not been sufficiently successful in dealing with bacterial resistance to antibiotic treatment [18–20]. In addition, ultrasound therapy [21,22] and extracorporeal shockwave therapy (ESWT) [23,24] are currently being explored, though each is associated with limitations [25–27].

An alternative approach is offered by Laser-Generated Shockwaves (LGS) [28–31]. In contrast to ESWT, LGS primarily uses compressive stress waves to delaminate biofilms [32], thereby eliminating the damage that could be caused by cavitation bubbles. In addition, LGS have been shown to both disrupt biofilm *in vitro* on a variety of targets [30], and enhance the permeability of bacterial biofilms [28,29].

The goals of this paper are to try and understand the effects of LGS on the wound bed underlying the biofilm infection. Freshly harvested porcine skin is used as the model in the experiments.

Studies Showing Effect of Laser and Laser Generated Shockwaves on Tissue and Cells

The mechanical effects of laser and LGS on various biological tissues have been studied and put to use extensively in the past few decades [33–36]. In as early as 1984, excimer lasers were demonstrated to ablate tissue with minimal thermal injury [37]. Subsequently, Puliafito showed that 193 nm radiation produced smaller damage zones than 248 nm radiation when ablating the crystalline lens [33]. On the whole, excimer lasers were found to be effective in producing controlled ablation of the crystalline lens *in vitro* with effects similar to those seen in the cornea. Vogel et al. [34] compared the effects of picosecond (ps) and nanosecond (ns) pulses while performing Nd:YAG laser surgery on intraocular tissue. Laser- induced cavitation effects were seen to be the major drawback of intrastromal corneal surgery. Cavitation causes tissue displacement and produces unpredictable refractive changes in the lens. The investigators concluded that ps pulses increased precision and diminished unwanted disruptive side-effects when compared to ns pulses, whereby the damage range decreased by a factor of three.Thus, ps pulsed lasers could be used for applications requiring great precision, although ablation rates are slow. In urology, the use of laser induced shockwaves for lithotripsy began to be explored both in combination with ultrasound [35] as well as exclusively with LGS [36]. By 1990, a variety of laser lithotripsy systems were in clinical use for urinary stone fragmentation [38]. The combination of small fiber diameters, mechanical stiffness of the endoscopes, and difficulty aiming the laser fibers led to perforation rates of approximately 10% of transurethral lithotripsy procedures [38]. Subsequent Holmium–Yag laser-based systems were developed and supplanted the original 504 nm dye-laser-based systems.

Safety Concerns Associated with Laser Generated Shockwave Treatment

Concerns regarding the safety of the LGS system understandably persist based on knowledge of safety issues in a variety of laser treatments. Some of the common deleterious effects that result from the interaction between the laser and the tissue seen with other laser treatments include ablation of the top layers of the skin [39,40], coagulation of connective tissue [41], tears and trauma in the various layers of the skin [42], erythema [43], hyperpigmentation and hypopigmentation [44]. It should, however, be noted that LGS treatment is unique in comparison to other techniques that involve treatment with laser energy, in that with our proposed technique the laser is not made incident on the skin directly. Since there is a lack of direct interaction between the laser and skin, it is possible that tissue will not be susceptible to typical laser damage. In addition, although minor deleterious effects were observed when using LGS for drug or gene transfer under certain conditions [45–46], the effects appeared to heal within a short period of time post-treatment. Therefore, due to the lack of laser interaction in the skin and the minor effects of the few studies exploring the safety of LGS on tissue, it is possible that LGS treatment can be performed with minimal damage to the underlying skin structures.

To test this hypothesis, the present study investigates the damage thresholds of LGS, specifically the effects of the laser system and the resulting shockwave, on the epidermal and dermal tissue structure in an *ex vivo* model. LGS with different energy fluences were made incident on freshly harvested porcine skin samples. The samples were immediately fixed, sectioned and stained using H&E and Masson's Trichrome stains. The tissue sections were then analyzed using light microscopy.

MATERIALS AND METHODS

Specimen Preparation

The porcine model was chosen as it has been used as a model for human skin in the past due to its similarities to human skin both physiologically and anatomically [47,48]. Porcine skin specimens were harvested from the abdominal region of a pig immediately post-mortem for a total of 15 specimens. The specimen was cut into square shaped pieces of 5 mm length using a scalpel and blade. Each resulting specimen was maintained at room temperature (25°C) throughout the experiment, and not frozen, so as not to alter the structure or mechanical properties of the collagen fibers. LGS treatment was carried out no more than 30 minutes after specimens were harvested.

Substrate Preparation

Mylar sheets with dimensions of $80 \times 30\,\text{cm} \times 0.1\,\text{mm}$ were RF sputtered (Denton Discovery II 550, Denton Vacuum, NJ USA) with 0.5 μm thick layer of Ti. A layer of waterglass was then spin-coated on top of the Ti to achieve a uniform layer of 50–100 μm. The waterglass layer acts as

the constraining layer and is transparent to the Nd:YAG laser wavelength of 1.064 μm.

Shockwave Generating System and Laser Parameters

A Q-switched, Nd:YAG laser was used to generate LGS. A 3–6 nanosecond (ns) long Nd:YAG laser pulse is impinged over a 3 mm diameter area on the 0.5 μm Ti sandwiched between the back surface of the Mylar sheet and the layer of waterglass. Laser-generated pulses impinging upon the thin metallic surface generate stress waves within the material. The laser energy then ablates the thin metallic film, thereby causing a rapid thermal expansion of the film resulting in a compressive wave propagating through the substrate. The resulting compressive stress wave is made incident on the biofilm-wound interface. Upon encountering the interface, a component of the wave reflects back as a tensile stress, while a second component of the wave propagates through the tissue as a compressive stress. The component reflecting as a tensile wave from the biofilm's free surface causes its spallation at sufficiently high amplitudes without propagating down through the tissue [49]. The compressive component, however, travels through the tissue and is absorbed. The laser fluence, pulse width and the substrate material properties contribute to the temporal characteristics of the stress wave. The peak stress, rise time of the wave, and the stress profile generated are dependent on the above-mentioned parameters [50–55].

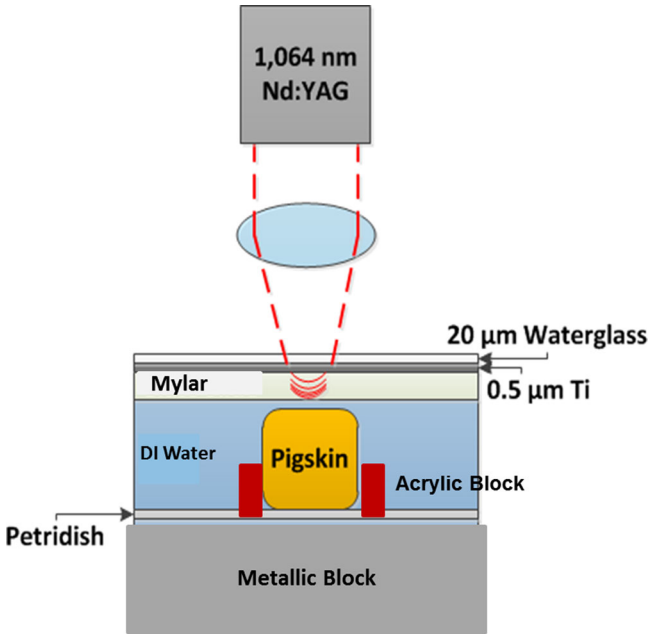

Fig. 1. Schematic of the experimental setup. The porcine skin specimen is placed between two acrylic blocks to prevent movement during irradiation. A sheet of Mylar, coated with Ti on one side, and waterglass on the other, us irradiated by a 1.064 μm Nd:YAG laser to generate LGS. The Mylar substrate is coupled to the tissue specimen with DI water.

Experimental Procedure

The porcine skin sample was immersed in a petridish containing deionized water, such that there was a 1 mm thick layer of deionized water over the sample. The deionized water was used as a coupling agent between the sample and the Mylar sheet. The pigskin was held in place by placing it in between two acrylic blocks glued to the base of the petridish. The shockwaves were made to be perpendicularly incident on the porcine skin sample (Fig. 1). Each sample was subjected to LGS of a particular laser fluence ranging from 100 to 500 mJ. The precise laser fluences and corresponding energy density generated by the laser are shown in Table 1. For this pilot investigation, there were two samples per energy level and one control sample.

Sample Preparation for Analysis

Immediately after shocking, the samples were fixed in formalin and prepared for paraffin histology. Specimens were sectioned sagitally at 5 μm thickness along the midline, coinciding with the center of 3 mm shocked region. This region should correspond to the maximum mechanical impact generated by the laser shockwaves. The sections were then stained using H&E and Masson's Trichrome stain. The tissue sections were scored on the basis of their overall appearance (O) and linear/slit- like spaces roughly parallel to the surface of the skin (S) when compared with other samples, on a scale from 0 to 3. An O score of "0" indicates that the sample is very different from the rest of the samples whereas an O score of "3" indicates that the sample is very similar to the rest of the samples on the basis of overall appearance. A S score of "0" indicates very small number of linear/slit-like spaces in the sample while a S score of "3" indicates very large number of linear/slit-like spaces. While assessing the overall appearance, the tissue sections were examined to see whether the stratum-corneum, epidermis, dermis and the epidermal-dermal junctions were intact. Indications for ablation of the top layers of the skin, congealing of the collagen fibers, changes to collagen structure or orientation, and mechanical trauma to the various layers of the skin were investigated.

TABLE 1. Laser Fluence and Energy Density Generated by the Laser at the Mylar Sheet

Laser Fluence (mJ)	Energy Density (mJ/mm^2)
118	16.68
149	21.07
228	32.24
264	37.33
350	49.49
400	56.56
498	70.42

Characterization of Laser Generated Shockwave System

The laser generated shockwave system used for the present experiments had been previously characterized across five flexible substrates under a range of laser fluences [56]. Materials examined were polyethylene film, polystyrene film, polyvinyl film, polycarbonate film, polypropylene film, and PEEK (polyetheretherketone) film (Fig. 2). A Michelson interferometer was used to measure the displacement caused by the shockwave exiting the material. Using the deflection measurements and the material's mechanical properties, the velocity and stress profile of the wave exiting the material was calculated. However, these values cannot be used to estimate the exact stress profile for mylar (essentially a polyester) used in the current study because the thickness of the mylar used was 0.1 mm compared to the 0.254 mm used for the characterization study. In addition, several other experimental conditions varied between the two studies including the energy density of the laser beam and the manner in which the LGS was coupled to the target. In the current study the range of energy density used was 16.68–70.42 mJ/mm^2 and the coupling agent was deionized water, whereas, in the characterization study the energy density used was 11 mJ/mm^2 and the coupling agent used was air. Therefore, we will be reporting the laser fluence as opposed to the peak stresses for this preliminary investigation.

RESULTS

Qualitative Observations of Tissue Sections Under Microscope

The tissue sections, including control, were viewed by an experienced pathologist (Dr. William Yong) in a comparative blind study. The stratum corneum, epidermis, dermis, and the epidermal-dermal junction were similar across all the irradiated specimens and control. There was no observable ablation of the top layers of the skin sample, congealing of the collagen fibers, or mechanical trauma in the various layers of the skin. The collagen structure and orientation remained intact, and no differences could be observed when compared to the control sample. There were some regions where the collagen fibers seem to have larger spaces or air pockets in between them, but such regions were also found in the control sample indicating that it is most probably an artifact related to preparation of specimens or sectioning (Figs. 3 and 4).

Tissue Section Scoring

The scores for the tissue sections are shown in Table 2. The control samples were given the highest slit/space and overall score. This suggests that the slits/spaces seen in the collagen structure are most probably a sectioning or preparation artifact. The high O score given to the control sample indicates that the control sample appeared the most damaged when compared to the irradiated samples. Therefore, it is unlikely that LGS is having a detrimental effect. In addition, Table 2 reveals that all the samples received a high O score of either 2 or 3 suggesting that the overall appearance of all samples was similar. Nine of the 15 samples received S scores of 3. Furthermore, three samples received the lowest S score of 4 (Table 2). If the the slits/spaces were indeed caused by LGS then we would expect samples shocked with 498 mJ to have the highest S score and samples shocked with 118 mJ to have the lowest S score. However, our results reveal that there is no clear pattern that can be observed in the S scores. This again suggests that the slits/spaces in the tissue sections are

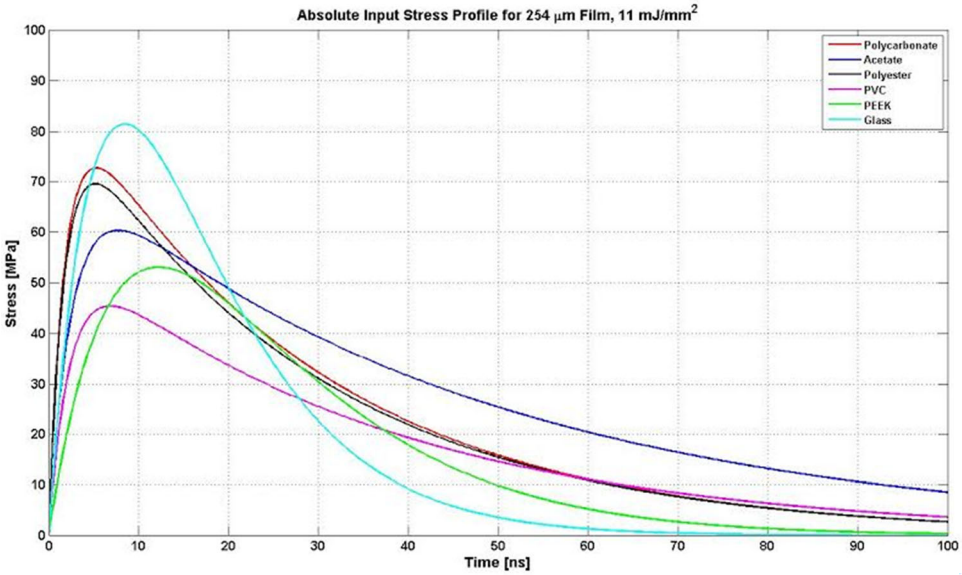

Fig. 2. The stress wave profile of five flexible substrates under a range of laser fluences, all coated with 0.5 μ thick Titanium film. Materials included were polyethylene, polystyrene, polyvinyl, polycarbonate, polypropylene and PEEK (polyetheretherketone).

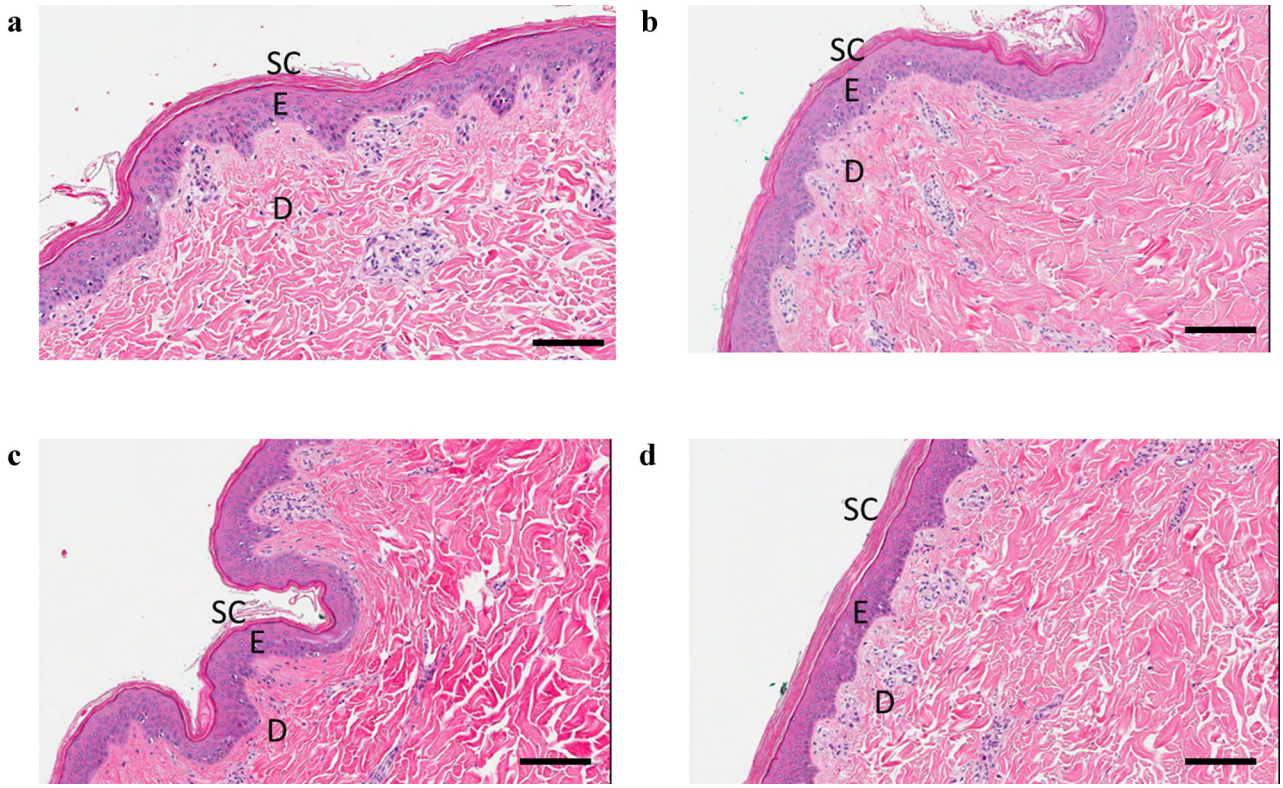

Fig. 3. Sagital sections of *ex vivo* porcine samples after treatment for (a) control (b) 118 mJ (c) 264 mJ and (d) 498 mJ of laser energy. Specimens were sectioned at 5 μm thickness and stained using H&E stain. Photomicrographs were attained at 10× magnification. Sample bars in each image represent 100 μm. Abbreviations: SC, stratum corneum; E, epidermis; D, dermis.

either inherently present in the tissue or are an artifact related to histology. Finally, there was no apparent relationship between S and O scores and the energy level with which the samples were shocked. While further investigation and a full statistical analysis is necessary to confirm this result, it appears that treatment of the samples with LGS did not alter the overall appearance and structure of the *ex vivo* pigskin samples.

DISCUSSION

The present study provides preliminary evidence that LGS technology has a negligible effect on *ex vivo* pigskin. Histological analysis demonstrates that, at the energies tested, various structural components of the skin; the epidermis, epidermal-dermal junction and the dermis remain intact after subjecting the specimens to LGS. Blinded evaluation by a trained pathologist showed that there was no change in the structure and orientation of the collagen fibers in the pigskin samples.

Studies Showing the Effect of Laser and Laser Generated Shockwaves on Skin Tissue

A wide range of skin conditions are amenable to treatment with lasers [57]. Initial studies show that removal of the upper layers of the skin has a positive cosmetic effect. CO_2 and Er:YAG lasers are currently the most popular for this technique called skin rejuvenation. Newer rejuvenating laser systems are being introduced to reduce the risk of side effects and unpleasant postoperative recovery period that are associated with CO_2 and Er:YAG lasers. These effects have also been achieved with mechanical systems. Histologic studies on the long-term effects of laser-resurfaced skin demonstrate elimination of epidermal atrophy and atypia, new collagen development in the dermis, proliferation of elastic fibers, homogenization of melanin distribution and a reduction in the amount of glycosaminoglycans [58]. These histological changes correlate with clinical findings of diminished fine wrinkles, enhanced color and texture and overall skin rejuvenation. However, a common side effect of these skin resurfacing methods is post-treatment erythema [57]. Sublethal thermal damage, increased vascular permeability, and collagen alterations have been proposed as etiologies for post laser erythema [57]. Recent developments in laser resurfacing technology have aimed at minimizing thermal damage to the dermis. Pigmentary disorders such as hyperpigmentation and hypopigmentation are some of the additional complications associated with laser resurfacing. All of these must be considered when evaluating the shockwave system. Pigmentary disorders are thought to be due to the influence of the treatment on the complex

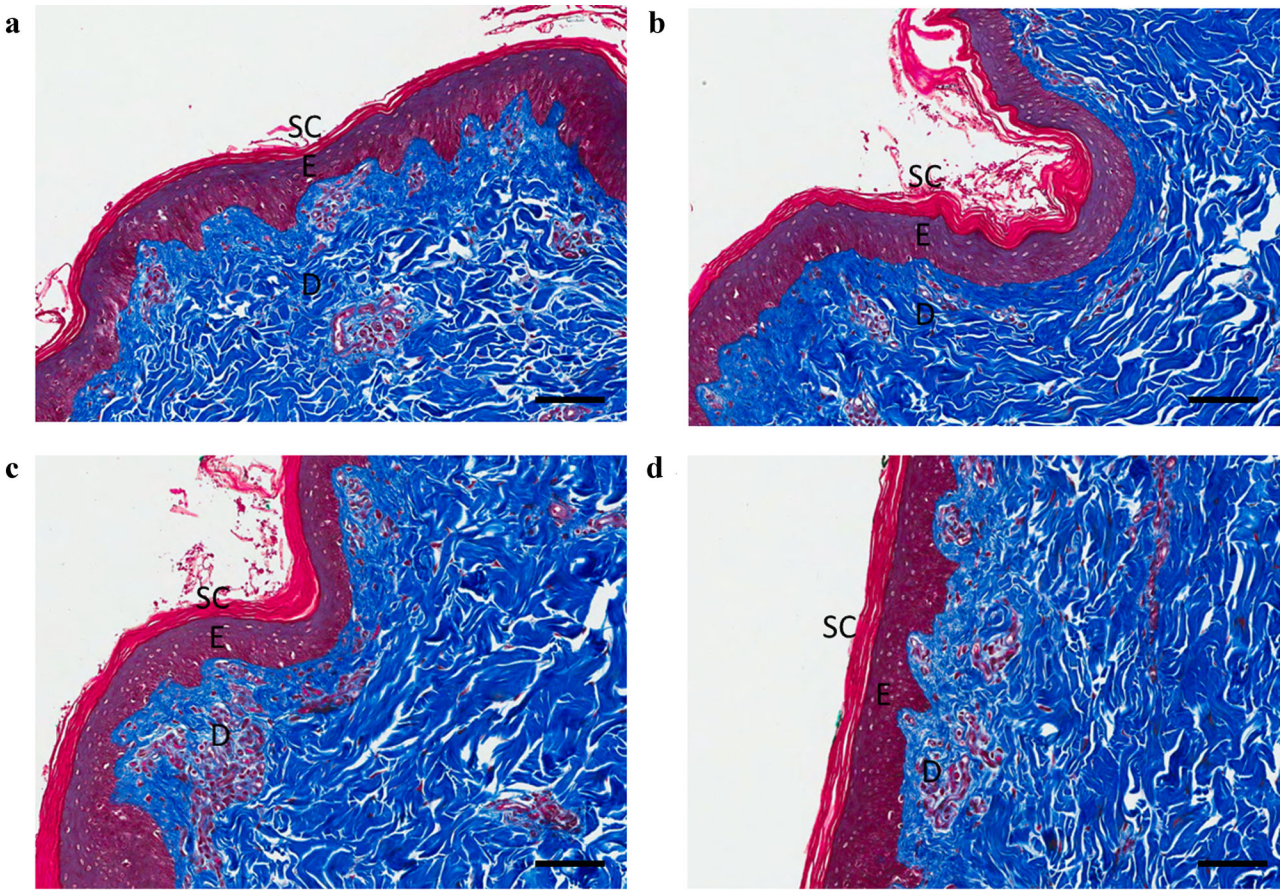

Fig. 4. Sagital sections of *ex vivo* porcine samples after treatment for (a) control (b) 118 mJ (c) 264 mJ and (d) 498 mJ of laser energy. Specimens were sectioned at 5 μm thickness and stained using Masson's Trichrome stain. Photomicrographs were attained at 10 × magnification. Sample bars in each image represent 100 μm. Abbreviations: SC, stratum corneum; E, epidermis; D, dermis.

TABLE 2. O Score (Overall appearance) and S Score (Linear slit like spaces roughly parallel to the surface of the skin) Assigned to the Specimens During Comparative Blind Evaluation on a Scale of 0–3

Energy Level (mJ)	Sample	O score	S Score
118	a	2	1
	b	2	1
149	a	2	2
	b	2	0
228	a	3	2
	b	3	2
264	a	2	0
	b	2	2
350	a	3	2
	b	2	2
400	a	2	2
	b	3	2
498	a	3	1
	b	2	2
Control	a	3	3

microenvironment of keratinocytes, melanocytes, and collagen fibers [44]. The effect of LGS on the structure of skin is, however, not very well known. There have been studies whereby the use of LGS for drug [45] and gene [46] delivery through skin has been explored. Doukas *et al.* showed that LGS produced using a Q switched ruby laser (694 nm wavelength and ~28 ns pulse duration) increases the permeability of the stratum corneum *in vivo* in humans [45]. The onset of permeabilization was observed at 35 MPa and increased with increasing peak pressure. During the transient period of increased permeability, macromolecules diffused through the stratum corneum to the epidermis and dermis. While pressure waves of 300 ns duration did not produce any negative sensation, pressure waves of 1 μs duration generated a noticeable sensation, but not pain [45]. With respect to skin changes, after the application of a pressure wave, the 300 ns pressure wave did not produce significant changes to the appearance of skin, while the 1 μs pressure wave produced a minor erethyma, which disappeared within 10–15 minutes. The stratum corneum eventually did recover its barrier function within 15 minutes when water was used as the

coupling medium [45]. Ogura et al. [46] demonstrated *in vivo* gene transfer using LGS generated by a Nd:YAG laser. Results revealed that rats injected with plasmid DNA and subsequently irradiated with LGS had luciferase activity at two orders of magnitude higher than controls. The peak pressures used in the study were estimated to be in the range of 15–75 MPa, with energy densities corresponding to 4–19 mJ/mm^2. At this fluence, one third of the rats showed erythema in the shockwave-exposed skin, but the effect disappeared within a week. Irradiation with more than three pulses caused erythema in all shockwave-exposed skin which also disappeared within a week. Thus, no major side effect on skin was observed. The energy density and peak stresses generated in the present experiments were significantly higher than those used in previous experiments [45–46,59]. Yet, the current study shows that a single Nd:YAG laser generated shockwave pulse (3–6 ns) ranging from 16.68–70.42 mJ/mm^2 of energy can be made incident on skin without damaging its collagen structure. The peak pressures of the LGS produced by Doukas [45] and Ogura [46] lie between 15–75 MPa. Figure 2 shows that the peak stress generated by polyester is approximately 70 MPa with a laser fluence of 11 mJ/mm^2. Using this information it can be said that the peak pressures used in the current study are much higher than 70 MPa since much higher laser energies (16.68–70.42 mJ/mm^2) were used. However, the pulse duration of the pressure waves used in our experiment is significantly shorter than those used by Doukas [45] and Ogura [46], therefore the mechanism of interaction of the skin tissue with these waves may be different. Previous studies by our group have shown that it is possible to delaminate biofilm off surfaces using similar LGS pulses generated by Nd:YAG laser [49]. Based on these encouraging results, we plan to proceed with *in vivo* experiments to carry out delamination of biofilm from wound surfaces using LGS. The most notable advantage of using LGS to delaminate biofilm from wound surfaces compared to methods such as ultrasound is the absence of the deleterious effects of tensile wave components and cavitation bubbles on skin tissue. In addition, given the precision of lasers we can expect to deal with biofilm delamination in a more controlled manner as compared to debridement of the biofilm from the wound which is the current gold standard for treating biofilm infected wounds. It is not clear whether the LGS kill the bacteria embedded in the delaminated biofilm. This question remains to be answered. Further studies with a larger sample size are required to continue this investigation *of ex vivo* samples. In addition, safety investigations into the impact of the shockwaves *in vivo* are currently underway to assess the long-term effects on the collagen structure and the physiological response to LGS.

ACKNOWLEDGEMENTS

We would like to thank Telemedicine and Advanced Technology Research Center (TATRC) for providing funding for this project.

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