Fractional CO_2 Laser Treatment for Vaginal Laxity: A Preclinical Study

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Background and Objective: Various studies have investigated treatment for vaginal laxity with microablative fractional carbon dioxide CO_2 laser in humans; however, this treatment has not yet been studied in an animal model. Herein, we evaluate the therapeutic effects of fractional CO_2 laser for tissue remodeling of vaginal mucosa using a porcine model, with the aim of improving vaginal laxity.
Study Design/Materials and Methods: The fractional CO_2 laser enables minimally invasive and non-incisional procedures. By precisely controlling the laser energy pulses, energy is sent to the vaginal canal and the introitus area to induce thermal denaturation and contraction of collagen. We examined the effects of fractional CO_2 laser on a porcine model *via* clinical observation and ultrasound measurement. Also, thermal lesions were histologically examined *via* hematoxylin–eosin staining, Masson's trichrome staining, and Elastica van Gieson staining and immunohistochemistry.
Results: The three treatment groups, which were determined according to the amount of laser-energy applied (60, 90, and 120 mJ), showed slight thermal denaturation in the vaginal mucosa, but no abnormal reactions, such as excessive hemorrhaging, vesicles, or erythema, were observed. Histologically, we also confirmed that the denatured lamina propria induced by fractional CO_2 laser was dose-dependently increased after laser treatment. The treatment groups also showed an increase in collagen and elastic fibers due to neocollagenesis and angiogenesis, and the vaginal walls became firmer and tighter because of increased capillary and vessel formation. Also, use of the fractional CO_2 laser increased HSP (heat shock protein) 70 and collagen type I synthesis.
Conclusion: Our results show that microablative fractional CO_2 laser can produce remodeling of the vaginal connective tissue without causing damage to surrounding tissue, and the process of mucosa remodeling while under wound dressings enables collagen to increase and the vaginal wall to become thick and tightened. Lasers Surg. Med. © 2018 Wiley Periodicals, Inc.

Key words: fractional CO_2 laser system; vaginal laxity; vaginal tightening; preclinical study

INTRODUCTION

Vaginal laxity is common and may affect sexual function and quality of life [1]. Although reduced sexual sensation is the most common symptom, it is not clear that laxity is directly related to sexual dysfunction. However, vaginal relaxation syndrome (VRS) is a general medical condition characterized by damage to an optimum vaginal structure, and is usually associated with childbirth and natural aging [2]. Frequent pregnancy and labor may deteriorate vaginal structure and VRS may also be associated with decreased hormones, vaginal atrophy, and menopause [3]. Consequently, long-term physical and psychological consequences may drive loss of sexual satisfaction. There is a wide range of treatments for VRS, including invasive surgical procedures, behavior therapy, hormonal therapy, and pharmacotherapy with tightening creams and sprays [4]. Although behavior therapy and pharmacotherapy are safe and noninvasive, their effects are normally limited. On the other hand, surgical procedures are risky but ensure higher satisfaction. Surgical treatments require the vagina and surrounding tissues to be cut and rearranged to reduce the size of the vaginal canal; this process is very risky and can lead to scarring of sensitive vaginal tissues, neural damage, and decreased sensory

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function. Surgery procedures are not risk-free, and are associated with higher pain and a longer recovery period [5]. That is why clinical investigators search for non- or minimally invasive procedures that are safe, effective, and have a short recovery period to treat VRS [6].

Microablative fractional CO_2 laser systems have become popular for use in the dermatology and plastic-surgery fields because they have been scientifically verified to offer efficient, accurate, popular, safe, and effective outcomes [7,8]. They are applicable to a wide range of uses, including treatment for skin lesions, like seborrheic keratosis [9], syringomas [10], and xanthelasmas around the eyes [11], and for warts [12], toenail diseases [13], and anti-aging [14]. Although a multitude of methods have been described for applying CO_2 laser to VRS, the most common surgical technique is a form of tissue remodeling and so called "tightening" within the vaginal mucosa as a result of collagen contraction mediated by the ablative and thermal effects of the CO_2 laser [15]. Contraction of the connective tissues makes the fibrous tissues shorter and thicker, and contraction of collagen and tissues induces collagen remodeling [16], which ultimately contributes to improving vaginal laxity and reducing VRS.

Nevertheless, the therapeutic effect of microablative fractional CO_2 laser is still undefined and there are limits to how well it can be evaluated in human studies. This preclinical study provides new data on the safety and efficacy of microablative fractional CO_2 laser, as used in a porcine model.

MATERIALS AND METHODS

Animals

The test was conducted after obtaining the approval of the Institutional Animal Care and Use Committee at MediKinetics Co., Ltd., Pyeong-Taek, Korea (MK-IA-CUC:160512-001). Micro-pigs® weighing 70–80 kg with childbirth experience (as sow) were bought from MediKinetics and raised under SPF conditions in an optimal environmental (temperature: $22 \pm 3°C$, humidity: $50 \pm 10\%$, cleanliness: 1,500, ventilation: 15T/HR, internal pressure: 3 mmHg, air velocity: 5 cm/sec, and noise: 10 dB). Pigs were determined to be the most suitable model for this study because a pig's skin is similar to that of human skin with regard to absorption force, allergic reaction, and epithelial structure, and because the organ sizes in humans and pigs are very similar. Importantly, adult sows with pregnancy and childbirth experience were selected to be compared with humans who had undergone similar conditions. Sows with a healthy appearance were randomly allocated into four groups with two sows per group. The groups were as follows: Group 1 = non-treated, Group 2 = 60 mJ pulse energy, Group 3 = 90 mJ pulse energy, and Group 4 = 120 mJ pulse energy. Two sows from each group were sacrificed 30 days after the procedure, and their tissues were collected and fixed.

Laser Treatment

Before the experiment, each animal was checked for weight and other abnormal conditions. A 3 milliliter-solution of Zoletil 50 (tiletamine hydrochloride + zolazepam hydrochloride, Virbac, Carros, France) and Rompun (xylazine hydrochloride, Bayer, Leverkusen, Germany) was mixed at a 6:4 ratio and was injected into each animal's muscle for pre-anesthesia. The animals were then moved to the operating room. A proportionally mixed solution of 2 ml was injected intravenously to the animals on the operating table. For respiratory anesthesia, a laryngoscope was used to maintain airway patency and an 8.5 intubation tube was inserted. Next, a 2:1 gas mixture of a Terel solution (isoflurane, Piramal Critical Care, Inc., Bethlehem, PA) and oxygen was injected.

FRX-CO_2 GynoLaser™ (Fraxis™, Ilooda, Inc., Suwon, Korea) is a fractional CO_2 laser system with a handpiece and a 360° rotating scanner to gradually radiate ultra-fine micro-unit laser beams into tissues using a 10,600 nm wavelength. The treatment area was dressed so as to wipe off the remaining moisture on the area to be treated with the FRX-CO_2 GynoLaser™. With the handpiece rotated 360°, the laser radiated from the uterocervical canal up to the vaginal opening while moving back with 1 cm steps.

Photography and Ultrasound Measurement

To assess the vaginal mucosa irritation caused by laser treatment with FRX-CO_2 GynoLaser™, a dermatologist visually inspected the area immediately after the treatment and 30 days later. To assess vaginal tightening, a finger breadth (FB) test was conducted on the vaginal opening before the treatment and 30 days later, and the Acuson P300 ultrasound device was used to photograph and measure the inside diameter of the vagina, at 1 cm back from the uterocervical canal. To observe how the FRX-CO_2 GynoLaser™ laser penetrated the vaginal mucosa (coagulation points), a magnified imaging device (Folliscope®, LeadM Corp., Seoul, Korea) was used to take enlarged photographs. To assess how the coagulation areas recovered from thermal denaturation, the area was assessed immediately after treatment and 30 days later.

Histopathologic Examination

A histopathologic assessment of the treatment by microablative fractional CO_2 laser for the vaginal mucosa was conducted. A 6-mm punch biopsy was performed on the vaginal tissues immediately after treatment and 30 days later, and the tissues were fixed in a 10% neutral buffered formalin solution and solidified through paraffin embedding. Next, and 5-μm-thick sections were made and stained for photographing with an optical microscope, using three staining procedures: H&E (Hematoxylin and Eosin), MT (Masson's trichrome), and EvG (Elastic-van Gieson). Next, we checked how the collagen and elastic fibers on the lamina propria and the fibromuscular wall had increased or decreased and how they were distributed.

For immunohistochemistry, paraffin sections were incubated overnight at 4°C with a rabbit polyclonal Collagen I alpha 1 antibody (1/100; NB600-408, Novus biological, Littleton, CO) and mouse monoclonal HSP70 antibody (1/100; SC-66048, Santa Cruz Biotechnology, Santa Cruz, CA). Then biotinylated secondary antibodies and horse-radish peroxidase conjugated with streptavidin (DakoCytomation, Glostrup, Denmark) were added to the solution. Visualization was obtained with an AEC (3-amino-9-ethylcarbazole)+ chromogen substrate (Dako, Carpinteria, CA). A camera (DP 70®; Olympus BIOSCOPS, Central Valley, PA) connected to a microscope (BX51®) was used to capture the histologic images. After staining, three high-power fields (×400) were randomly selected in each slide, and the average proportion of positive cells in each field was counted using the true color multi-functional cell image analysis management system (Image-Pro Plus; Media Cybernetics, Bethesda, MD).

Statistical Analyses

All experiments were performed at least three times and data are reported as means ± SE. Statistical comparisons between the laser-treated groups involved one-way ANOVAs followed by Scheffe's post-hoc test using SPSS software (SPSS Inc., Chicago, IL). We considered P-values <0.05 statistically significant. Significance levels are designated as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

RESULTS

Clinical and Dermoscopic Changes

Minimally ablative fractional laser devices have gained acceptance as a preferred method for skin resurfacing. Considering that the device radiates laser energy directly into the tissue and generates heat, it is essential to evaluate the effects of wavelengths on biological tissues, to verify the accuracy of targeting focal points for treatment, and to assess the device's overall safety and performance. To assess how the extent to which laser treatment with microablative fractional CO_2 laser irradiates the vaginal mucosa, a dermatologist visually inspected the test area immediately after treatment and 30 days later. Immediately after treatment, a slight thermal denaturation due to the laser treatment was observed in the vaginal mucosa, but no abnormal reactions, such as excessive hemorrhaging, vesicles, or erythema, were observed. We macroscopically confirmed that the fractional CO_2 laser-induced thermal effect significantly increased after laser treatment with 60, 90, and 120 mJ. Also, immediately after fractional CO_2 laser treatment we observed that 120 mJ, which received higher fluence and energy, ablated significantly more than 60 and 90 mJ. On the 30 day post-treatment, the vaginal mucosa had recovered from the slight thermal denaturation (Fig. 1). Finally, we revealed that fractional CO_2 laser systems have shown vaginal tightning with threshold energy exposures generating energy values of 60–120 mJ.

Histological Changes

A histopathologic assessment of the microablative fractional CO_2 laser treatment to the vaginal mucosa was conducted. The thermal energy generated by laser penetration denaturalized and contracted collagen and induced neocollagenesis. According to H&E staining, the epithelium became thicker in all of the treatment groups except the non-treatment group by day 30, compared with the epithelium immediately after treatment, and these changes affected the entire mucosal architecture of the biopsy area. Immediately after fractional CO_2 laser, for all exposure energy (60, 90, and 120 mJ) epithelial and connective stroma proteins showed coagulation, with the stratum corneum layer being damage in the majority of cases. The zone of laser damage could be seen in histological specimens. In particular, these results confirmed that mild ablation and thermal damage observed after treatment (60 mJ) (Fig. 2A). Penetration depth and width appeared to be correlated with energy, and the penetration depth was measured (Fig. 2B). Vaginal rejuvenation performed with this technique is a minimally invasive procedure that stimulates internal tissues of the female lower genital tract to regenerate the mucosa, improving tissue remodeling. The results of MT (Masson's trichrome) and EvG (Elastic-van Gieson) staining showed an increase in collagen and elastic fibers on the lamina propria, as well as neovascularization due to increased contents of blood capillaries and vessels (Fig. 3A and B). In this study, these results showed that tissue regeneration observed by ablative treatment of all exposure energy (60, 90, and 120 mJ).

Increased Collagen Type I and HSP70 Expression by Fractional CO_2 Laser

To evaluate the mechanism underlying the activation of collagen synthesis in this model, we performed immunohistochemistry on the sow skin specimens using specific antibodies against collagen I and HSP70. Immunohistochemistry studies showed that all of the treatment groups had markedly increased collagen type I and HSP70 levels compared with the non-treatment group. By day 30 of the experiment, the optical density of Collagen I and HSP70 labeled areas was greater than that in the non-treatment groups (Fig. 4A and B). In the treatment groups, some HSP70-intensely positive cells were found in the epidermal layer, some were found in the upper part of the connective tissue papillae, and others were found in the deeper area of coagulation zone. Previous studies have revealed that HSP70 is an important factor for protein folding and play a protective role against harmful factors such as laser induction [17]. Furthermore, these results suggested that fractional photothermolysis treatment promotes a wound-healing process characterized by acute induction of an inflammatory reaction, followed by matrix remodeling as verified by new collagen synthesis.

Assessment Methods for Vaginal Tightening

Photographs from the ultrasound device showed a decrease in the inside diameters of the vagina from before the laser treatment to day 30 (Fig. 5). To assess vaginal tightening, a finger breadth (FB) test was conducted on the vaginal opening. No changes in FB values were observed from immediately before the laser treatment to 30 days after (data not shown). The reason for such a decrease is

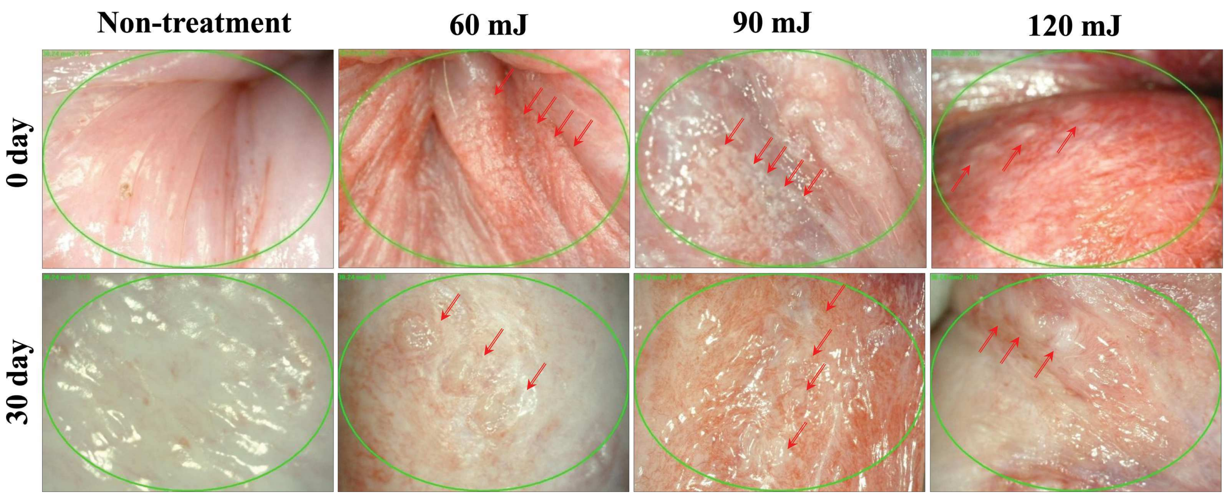

Fig. 1. Microscopic view of the surface of the vaginal mucosa immediately after treatment with the fractional CO_2 laser system: The Folliscope® (Original Magnification, ×15) was used to determine morphological changes in the mucosal surface. Red arrows indicate macroscopic ablation zones (immediately after: laser irradiation effect on the surface of vaginal mucosa; day 30: restored vaginal mucosa).

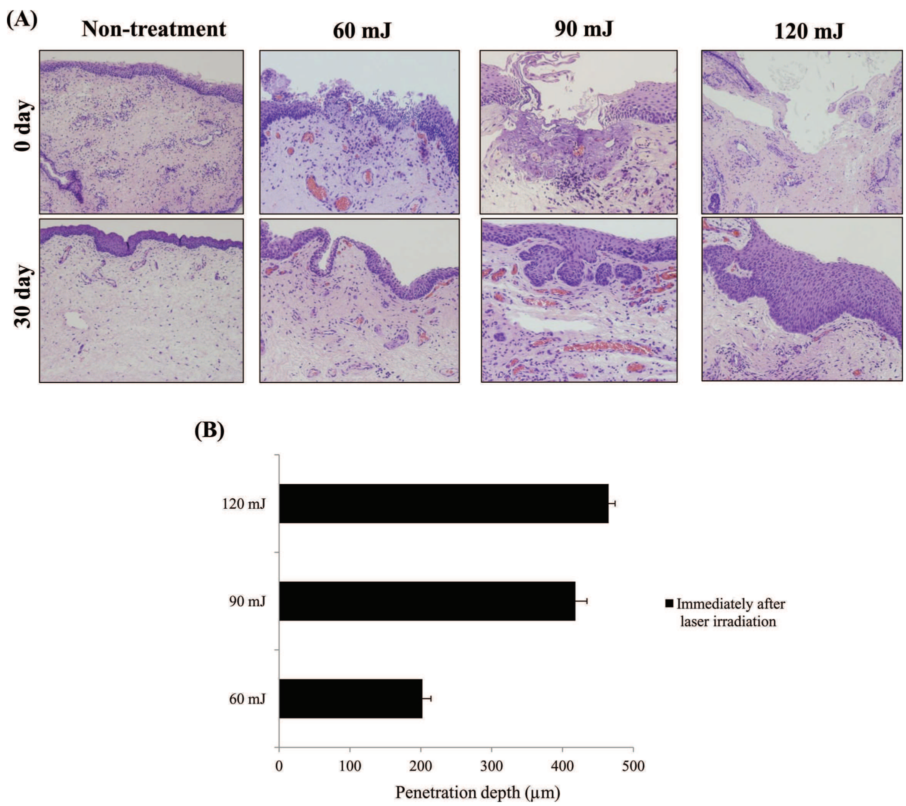

Fig. 2. Histologic changes after treatment with the fractional CO_2 laser system: (**A**) Hematoxylin and eosin staining of sow vaginal mucosa before and after treatment. Fractional CO_2 laser produces hyperthermia in the epithelium and the lamina propria. (**B**) Penetration depth was measured after the laser application. Data are expressed as means ± standard deviations of triplicate samples. Bars are representative of five individuals. Original magnification, ×100.

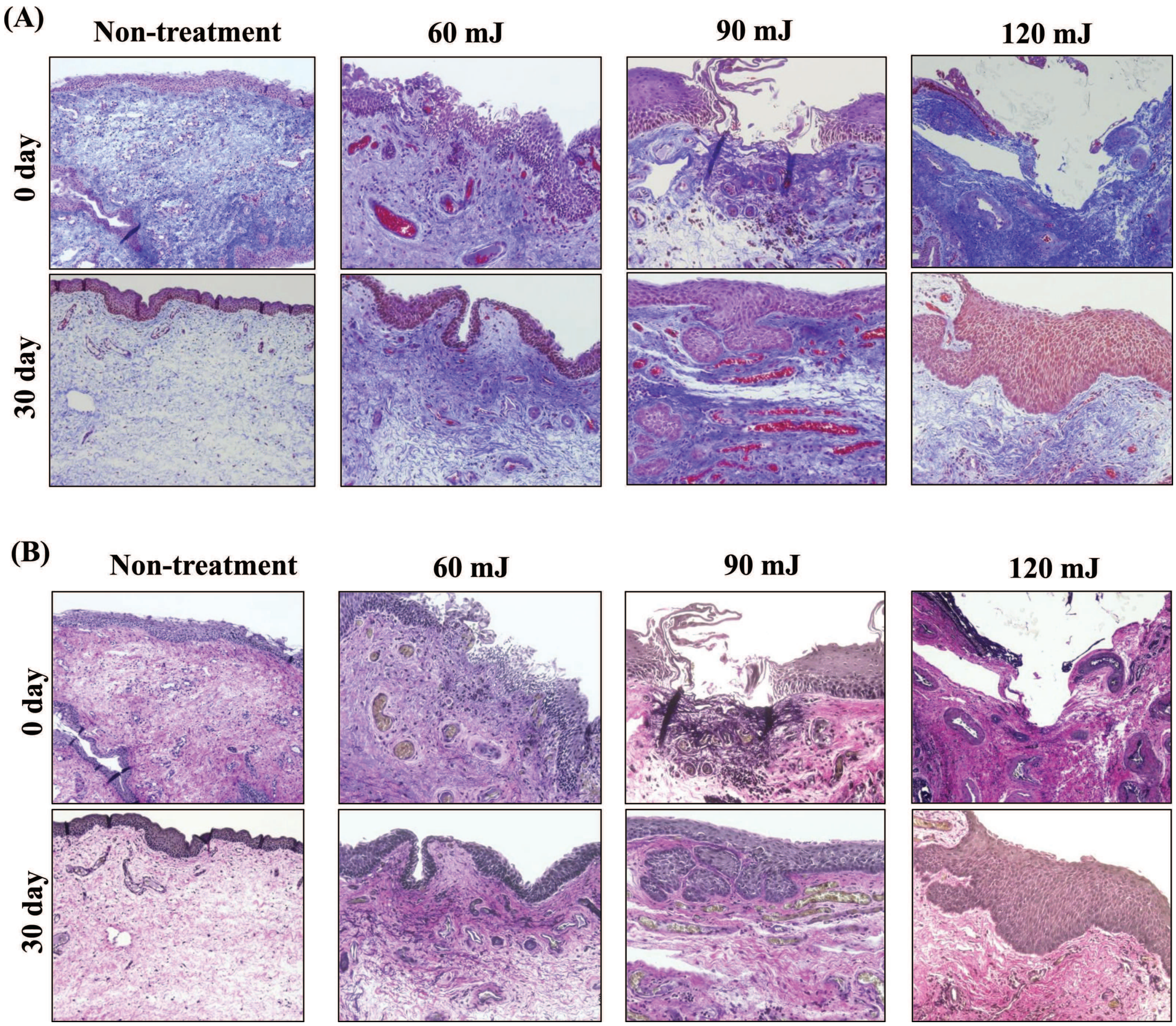

Fig. 3. Histological changes after treatment with the fractional CO_2 laser system in sow vaginal mucosa: (**A**) MT (Masson's trichrome), (**B**) EvG (Elastic-van Gieson) staining, Microscope (OLYMPUSIX71, Tokyo, Japan). MT (Masson's trichrome: collagen, blue; basement membrane and glomerular tuft, blue; nuclei, black–blue; cytoplasm and keratin, red), EvG (Elastic-van Gieson: collagen, rose pink; stratified epithelium, yellow; RBC inside vessels, yellow; nuclei, dark brown). Images are representative of five individuals. Original magnification, ×100.

not because of any change in the vaginal opening 30 days later but rather because treatment with microablative fractional CO_2 laser reduced the inside diameter of the vagina.

DISCUSSION

Vaginal rejuvenation has become a commonly used, yet poorly evidenced, intervention offered by surgeons, often driven by a high demand from patients requesting either vaginal tightening or management of sexual dysfunction [18]. Although various methods have been described for the application of microablative fractional CO_2 laser, the most common surgical technique is a form of tissue remodeling [19]. There are many approaches to treating vaginal laxity and vaginal atrophy, ranging from conservative methods to surgical interventions; however, most treatment options present critical limitations, including safety concerns and clinical question-based assessment methods that are not very robust [20]. Therefore, this paper provides a new model to evaluate quality of care in gynecology surgery, particularly vaginal laxity. Because vaginal laxity was the condition being evaluated, adult sows with pregnancy and childbirth experience were chosen as a model.

A 10,600-nm wavelength was strongly absorbed by the mucosal tissues and minor non-specific thermal damage was associated with a particular profile pulse [21]. This CO_2 laser system was developed on skin to achieve good clinical results from microablative fractional resurfacing [22]. This device can produce microablation of the

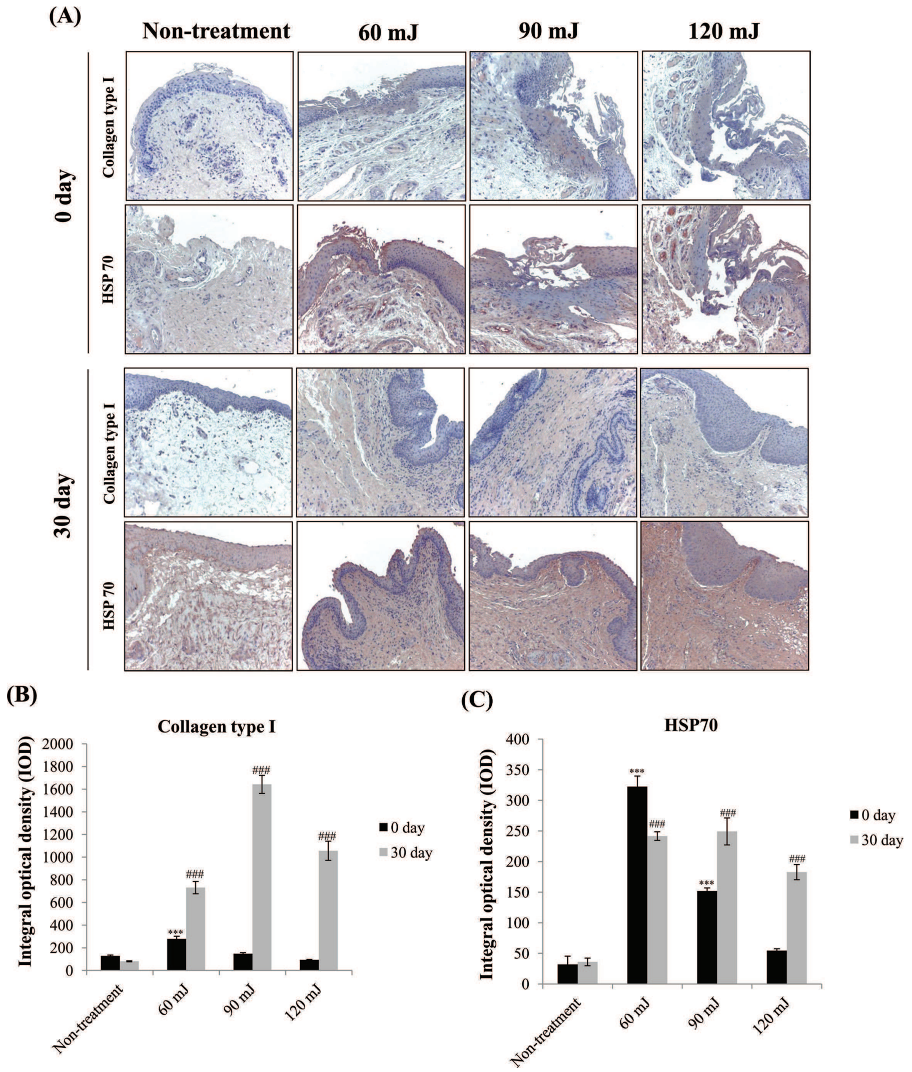

Fig. 4. Immunohistochemical staining for collagen type I and HSP70-positive cells in sow vaginal mucosa. Skin biopsy samples were harvested immediately after (day 0) and day 30. (**A**) Collagen type I- and HSP70-positive areas were stained with AEC+ chromogen substrate and hematoxylin counterstained to visualize nuclei. Bar graph of the values of the integrated optical density of collagen type I (**B**) and HSP70 (**C**) labeled areas. Results are expressed as the mean ± SEM. $^{***}P < 0.001$ as compared to the non-treatment group in day 0 and $^{\#\#\#}P < 0.001$ as compared to the non-treatment group in day 30. Images are representative of five individuals. Original magnification, ×100.

epidermis and thermal effects to the dermis are observable, although only microscopically. In the present paper, visual and instrumental assessments and histopathologic assessment were carried out immediately after treatment and 30 days later. Treatment with a microablative fractional CO_2 laser caused slight thermal denaturation in the vaginal mucosa but no abnormal reactions, such as excessive hemorrhaging, vesicles, or erythema. The efficacy of the microablative fractional resurfacing on the depth of the injury; deeper zones of thermal damage result in greater impact on clinical efficacy. Therefore, our data suggest that fractional CO_2 laser pulse energy between 60 and 120 mJ can be effective, but shorter energy pulse (>60 mJ) do not appreciably damage the vaginal wall. Therefore, this study provides data on the efficacy, safety, and performance of microablative fractional CO_2 laser for obstetric and gynecologic applications, including vaginal laxity and VRS, in sows.

Moreover, treatment with microablative fractional CO_2 laser caused thermal denaturation and collagen contraction of the vaginal canal, thus inducing collagen remodeling and neocollagenesis and increasing collagen and elastic fibers, which finally contributed to firming and tightening of the vaginal wall [23]. Fractional CO_2 lasers supply energy to the vaginal wall to superficially ablate the epithelial component (especially primary effects on epidermal keratinocytes and dermal fibroblasts) of atrophic mucosa, finally triggering the synthesis of new collagen

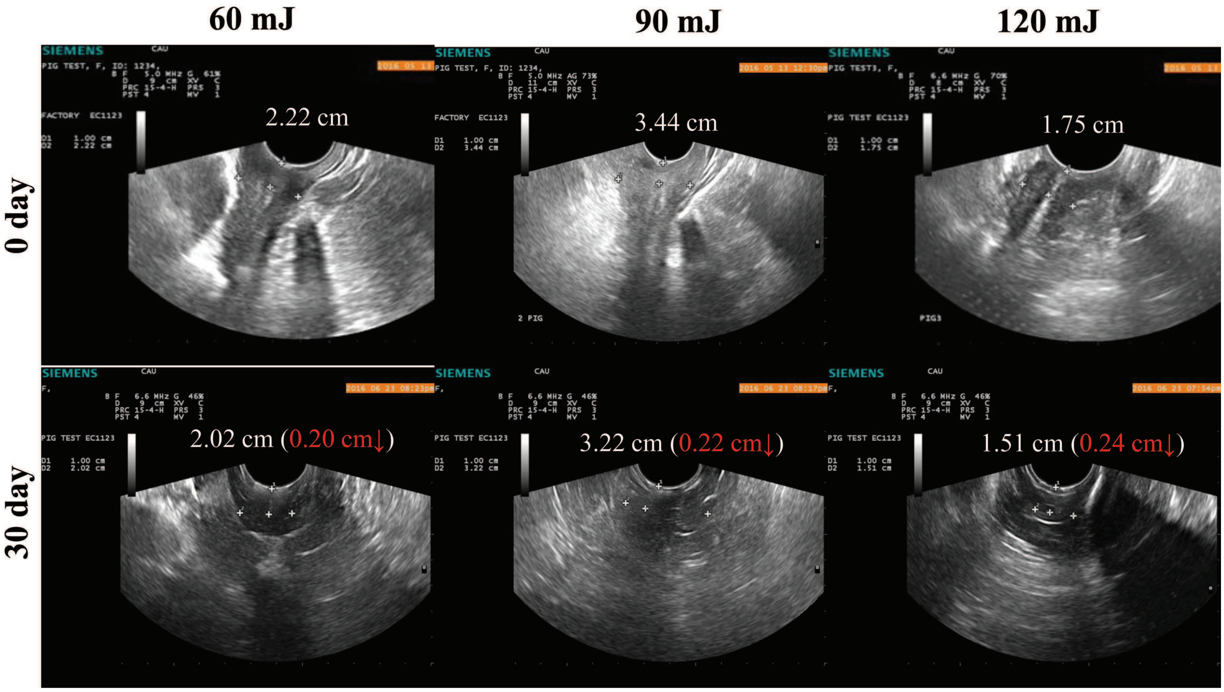

Fig. 5. Assessment methods for vaginal tightening in sow model. Ultrasound measurement. The inside diameter of the vagina, which is 1 cm back from the uterocervical canal, was photographed and measured with an ultrasound device (Acuson P300, Siemens Healthcare, Forchheim, Germany).

formation. Recent studies have clearly demonstrated that HSP70, which is over-expressed following laser irradiation, could play a role with a coordinated expression of other growth factors [24]. Herein, we have shown increased vaginal tightening after nonsurgical microablative fractional CO_2 laser treatment for vaginal laxity and confirmed that the denatured lamina propria induced by microablative fractional CO_2 laser was dose-dependently increased after laser treatment at 60, 90, and 120 mJ. Based on these results, we can establish and apply safe energy results at the clinical stage. Further studies with longer follow-up periods are required to evaluate the long-term results for vaginal tightening. Moreover, a limitation of our study is the failure to conduct surveys on individual satisfaction including patient assessment of postprocedural sexual satisfaction.

Collectively, our data suggest that microablative fractional CO_2 laser treatment is safe and effective for improving vaginal tightness. Based on our data, we suggest for the first time that microablative fractional CO_2 laser can increase vaginal tightening with consequent renewal of the vaginal mucosa in an animal model.

Recently diverse trials including radiofrequency and fractional laser irradiation have been studied related with vaginal tightening and orgasmic dysfunction [25–28]. When compared with classical surgical approaches these relatively non-invasive or semi-invasive technique successfully proved their efficacy and safety for vaginal tightening and orgasmic dysfunction [25–28]. We believe that convergence of new technology including laser, radiofrequency, and HIFU may open new era of external genital skin rejuvenation in a near future.

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