의학박사 학위논문

Effect of platelet-rich plasma combined with fractional CO_2 laser treatment for acne scars

여드름 흉터 치료에서 탄산가스 프락셀 레이저와 병합한 platelet-rich plasma 의 효과

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Effect of platelet-rich plasma combined with fractional CO_2 laser treatment for acne scars

by

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A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medical Science (Dermatology) at Seoul National University College of Medicine

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Abstract

Atrophic acne scars result from dermal inflammation caused by acne. Accordingly, destruction of dermal tissue followed by imperfect wound healing results in prolonged periods of time during which acne scars remain noticeable. Laser-mediated skin resurfacing induces regeneration and remodeling of dermal matrix, and thereby lessens the severity of acne scar. While fractional laser treatment reduces the chances of a patient experiencing adverse effects related to treatment, the resulting efficacy is also reduced compared to that obtained with classical laser therapy. Platelet-rich plasma (PRP) contains large amounts of growth factors which have the potential to enhance therapeutic efficacy of laser treatment. Furthermore, the rapid skin barrier recovery induced by PRP can reduce the chances of a patient experiencing certain adverse effects associated with laser treatment.

The present study was conducted to evaluate the efficacy of PRP injections combined with fractional laser treatment for treating acne scars. Moreover, the underlying mechanism of this therapy was investigated using samples of human skin and an experimental cell model.

Twenty-five subjects with mild to moderate acne scars were treated with two sessions of fractional CO_2 laser therapy given with and without co-administration of PRP at a four-week interval in a split-face manner. Normal saline was injected intradermally on the control side of each patient's face. Objective and subjective assessments of treatment

results were conducted at baseline, 1, 3, 7 days after each treatment, and 4 weeks after the final treatment. Skin biopsy specimens were obtained at baseline, 1, 3, and 7 days after the first treatment session for investigation of molecular profiles associated with acute skin changes produced by laser plus PRP treatment. Additionally, the biological effects of combined fractional CO_2 laser/PRP on fibroblast were explored in a fibroblast cell line model. The time-dependent expression of collagen and various growth factors was evaluated by western blot.

Fractional CO_2 laser treatment produced a significant improvement in acne scars; however, improvement on the PRP-treated side of the patient's face was better than that on the control side. Evaluations based on the Investigators' Global Assessment showed average improvements of 75% and 50% on the PRP-treated side and control side, respectively. Adverse effects such as erythema, swelling and oozing occurred with limited severity on the PRP-treated side. Scores on patient self-assessments of their satisfaction with treatment effectiveness were higher for the PRP plus laser treated side of their face. Patient skin biopsy specimens showed reactions to laser treatment. Compared with specimens of control skin, biopsy specimens of combined laser/PRP treated skin showed more compact and denser depositions of collagen in the papillary dermis. Expressions of TGFβ1 and TGFβ3 proteins as well as transcription of TGFβ 1, TGFβ 3 and collagen I mRNA were more highly elevated on

the PRP-treated side of the face compared to the control side. Mediators such as β-catenin and αSMA, which are associated with the TGFβ fibrogenetic signaling pathway, showed greater upregulation on the PRP-treated side compared to the control side. In a cell model, PRP administration increased expressions of p-Akt, TGFβ1, TGFβ3, β-catenin, collagen I and collagen III in both dose-dependent and time dependent manners. Inhibition of p-Akt by pretreatment of the PI3K/Akt pathway did not limit expression of TGFβ1, but limited expression of β-catenin.

Administration of PRP combination increased the efficacy of CO_2 laser for treating acne scars and decreased the incidence of adverse effects. Elevated levels of TGFβ1 and TGFβ3 after combined fractional CO_2 laser/PRP therapy may be a mechanism for the improvement in acne scars. Our clinical data combined with the data from cell experiments suggest that TGFβ induced by PRP treatment resulted in increased expression of αSMA in fibroblasts and collagen production via activating β-catenin through stimulation of p-Akt. This molecular signaling pathway is suggested as a mechanism for the clinical efficacy of combined fractional CO_2 laser/PRP treatment.

Keywords: acne scar, fractional CO_2 laser, platelet-rich plasma, TGFβ, β-catenin, collagen

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Introduction

Scaring as a result of acne has been generally reported to occur in 0.17 to 14% of acne cases; however, another study reported scaring in 95% of cases (1-3). Acne scar can produce profound psychological disfigurement resulting in impaired self-esteem and social interactions (4), as well as difficulty in obtaining employment (5). As a result of such psychological insults, there is an increasing need to improve the appearance of scars and reduce their occurrence.

Atrophic acne scars occur as a result of severe acne inflammation, and are best treated by preventive methods of acne control. However, such preventive methods have limitations resulting from their delayed implementation and the low therapeutic efficacy of medications used for treating severely inflamed acne.

Unlike other ordinary scars created by a wound trying to heal itself and the resulting overproduction of collagen, atrophic acne scars are produced by destruction of the dermal matrix, followed by imperfect matrix synthesis or repair (6). Therefore, most acne scar treatments work by inducing regeneration and remodeling of the dermal matrix.

Acne scars are very resistant to most treatment modalities, and numerous clinical studies have been conducted with the goal of finding new treatments and increasing the effectiveness of existing therapies. The treatment of acne scars was revolutionized by the development of

laser therapy, and laser-tissue interactions can be explained by selective thermolysis. Furthermore, laser pulses of shorter duration are now used to deliver energy sufficient to induce the desired degree of thermal injury without severely damaging surrounding tissue.

Until now, ablative laser resurfacing has been regarded as the gold standard treating atrophic acne scars. However, resurfacing provided by conventional ablative laser treatment produces additional cutaneous maladies such as hypertrophic scarring or permanent hyperpigmentation. Fractional laser therapy was developed to reduce these adverse effects. Although the remaining viable epidermis often accelerates postoperative recovery, such therapeutic effects are often limited. While high-energy laser treatment can increase therapeutic efficacy, it can also increase the chance that a patient will experience adverse side effects (7).

Platelet-rich plasma (PRP) is generated from autologous blood, and consists of highly concentrated platelets which contain large amounts of various growth factors and cytokines which play key roles in tissue repair (8). Additionally, platelets can also induce the synthesis of various growth factors which promote tissue regeneration (9, 10). A study which used PRP to treat equine leg wounds revealed the superior wound healing effect of PRP compared to that of a control substance (11). PRP was also demonstrated to accelerate epithelization and reduce inflammation after wounding in rabbits (12), and has been suggested as a supplement to diabetic foot surgery due

to its regenerative capabilities (13).

In addition to its use in problematic wounds, autologous PRP has been increasingly used in cosmetic surgery, spinal surgery, and treatment of bone defects (9, 14-16). With regards to the skin, PRP has been reported to induce rapid skin barrier recovery and reduce erythema after carbon dioxide laser therapy (17). Such reports suggest PRP's potential for enhancing the efficacy and reducing the adverse effects associated with fractional laser therapy used for treating acne scars.

Objectives

We conducted the current study to evaluate the efficacy of PRP combined with high-fluence, fractional CO_2 laser therapy in treatment of acne scars. Additionally, the mechanism of this combined treatment was investigated using human skin samples and by experiments conducted with cultured fibroblasts.

MATERIALS AND METHODS

Ethics statement

This study protocol was approved by the Institutional Review Board of the Seoul National University Hospital (No. H-1206-005-412). All experimental procedures including clinical trials and investigation using human samples were conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all subjects prior to enrollment.

Clinical study design and subjects

We conducted this 12-week, prospective, single-blind, comparative (randomized split-face) clinical trial at the Department of Dermatology, Seoul National University Hospital, during October 2012-May 2013. A total of 25 subjects with Fitzpatrick skin types III-IV and moderate-to-severe acne scars on both sides of the face were enrolled in the study. The subjects did not receive retinoids or other acne scar treatments during the study period.

Devices and laser treatment techniques

To ensure consistency between treatment sessions, all laser procedures were conducted by the same surgeon. Following a 30-minute treatment with a topical anesthetic, the whole face of each subject was irradiated with a fractional CO_2 laser (COFRAX®, AMT

Engineering Co, Seongnam, Korea). After each laser treatment, one half of the subject's face was injected intradermally with platelet-rich plasma (PRP), and the other half of the face was injected with normal saline (NS) spaced at 1 to 1.5 cm intervals. Each sites received about 0.02 ml of PRP or NS. A random computer-generated allocation method was used to assign the treatment modality. Fractional CO_2 10600-nm laser irradiation was applied at a fluence of 30-70 mJ/cm^2 with a 150 microthermal treatment zone (MTZ) and 12 mm spot size via a 1 ms pulse duration in a non-overlapping manner, and using a single pass. The laser irradiation induced a clean, pink hue on the skin, with minimal bleeding and edema. Volunteers were subjected to two identical sessions of treatment, with a 4-week interval between treatments. Each patient was instructed to apply a mupirocin ointment to the face several times a day until the crust had completely fallen off.

Preparation of PRP

PRP for treating acne scars was prepared from the autologous blood of each Individual subject. A sample of venous blood (10 mL) was drawn from the antecubital vein with a syringe prefilled with 1.5 mL of anticoagulant agent (citrate dextrose solution formula A, Baxter, Deerfiled, IL, USA), and then centrifuged at 160g for 10 minutes. After one centrifugation, the supernatant portion was collected and centrifuged at 400g for 10 minutes. The PRP fraction was made from the resulting pellet mixed with 1.5 mL of supernatant. Platelets in the

PRP preparation were activated by addition of 1 mL of 3% calcium chloride. Platelet depleted plasma (PDP) was prepared from the supernatant without addition of the pellet.

Assessments of clinical outcome

Subjects were followed-up on days 1, 3, and 7 after each session and at 1 and 2 months after the final session. Digital photographs were taken at each follow-up visit using identical lighting conditions and camera settings (EOS 600D®, Canon, Tokyo, Japan). Random assignment codes were secured until all data entry was completed. Efficacy was assessed using the 5-point Investigator's Global Assessment (IGA; Table 1), Echelle d'évaluation clinique des Cicatrices d'acné (ECCA) scores, and a subtype (icepick, boxcar, rolling scar) analysis (18). ECCA grading scales are a semi-quantitative tool used for evaluating acne scars, and then to calculate a weighted value according to six types: V-shaped, U-shaped atrophic, M-shaped atrophic, hypertrophic inflammatory, keloid scars, and superficial elastolysis. The response of the skin to treatment was evaluated using a six-point epithelization scale (Table 2), and degrees of erythema were measured using two photometric devices (Spectrophotometer CM-2002®, Konica Minolta, Tokyo, Japan; Derma-spectrometer®, Cortex Technology, Hadsund, Denmark). Each patient provided a subjective treatment satisfaction score, a pain score, convenience score, and a therapeutic effectiveness score starting on

day 1, and then on a continual basis throughout the study.

Histopathology and immunohistochemistry

Skin biopsy specimens (2 mm) were obtained on days 0, 1, 3, and 7 after the first treatment session. Sections were stained with hematoxylin-eosin (H&E), Masson's trichrome (MT), and Herovici stains. Samples were processed for immunohistochemical staining for transforming growth factors TGFβ1, TGFβ2, and TGFβ3, as well as collagen I, collagen III (Abcam, Cambridge, MA, USA), MMP2, β-catenin (Cell Signaling Technology, Beverly, MA, USA) and α-smooth muscle actin (SMA) (DAKO, Carpinteria, CA, USA). The intensity of immunohistochemical staining was evaluated using an image analysis program (Leica QWin version 3.5.1, Leica Microsystems, Wetzlar, Germany).

Quantitative PCR amplification

Punch biopsy specimens of skin were frozen at days 0, 1, 3, and 7 after the first treatment session. RNA isolation and cDNA synthesis were conducted using published methods (19). cDNA (1 ug) was amplified by real-time PCR. Primer sequences used for TGFβ1, TGFβ3, collagen 1, and GAPDH are shown in Table 1, and were based on the manual used for the SYBR Green/ROX q-PCR kit (Fermentas, Vilinius, Lithuania). Quantitative estimations of mRNA expression were obtained by polymerase chain reactions (PCR) performed using a

7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). All experiments using SYBR Green were performed in triplicate.

Cell culture

Human dermal fibroblast cells (CCD-986Sk) were purchased from the Korean cell line bank, and cultured in DMEM supplemented with glutamine (2 mM), penicillin (400 U/mL), streptomycin (50 mg/mL), and 10% FBS in a humidified 5% CO_2 atmosphere at 37°C. Fibroblasts at 20 passages were cultured to 80% confluence and then starved in DMEM containing 0% FBS for 24 hours prior to intervention.

Cell treatment with PRP and laser irradiation

Fibroblast cells were cultured in several 30-ϕ dishes, after which, six dishes were prepared as a set for later comparisons. After removing the culture media, five of the six dishes were irradiated with a fractional CO_2 laser at a fluence of 10 mJ/cm^2 in 150 MTZs. The dose of laser irradiation was selected based on results in a previous pilot study. Doses of laser irradiation were given starting with a fluence of 60 mJ/cm^2, and continued in a descending manner. The fluence of laser irradiation was selected to permit an evaluation of the effects of combined laser/PRP treatment. Following laser irradiation, a culture medium without FBS, PRP (5% and 20%), and PDP (5% and 20%) was added, and then replaced with a medium lacking only FBS 30

minutes later. Molecular changes in the cultured cells over time were investigated in 3 sets of cells at 30 min, 254 minutes, and 48 hours, respectively. The effect of Akt inhibition was explored in cells pretreated with 50 μM LY294002 (a cell permeable PI3-K inhibitor) for 30 minutes prior to laser irradiation. This concentration of LY294002 has been commonly used in other cell systems. All experiments were conducted in triplicate.

Western blot

To determine the amounts of collagen I and collagen III secreted into culture media, equal aliquots of conditioned culture media obtained from cultures with equal numbers of cells (8×10^4 cells/cc) were fractionated. To investigate changes in expression of cell signaling proteins, total protein was extracted using a cell lysis buffer (Cell Signaling), and the total protein amounts in lysates were determined using the BCA Protein Assay (Pierce, Rockford, IL, USA). Equal amounts of protein were separated on 10% SDS-PAGE gels, and then transferred to a polyvinylidene difluoride membrane. The blots were primarily probed with t-Akt, Phospho-Akt (Ser473), MMP2, and β-catenin rabbit antibodies (Cell Signaling), β-actin mouse antibody, collagen I and EGFR rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), collagen III, TGFβ1, and TGFβ3 rabbit antibodies (Abcam), αSMA mouse antibody (DAKO), and IL-1β mouse antibody (R&D Systems™; Minneapolis, MN, USA). Secondary anti-

rabbit IgG and anti-mouse IgG antibodies (Cell Signaling) were used to detect primary antibodies. Blots were developed with EzWestLumi (ATTO) and exposed to the Kodak x-ray film. Films of blots were analyzed and quantified using NIHs ImageJ software (Version 1.48).

Statistical analysis

The paired *t*-test and Wilcoxon signed-rank test were used to compare differences between treatment modalities. In vitro data were analyzed with the Mann-Whitney test. All analyses were conducted using SPSS for Windows, Version 12.0 (SPSS Inc, Chicago, IL, USA). *P*-values < 0.05 were considered statistically significant.

Table 1. Investigator’s global assessment

Grade	Degree of improvement
0	No improvement
1	0-25%
2	25-50%
3	50-75%
4	75-100%

Table 2. Epithelization Scale

	0	1	2	3	4
Erythema	Normal	Pink	Bright red	Dark red	Purple
Oozing	None	Slight	Moderate	Marked	Very marked
Swelling	None	Slight	Moderate	Marked	Very marked

Epithelization Scale is a total sum of the individual score of erythema, oozing and swelling.

Table 3. Real time PCR primers

Gene Target	Forward Primer	Reverse Primer
GAPDH	CCG TCT AGA AAA ACC TGC C	GCC AAA TTC GTT GTC ATA CC
TGFβ1	CCC AGC ATC TGC AAA GCT C	GTC AAT GTA CAG CTG CCG CA
TGFβ3	GCG GAG CAC AAC GAA CTG	CTG CTC ATT CCG CTT AGA G
collagen1	CTC CCC AGC TGT CT ATG GC	CAC CAT CAT TTC CAC GAG CA

Results

Clinical trial

Of the 27 subjects who were initially enrolled in the study, 25 subjects (mean age 31.9 years; range, 20~34) completed the study, and two subjects dropped out for personal reasons. No patients were lost to the study due to serious adverse effects.

Scar improvement

The grade of acne scarring improved on the treated side of the face (laser treatment plus PRP) in all patients. No patient had a worse acne score after receiving either type of treatment (combined laser/PRP treatment or combined laser/NS treatment). An evaluation of inter-rater agreement by kappa statistics showed high congruence between the two independent raters (kappa value = 0.7, $P < 0.001$). The mean IGA scores indicated that combined fractional CO_2 laser/PRP treatment and fractional CO_2 laser/NS treatment resulted in ~ 75% and 50% improvements in acne scaring, respectively ($P < 0.001$) (Figure 1a). Additionally, ECCA scores indicated a significant improvement following treatment with either regimen (combined factional CO_2 laser/PRP, $P < 0.001$; combined fractional CO_2 laser/NS, $P < 0.001$) (Figure 1b). However, treatment with combined fractional CO_2 laser/PRP was superior to treatment with combined fractional CO_2

laser/NS for improving all scar subtypes (Figure 1c). Clinical photographs also showed greater improvement of acne scars on the side treated by combined fractional CO_2 laser/PRP therapy (Figure 2).

Epithelization and adverse effects

Treatment with the combined fractional CO_2 laser/PRP modality produced less erythema than treatment with the laser plus NS (Figure 2). Additionally, skin recovery rates after treatment as assessed using the epithelization scale showed a significant difference between the two treatment modalities on day 1 (P = 0.01, Figure 3a). PRP treatment effectively limited swelling due to laser treatment. The swelling began to decrease starting on day 1, and continued to decrease throughout the study. However, swelling on the NS-treated side increased after each treatment session, and lasted for > 7 days. Both the erythema index (Figure 3b) and colorimetric measurements (Figure 3c) revealed consistently less erythema of the PRP-treated side compared with the control side. Erythema and pigmentation at 84 days were evaluated using a 3-degree visual analogue scale (VAS). Erythema and hyperpigmentation remained on four PRP-treated sides and five control-sides. The mean values for erythema on the PRP-treated side and control side were 1.2 and 2.2, respectively. The mean VAS score for hyperpigmentation was 1.0 on the PRP-treated side and 2.4 on the control side. Serious adverse effects such as scarring or ectropion were not observed on either side of the face.

Subjective assessments by patients.

The subjects reported similar satisfaction scores for their two treatment regimens both immediately after treatment and on day 1 after treatment (Figure 4a). However, on days 7 and 84 after treatment, the patients reported higher satisfaction scores for the PRP combination therapy (*P*-value on day 84 = 0.016). Additionally, the satisfaction scores were strongly correlated with scores on the improvement scale. The subjects reported significantly higher scores for improvement by PRP combination treatment compared to NS combination treatment on days 7 (*P* = 0.03) and 84 (*P* = 0.02) (Figure 4c). However, the patient-reported pain and convenience scores were not significantly difference for the two treatment modalities (Figures 4c, 4d).

Tissue reaction after treatment and collagen deposition evaluated by histology

The fractional CO_2 laser treatment induced formation of necrotic columns. Oozing and crust formation were observed on days 1 and 3. PRP treatment induced epidermal thickening on day 7 (Figure 5a). Both treatment modalities induced collagen deposition an day 7 as compared with the baseline. Collagen deposits were more compact and dense in the papillary dermis after PRP combination treatment than after NS combination treatment (Figure 5b). Herovici staining showed that type III collagen (bluish color) was increased in the

papillary dermis on the PRP-treated side of the face, while type I collagen was increased in the papillary dermis on the control side (Figure 5c).

Expression of TGFβ, collagen and MMP2 evaluated by IHC

Image analyses of IHC results on day 7 revealed significantly higher TGFβ1 expression after combined fractional CO_2 laser/PRP treatment compared to expression following the control treatment (Figure 6a, P = 0.01). On the PRP-treated side, TGFβ1 expression peaked on day 3 and then decreased on the following days; however, it still remained elevated compared with its baseline expression. There was no significant difference in TGFβ2 expression following treatment with the two regimens (Figure 6b). On day 1, significantly greater TGFβ3 expression was found on the PRP-treated side compared with the control side (Figure 6c, P = 0.03), and TGFβ3 expression continued to increase with time and remained higher than expression on the control side. Collagen I and collagen III were more prominent on the PRP-treated side compared with the NS-treated side; however, the difference was not statistically significant (Figures 6d, 6e). The overall patterns of changes in collagen I and collagen III expression were similar. PRP induced a reduction in MMP2 expression, which was usually significantly increased by laser irradiation on day 3 (Figure 6f, P = 0.01).

mRNA transcription evaluated by quantitative PCR

Molecular changes in skin tissue which occurred during the acute wound healing stage after treatment were investigated by examining the mRNA levels of biomolecules associated with wound healing processes and collagen production. We found that amounts of mRNA showed some degree of correlation with the respective degrees of protein expression as determined by immunohistochemical staining. Expression of TGFβ1 mRNA was significantly higher on the PRP-treated side than on the control side on days 3 and 7 (Figure 7a, both *P*-values < 0.001), and a similar pattern of expression was shown for TGFβ3 mRNA (Figure 7b). Type I collagen mRNA increased with time, and there was a significant difference in type I collagen mRNA levels between the two treatment modalities at day 3 (Figure 7c, $P = 0.005$).

Assessments of β-catenin and αSMA expression

Expression of β-catenin, which is thought to be a mediator of TGFβ signaling, was assessed by IHC methods. On days 1 and 7, expression of β-catenin was significantly higher on the PRP-treated side than on the control side (Figure 8a, $P = 0.026$). Additionally, IHC results showed robust expression of αSMA on the side treated by combined laser/PRP therapy (Figure 8b).

Effects of laser irradiation, PDP, and PRP on cell morphology

We next investigated the molecular mechanism for the effects of

combined laser/PRP treatment on fibroblasts. Fibroblasts received laser irradiation for 30 minutes, after which the culture medium was replaced by serum-free culture medium. Examinations conducted 24 hours later showed that irradiated fibroblasts displayed morphologies different from those of non-irradiated fibroblasts (Figures 9a, 9b). Treatment with either PDP (Figure 9c) or PRP (Figure 9d) resulted in the rapid recovery of normal fibroblast morphology and increased cell proliferation. Treatment with PRP increased cell proliferation to a greater extent than treatment with PDP.

Molecular changes in cells as evaluated by western blot

We looked for evidence of quantitative changes in cell signaling molecules related to fibrogenesis. Two different cell-lines (HaCaT and CCD-986sk) were treated with either PDP or PRP in time and dose-dependent manners following laser irradiation. PRP treament produced increased EGFR expression in HaCaT cell at 48 hours (Figure 10a).

The amounts of phospho-Akt, TGFβ1, TGFβ3, β-catenin, collagen I, collagen III, IL-1β, and MMP2 were quantified in the cell lysates of fibroblasts. Both PDP and PRP increased cellular expressions of phospho-Akt, TGFβ1, TGFβ3, β-catenin, collagen I, and collagen III compared to cells without PDP or PRP treatment (Figure 10b, 10c). Expression levels of these molecules were higher after treatment with 20% PDP or PRP than after treatment with 1% PDP or PRP. PRP

induced higher expressions of these molecules than PDP at the same concentration; however, the molecular expression patterns induced by PDP and PRP were nearly the same at 24 hours or 48 hours after treatment. PRP treatment appeared to induce higher expressions of secreted collagen I, collagen III, β-catenin, and αSMA than PDP treatment. The expression patterns of TGFβ1 and TGFβ3 shown after 24 hours were not as clear as those shown at 30 minutes. Expression of β-catenin peaked at 24 hours, and then decreased at 48 hours, while αSMA expression increased and then peaked at 48 hours. With PDP treatment, expression of secreted collagen I peaked at 24 hours, but did not increase until 48 hours with PRP treatment. Expression of secreted collagen III peaked at 24 hours, and then decreased with either PDP or PRP treatment. We next evaluated the effect of p-Akt by pretreating fibroblasts with LY294002 (a PI3K/Akt inhibitor). Inhibition of the Akt pathway resulting in decreased β-catenin expression; however, expression of TGFβ1 was not affected by p-Akt inhibition (Figure 10d).

Unlike p-Akt/β-catenin signaling, IL-1β was decreased by PRP treatment at 24 hours. MMP2 expression was also reduced by PRP treatment (Figure 10e).

Discussion

Although numerous treatments exist for acne scars, most produce disappointing results. While the development of laser treatment has increased the safety and efficacy of treatment, many treatment sessions can be required, and patients often express dissatisfaction with the results. The basis for this unsatisfactory response to treatment seems to lie in the pathogenic process involved in formation of acne scars. Acne scars are strongly associated with an incomplete recovery from dermal matrix destruction caused by acne inflammation (6). While acne inflammation does not always lead to scarring, patients with severe degrees of scarring tend to show a poor response to treatment. This phenomenon suggests that the generation and correction of acne scars are strongly related to an individual's regenerative capabilities. Thus, raising a patient's regenerative abilities may represent a novel approach to treating acne scars.

One such method is the application of growth factors to enhance tissue regenerative properties and increase the speed and success of wound healing. Various growth factors are known to be contained in platelets. Activated PRP contains high concentrations of various growth factors (20), and is often used in the post-operative care of problematic wounds in diabetic patients (9, 10, 15, 16, 21, 22). The growth factors in PRP are assumed to increase the regenerative ability of damaged

tissue in acne patients undergoing laser treatment, and thus increase its efficacy.

The superior clinical efficacy of PRP combination treatment shown in our current study concurred with results reported in a previous study (23). In the present study, the superior clinical efficacy of combined laser/PRP treatment was confirmed using two different evaluation tools. Additionally, our study showed the superiority of the PRP combination therapy in treating all types of acne scars. Numerous studies have reported improved results when treating acne scars with a fractional laser (24-26). Our data also indicated that 2 sessions of fractional CO_2 laser therapy produced a significant improvement (~50%) in scars compared with baseline evaluations. Molecular studies on the mechanism of fractional laser therapy are rare. However, elevated levels of HSP70 and HSP47, which are markers of heat damage, have been reported after laser treatment (27, 28). Similar to the traditional ablative CO_2 laser, dermal remodeling and regeneration by heat stimulation via laser penetration are also suggested as mechanisms for the clinical efficacy provided by the fractional CO_2 laser (29).

PRP is known to elevate levels of TGFβ, which is thought to be an important modulator of fibrosis (30-32). TGFβ purified from human platelets has been shown to promote collagen formation and increase fibroblast proliferation; however, these effects have not been demonstrated by PDGF or EGF (32). Especially, fibroblast proliferation positively correlated with TGFβ1 expression during the early stages

after fractional CO2 laser (33). Especially, fibroblast proliferation was positively correlated with TGFβ1 expression during the early period after fractional CO_2 laser treatment. The in vivo data from our current study suggest that the effects of PRP may be mediated by TGFβ. PRP-treatment induced elevated expressions TGFβ1 and TGFβ3 as determined by both IHC studies and quantitative PCR, and also led to dense collagen depositions and upregulated collagen I mRNA transcription. Additionally, TGFβ1, TGFβ3, and collagen I showed similar overall expression patterns.

Wnt/β-catenin signaling has recently been implicated in fibrotic reactions (34-36). Furthermore, crosstalk between TGFβ and the Wnt/β-catenin pathway during the process of fibrosis has also been demonstrated (37, 38). Both TGFβ and Wnt/β-catenin signaling can stimulate fibroblasts, induce myofibroblast differentiation, and thus stimulate collagen production. In our study, the dermal expressions of β-catenin and αSMA were higher on the PRP-treated side of faces compared to the control sides. This result suggests that β-catenin may help mediate myofibroblastic activation and increase collagen deposition following PRP injection.

Our in vitro experiments were conducted to clarify the molecular mechanism for the efficacy shown by combined fractional CO_2 laser/PRP treatment. Laser irradiation induced morphological changes in fibroblast cells, presumably due to heat energy transfer. To exclude the simple effect of serum, the effects of PDP treatment were

compared with those of PRP treatment. Although PDP induced a rapid recovery from laser insult, PRP induced a more rapid recovery, and also the proliferation of fibroblasts. When examined from the standpoint of cellular proliferation, the results of our current study support those reported in earlier studies which suggested the proliferative effect of PRP on fibroblasts (39, 40).

The overall expression patterns of TGFβ, collagen, β-catenin, and Akt were similar to their respective protein expressions. Such results suggest that these molecules which were increased by PRP treatment may have a common connection. Akt signaling is known to be involved in cell survival (41), and a recent study revealed its role in wound healing through matrix regulation (42). Furthermore, Akt signaling was reported to mediate αSMA expression, myofibroblast differentiation, and regulate Wnt/β-catenin signaling in association with Dishevelled (43). In the present study, as blockade Akt signaling resulted in inhibition of β-catenin expression. However, the Akt signal blockade did not affect expression of TGFβ, suggesting that PRP may increase TGFβ levels. Thus Akt may act as a mediator of TGFβ and upregulate collagen production via β-catenin.

PRP has been reported to reduce the incidence of adverse effects produced by fractional CO_2 laser therapy (17, 23). In the current study, erythema and edema were less severe on the PRP-treated side of each face compared to the NS-treated side. Upregulation of EGFR in keratinocytes and downregulation of IL-1β in fibroblasts by PRP can be

suggested as possible mechanisms for reduced erythema and edema. Moreover, reduction of inflammation may lead to decreased levels of MMP2, and subsequent decreased collagen degradation.

In summary, PRP injections increased the efficacy of fractional CO_2 laser treatment for acne scarring. Increased levels of TGFβ can be suggested as a mechanism for the clinical improvement shown when using combined fractional CO_2 laser/PRP treatment. Akt and β-catenin might be mediators of a signaling pathway associated with TGFβ. Furthermore, reduction of inflammation by PRP may accelerate collagen deposition by decreasing collagen degradation.

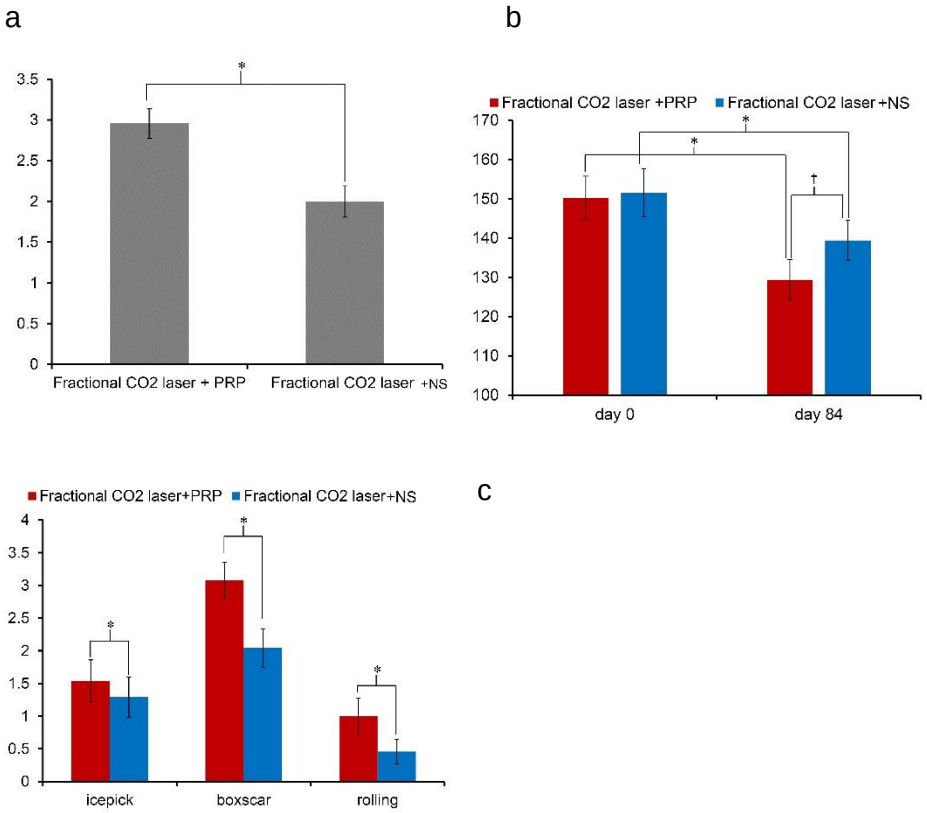


Figure 1. Fractional CO_2 laser combined with PRP treatment induced significant improvement of acne scar. The acne scars received two sessions of treatment. (a) IGA scores suggested that combined laser plus PRP treatment produced significantly better results for treating acne scars (*, $P < 0.001$). (b) Both treatment modalities resulted in the significant improvement of acne scars when compared with baseline (*, $P < 0.001$). PRP combination treatment was superior to NS treatment (†, $P < 0.001$). (c) An acne scar subtype analysis showed that combined fractional CO_2 laser/PRP treatment was superior to fractional CO_2 laser plus NS treatment in all scar types (*, $P < 0.001$).

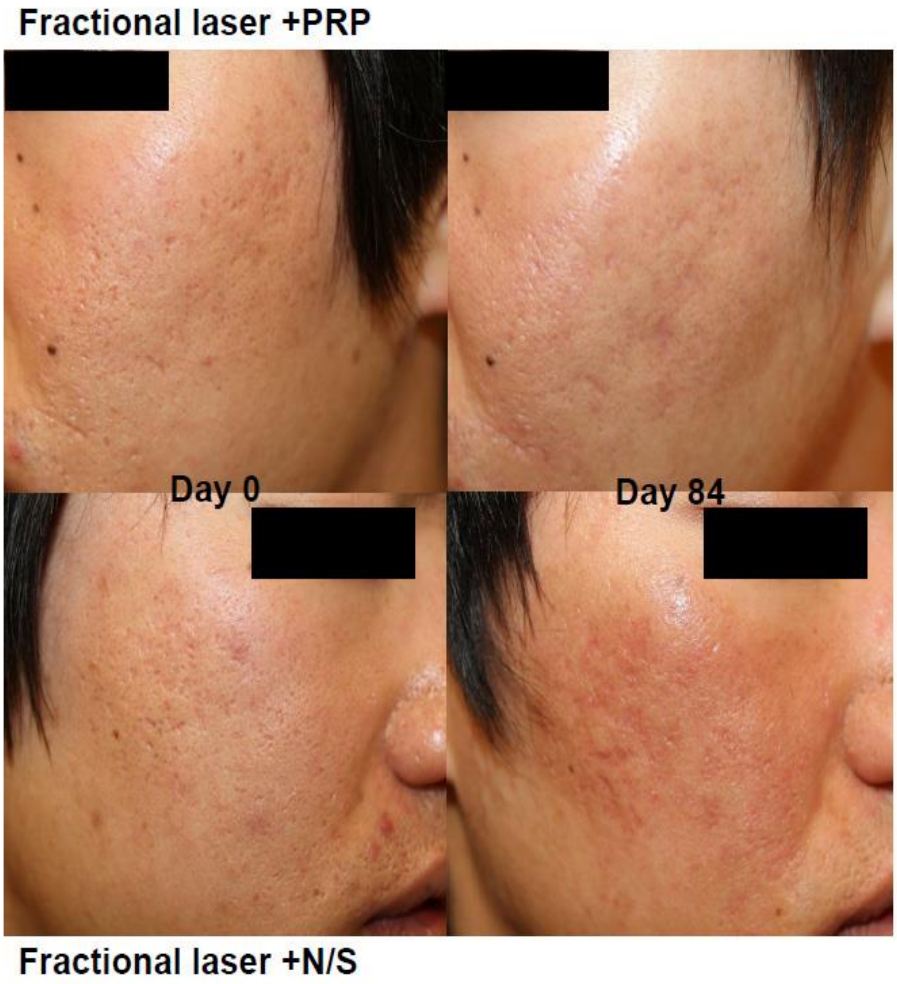


Figure 2. Combined fractional CO_2 laser/PRP treatment can increase efficacy and reduce adverse effects. The clinical photo shows the higher efficacy of combined laser/PRP treatment compared to combined laser/NS treatment. Erythema induced by laser treatment was less on the PRP-treated side compared to the NS-treated side.

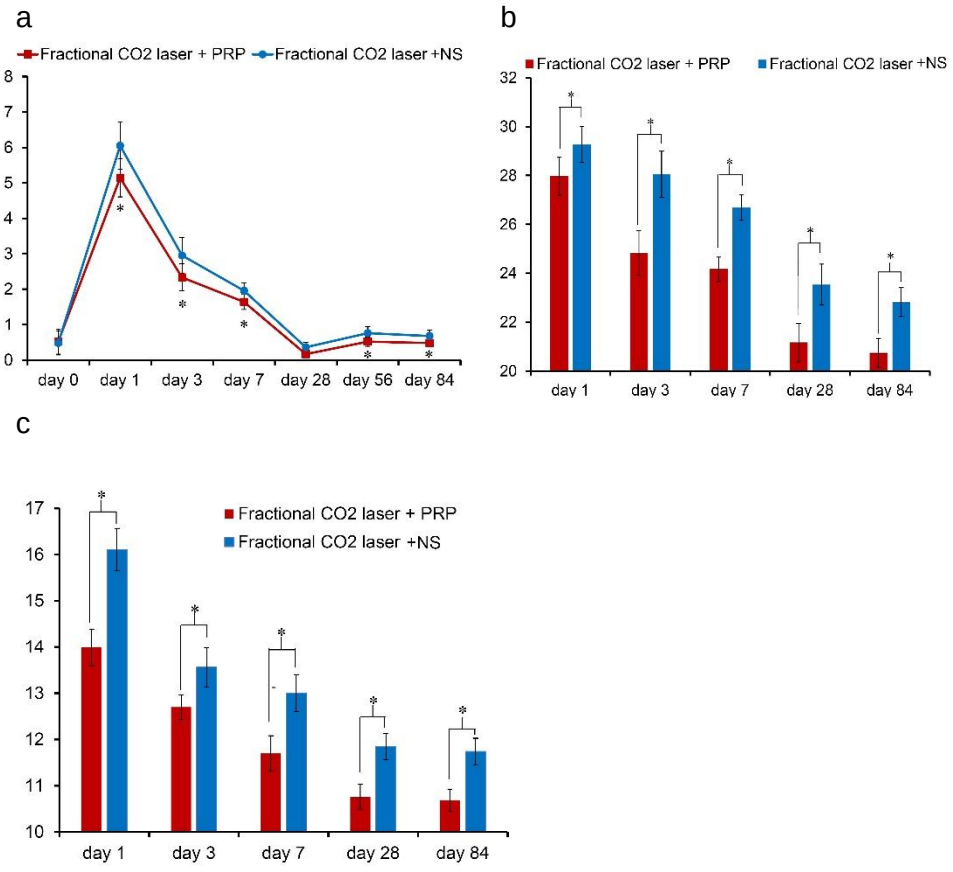


Figure 3. PRP can reduce the adverse effects of laser treatment. (a) Scores on the epithelization scale which totaled the degrees of erythema, swelling, and oozing were significantly lower for PRP-treated side compared to the NS-treated side starting from day 1 and throughout the study (on day 1, P = 0.01). (b) The erythema index determined by spectrophotometry showed consistent elevation of erythema on the side treated with combined fractional CO_2 laser/NS throughout the study period. (c) Colorimetric measurements (a*) revealed a similar erythema pattern.

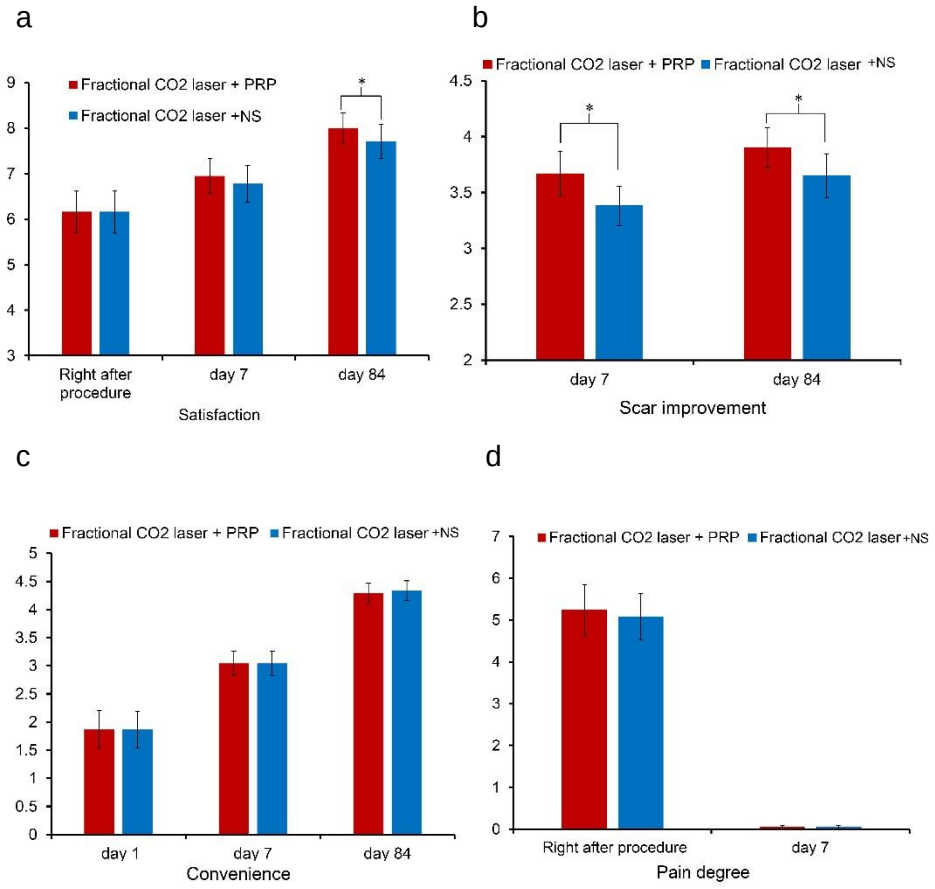
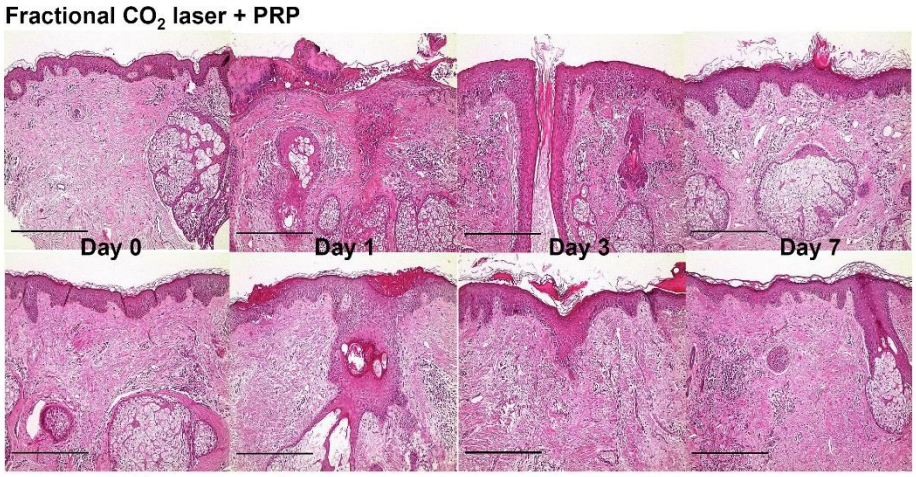


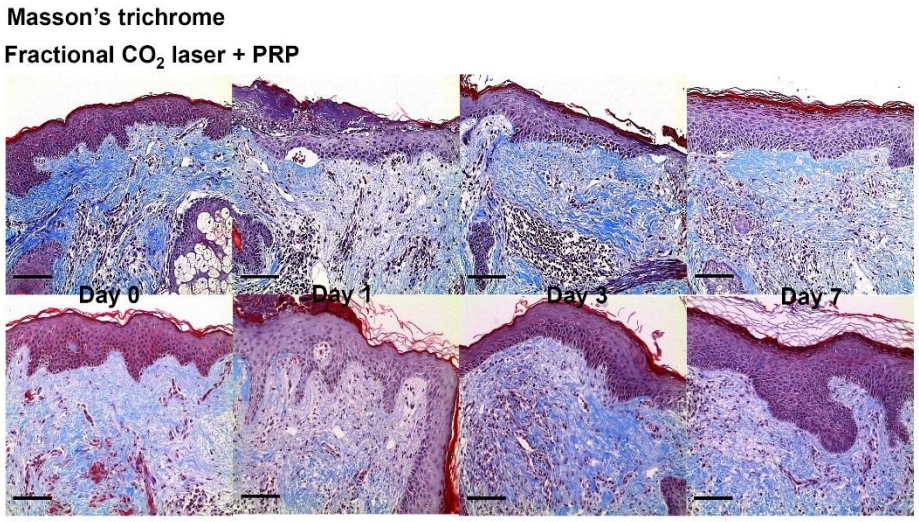
Figure 4. Subjects were more satisfied with the results of combined fractional CO_2 laser/PRP treatment. (a) Subjective assessments made by subjects indicated significantly higher satisfaction with treatment results on the PRP-treated side compared with those on the NS-treated side on days 7 and 84 (*, $P = 0.016$). (b) Scores for scar improvement were significantly higher for PRP-treated side compared to the NS-treated side on days 7 (*, $P = 0.03$) and 84 (*, $P = 0.02$). (c) There were no differences in the convenience scores or pain scores. (d) reported for the two treatment modalities.

a



Bar=100μm

b



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c

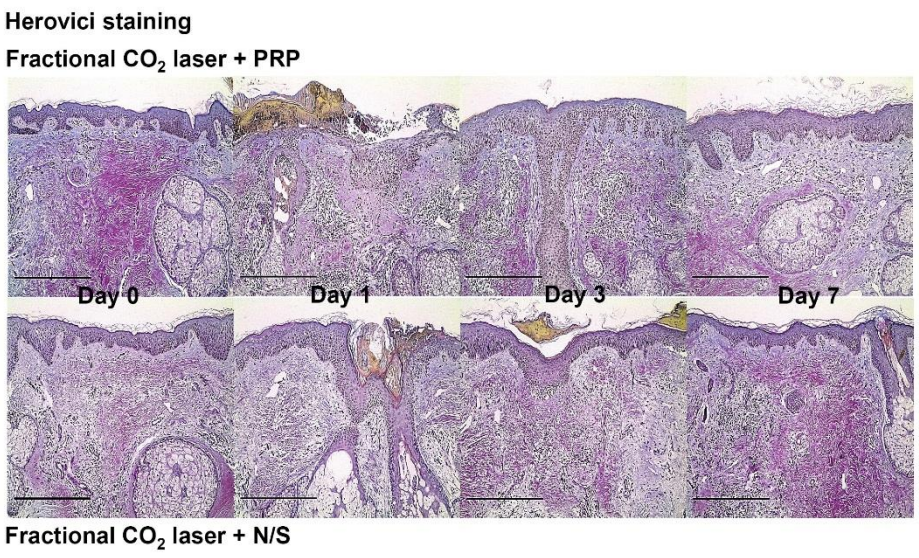
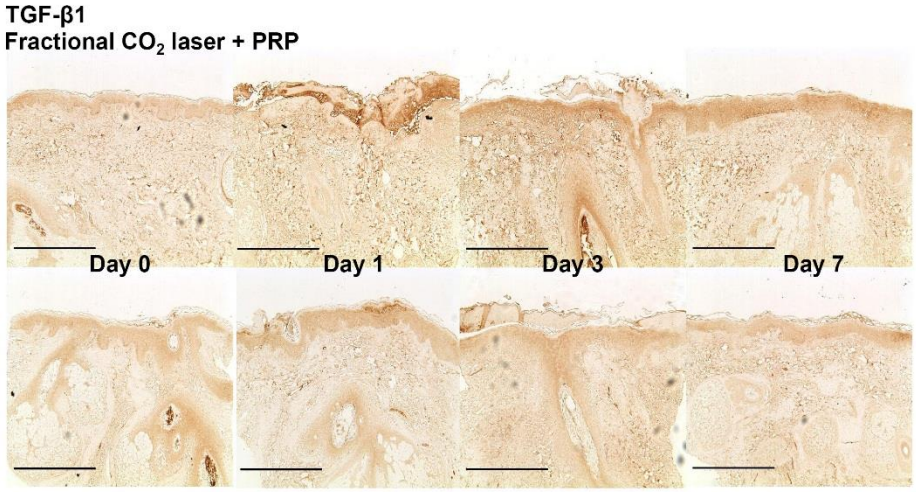
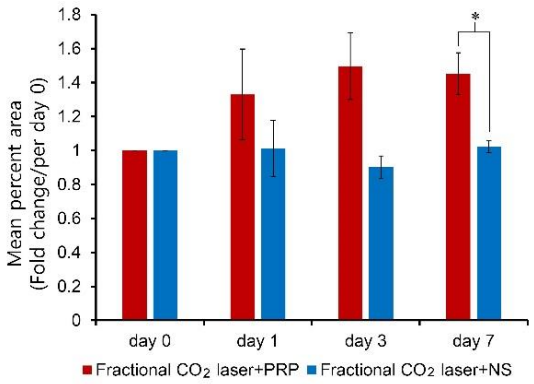


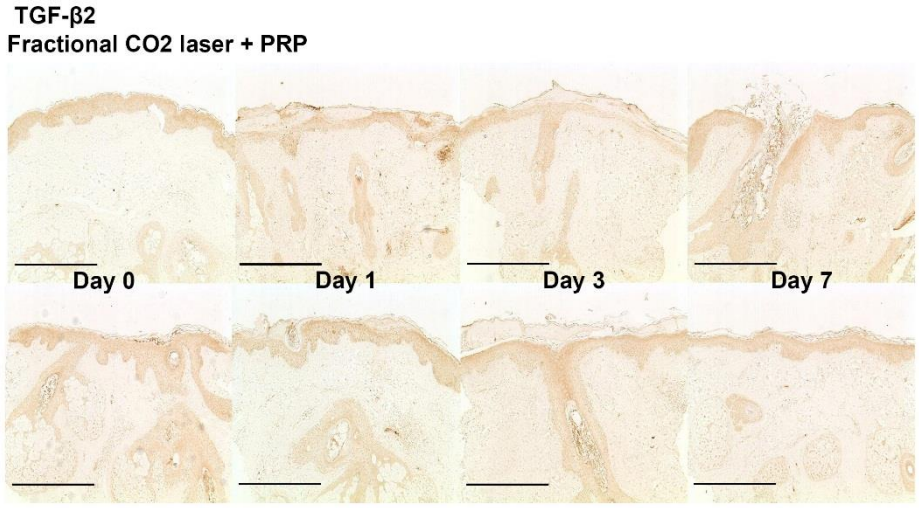
Figure 5. Histochemical staining revealed tissue reaction against laser irradiation and PRP treatment. (a) Necrotic columns and crust induced by laser irradiation. Greater degrees of epidermal thickening were observed on the PRP-treated side compared to the NS-treated side (H&E staining, x100). (b) PRP treatment resulted in thicker and denser collagen depositions on the PRP-treated side (Masson's trichrome staining, x200). (c) Type III collagen was relatively increased in the papillary dermis after PRP treatment compared with NS treatment (Herovici staining, x100).

a


TGF-β1
Fractional CO_2 laser + PRP
Day 0
Day 1
Day 3
Day 7
Fractional CO_2 laser + N/S
Bar=100μm


Mean percent area
(Fold change/per day 0)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
day 0
day 1
day 3
day 7
Fractional CO_2 laser+PRP
Fractional CO_2 laser+NS

b



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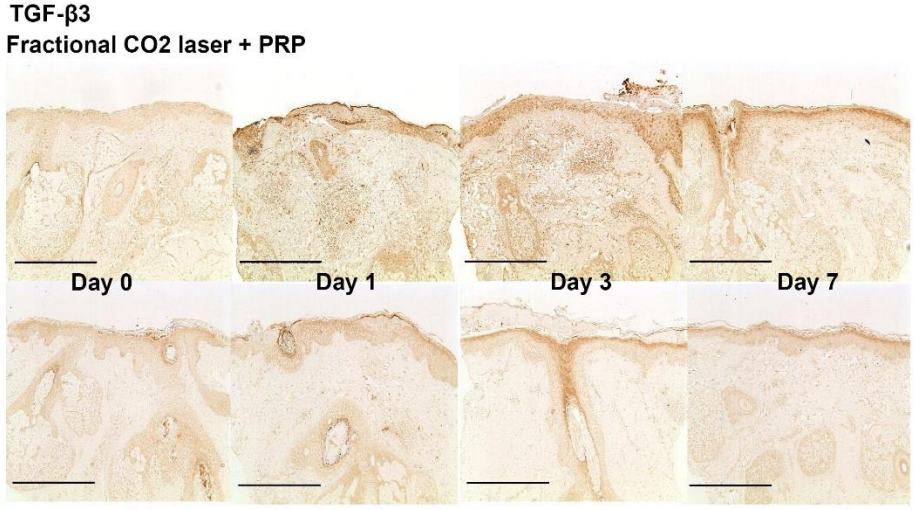
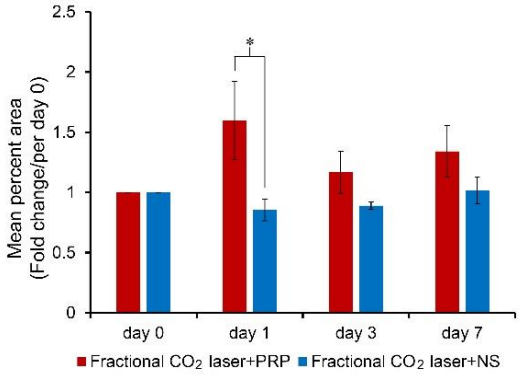
c



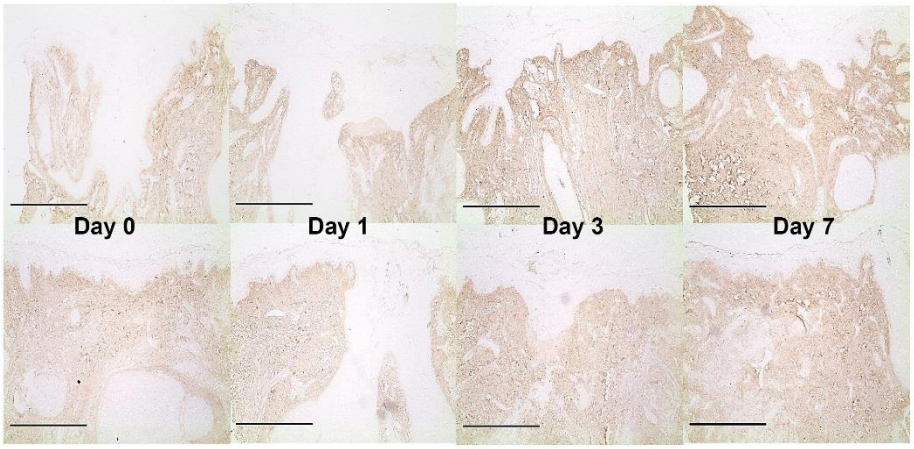
Bar=100μm



d

Collagen 1

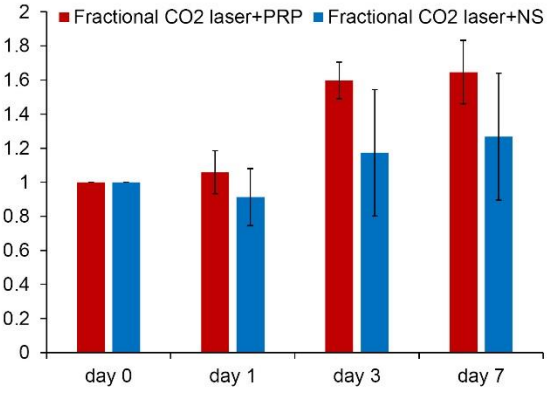
Fractional CO_2 laser + PRP



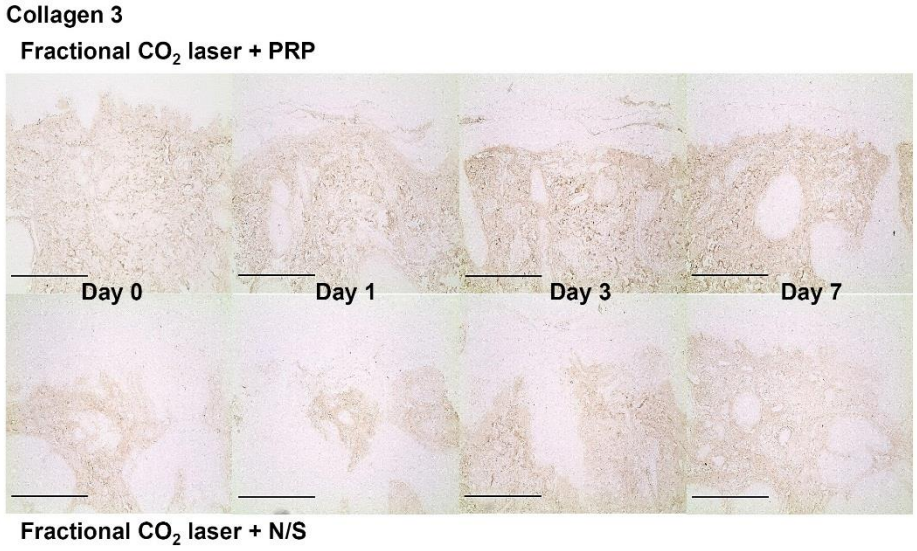
Fractional CO_2 laser + N/S

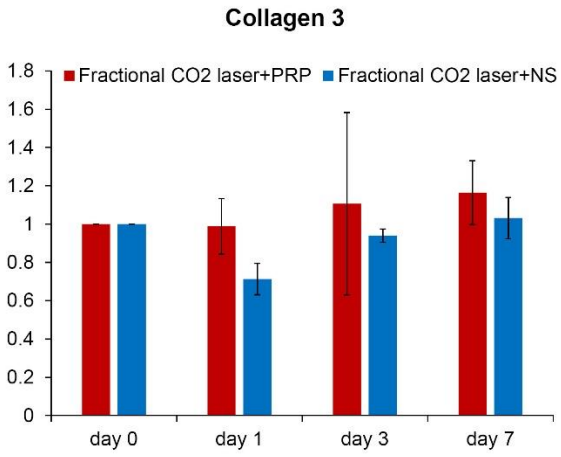
Bar=100μm

Collagen 1



e

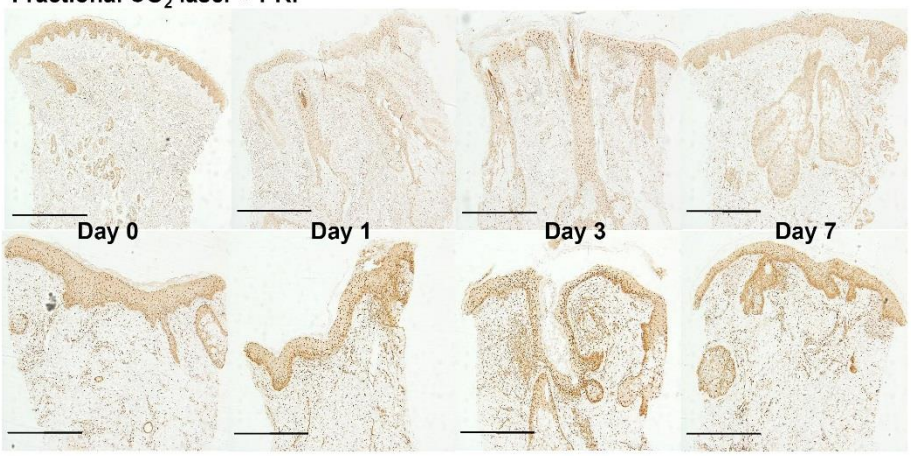

Collagen 3
Fractional CO_2 laser + PRP
Day 0
Day 1
Day 3
Day 7
Fractional CO_2 laser + N/S
Bar=100μm


Collagen 3
Fractional CO2 laser+PRP
Fractional CO2 laser+NS
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
day 0
day 1
day 3
day 7

f

MMP 2

Fractional CO_2 laser + PRP



Fractional CO_2 laser + N/S

Bar=100μm

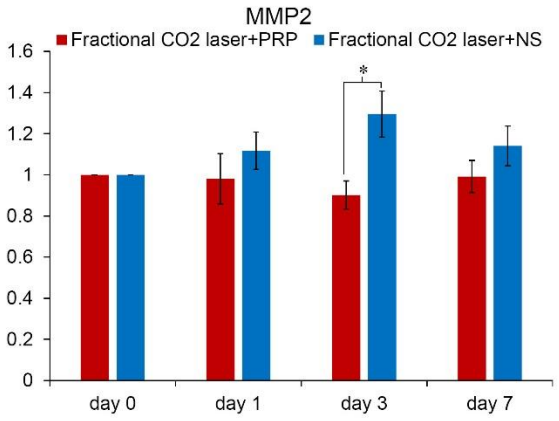


Figure 6. Different patterns of TGFβ, collagen and MMP2 expression were observed on the PRP-treated side compared to the NS-treated side after laser irradiation. Immunohistochemical staining and image analyses were conducted to evaluate the relative expressions of TGFβ1, TGFβ2, and TGFβ3. (a) On day 7, TGFβ1 showed significantly greater upregulation on the PRP-treated side compared to the NS-treated side (x100) (*, P = 0.01). (b) The two treatment modalities showed no significant difference regarding their effects on TGFβ2 expression (x100). (c) On day 1, TGFβ3 expression was significantly higher on the PRP-treated side compared to the NS-treated side (x100) (*, P = 0.03). (d) Collagen I expression was more prominent on the PRP-treated side than on the NS-treated side; however, the difference was not statistically significant. (e) Collagen III expression was higher on the PRP-treated side than on the NS-treated side; however, the difference was not statistically significant. The pattern of collagen III expression was quite similar to that shown for collagen I expression. (f) Unlike TGFβ and collagen, MMP2 expression was lower on the PRP-treated side than on the NS-treated side, and the difference was statistically significant on day 3 (*, P = 0.01).

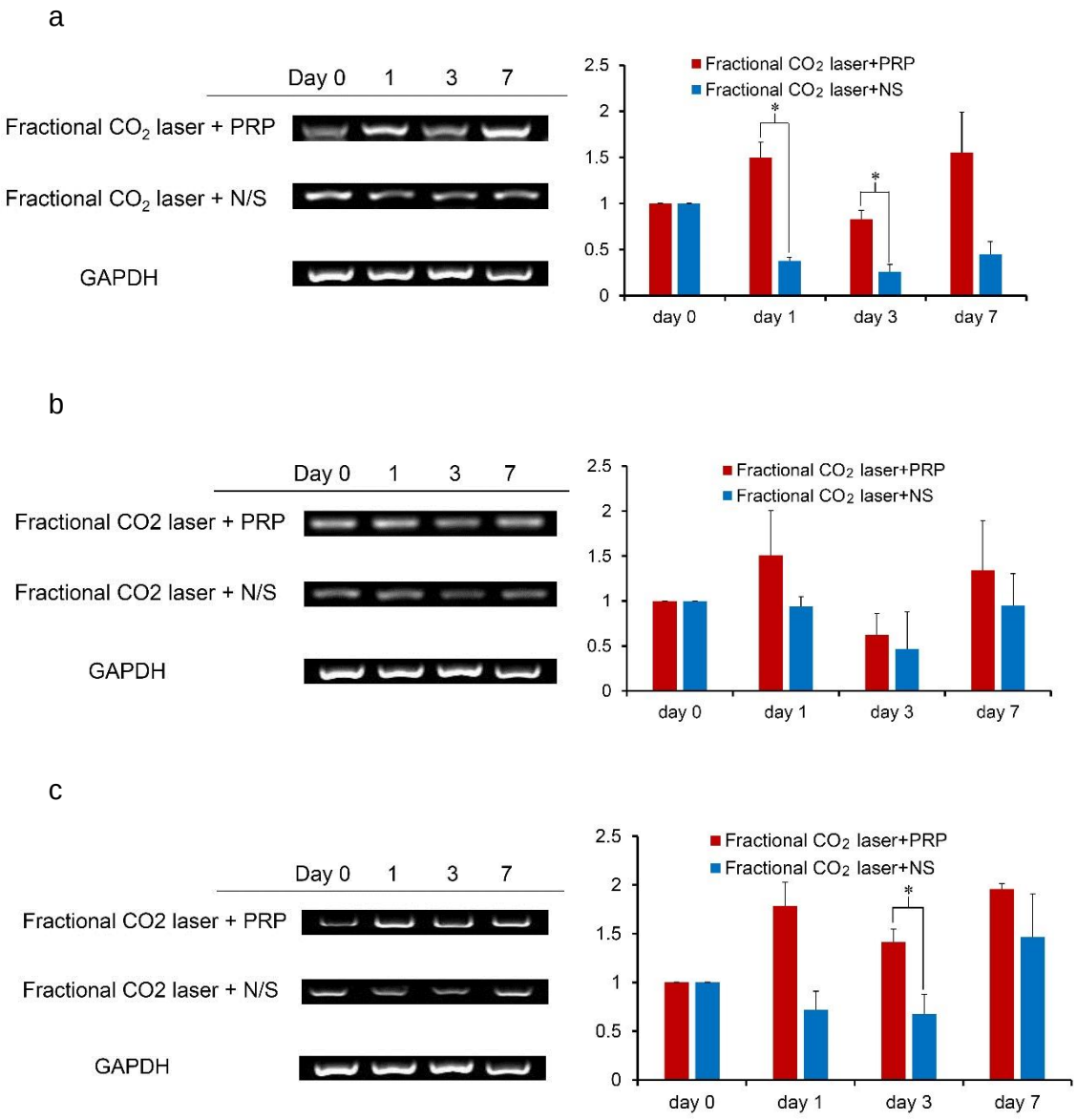


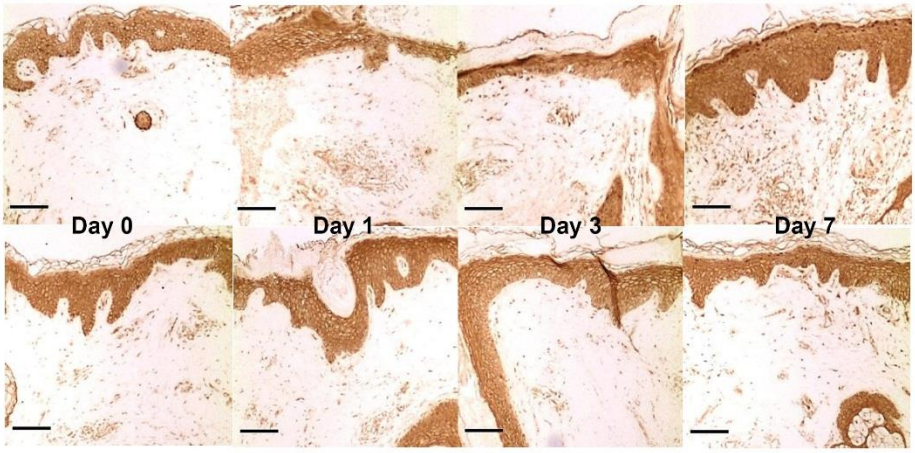
Figure 7. PRP treatment increased transcription of TGFβ1, TGFß3, and collagen I mRNA. Patient skin biopsies were subjected to quantitative PCR analysis. (a) Expression of TGFβ1 mRNA was higher in biopsies obtained from the PRP-treated side compared to the NS-treated side, and the differences on days 1 and 3 were statistically

significant (*, both *P*-values < 0.001). (b) TGFβ3 and TGFβ1 showed similar patterns of mRNA expression. (c) On day 3, collagen I transcription was significantly higher on the PRP-treated side compared to the NS-treated side (*, *P* = 0.005).

a

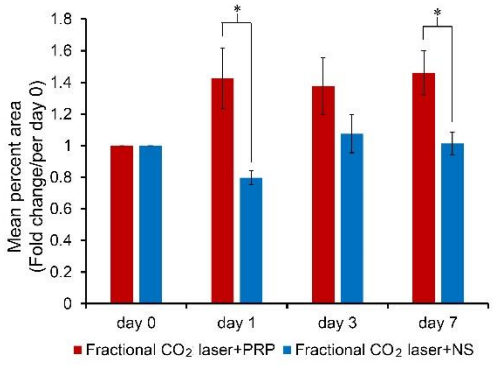
β - catenin

Fractional CO_2 laser + PRP



Fractional CO_2 laser + N/S

Bar=100μm



b

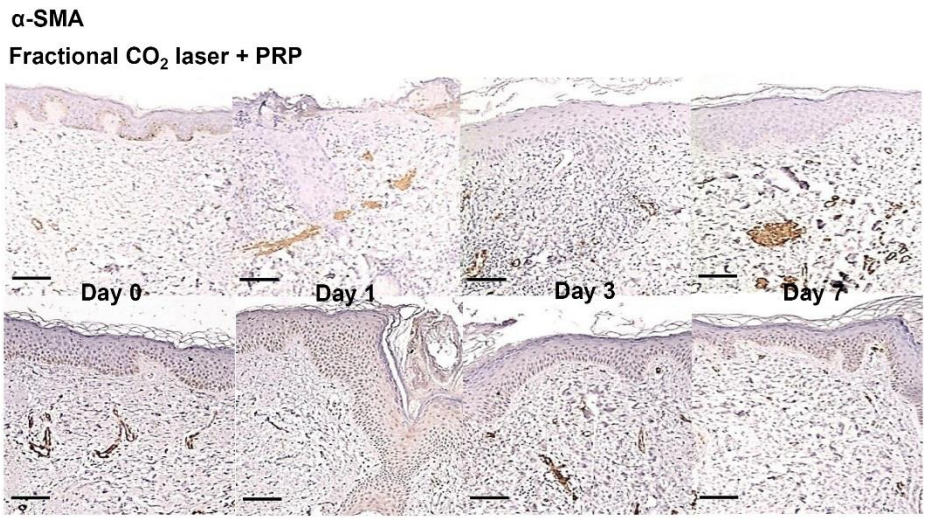


Figure 8. PRP treatment increased β-catenin and αSMA expression. (a) IHC revealed significantly higher β-catenin expression in the dermis of the PRP-treated side compared to the NS-treated side (x200) on study days 1 and 7 (*, both *P*-values = 0,026). (b) αSMA, a marker for fibroblast activation, was upregulated in the dermis of the PRP-treated side (x200).

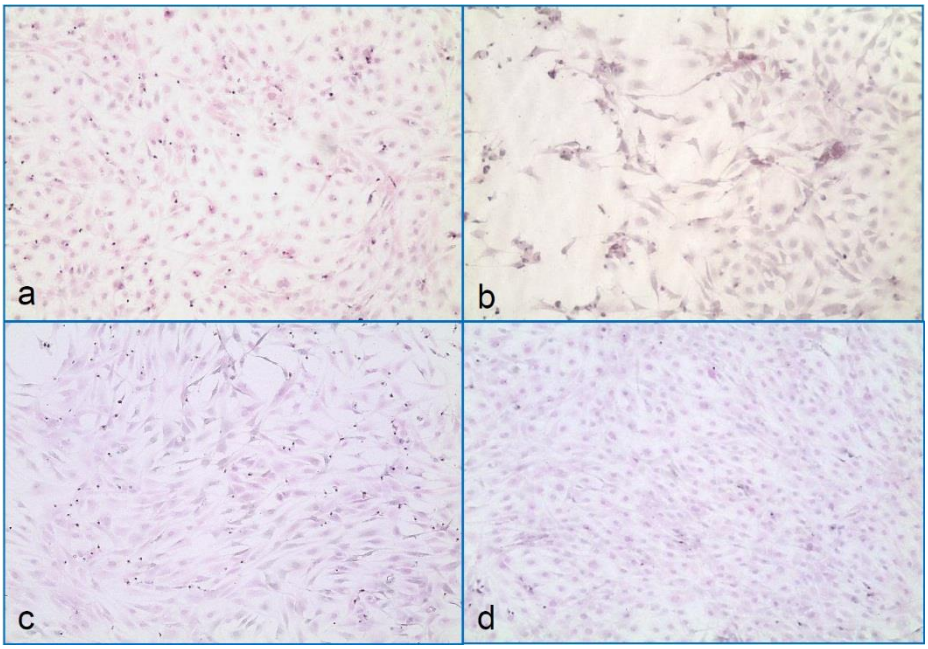
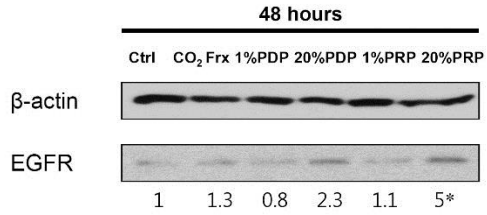


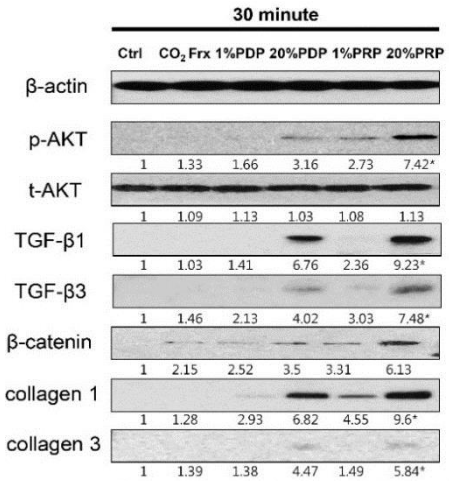
Figure 9. PRP accelerated recovery of cell morphology after insult by laser irradiation. Three culture plates with fibroblasts were exposed to laser irradiation. After 24 hours, morphological changes were observed by H&E staining. (a) Cells without any treatment had normal morphology. (b) After laser irradiation, morphological changes were observed. (c) Cells recovered their normal morphology with PDP treatment. (d) PRP accelerated morphological recovery, and cell density was similar to that for control cells.

a



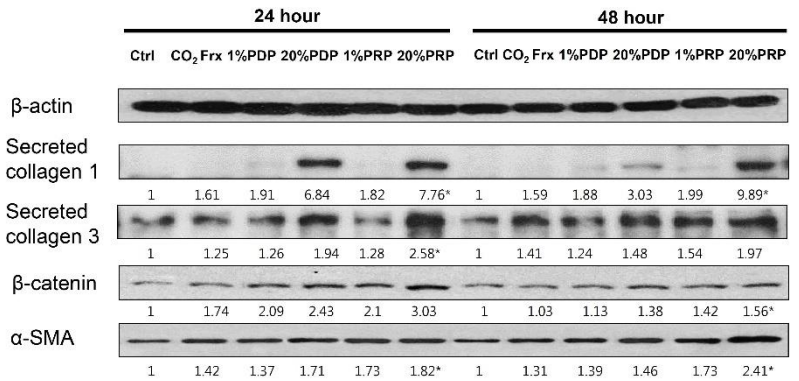
*P<.05, between fractional CO_2 and fractional CO_2 +PRP

b



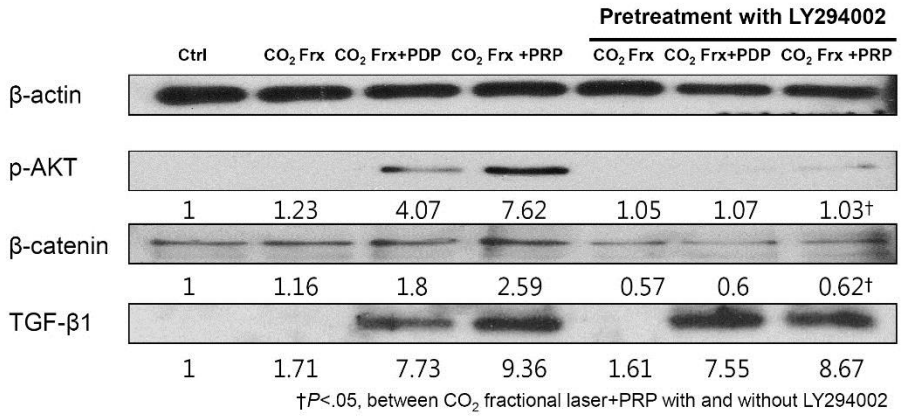
*P<.05, between fractional CO_2 and fractional CO_2 +PRP

c



*P<.05, between fractional CO_2 and fractional CO_2 +PRP

d



e

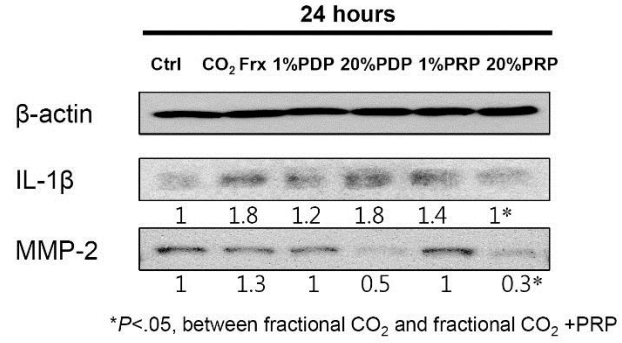


Figure 10. PRP upregulated EGFR expression in keratinocytes, TGFβ expression in fibroblasts, and downregulated IL-1β and MMP2 expression in fibroblasts after insult by laser therapy (a) PRP treatment increased EGFR expression in HaCaT cells at 48 hours. (b) Fibroblast cells were treated with low (1%) and high (20%) concentrations of PDP and PRP after laser irradiation. TGFβ1 and TGFß3 were upregulated by PDP and PRP at 30 minutes. Expressions of TGFβ1 and TGGß3 were higher at the high concentrations of PDP

and PRP compared to the low concentrations, When examined at the same PRP and PDP concentrations, expression of TGFβ was higher in PRP treated cells than in the PDP treated cells, and similar results were found for the expression patterns of phospho-Akt, β-catenin, collagen I, and collagen III. (c) These expression patterns remained unchanged at 24 hours. Collagen I, collagen III, β-catenin, and αSMA showed higher expression after PRP treatment than after PDP treatment. TGFβ1 and TGFß3 were not detected during the 24 hour period. β-catenin expression was strongest at 24 hours, and αSMA expression peaked at 48 hours. Expression of secreted collagen I peaked at 24 hours after PDP treatment, and was maintained for 48 hours by PRP treatment. (d) Inhibition of the PI3K/Akt pathway by pretreatment with LY294002 reduced β-catenin expression, but not TGFβ1 expression. (e) IL-1β, which is an inflammatory cytokine whose expression was increased by laser irradiation, was not elevated by treatment with 20% PRP at 24 hours. MMP2 expression was decreased by treatment with 20% PRP at 24 hours.

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국문초록

여드름 흉터는 여드름에 의한 염증으로 인한 진피 기질의 파괴와 불안전한 진피 내 기질의 회복으로 유발된다. 레이저에 의한 흉터 치료는 진피 기질의 재생과 재형성 과정을 유도하여 흉터를 호전시킨다. 탄산가스 프락셀 레이저는 기존의 레이저에 비해서 치료에 의해 나타나는 부작용이 나타날 가능성은 줄어들지만, 여드름 흉터에 대한 효과는 기존의 탄산가스 레이저 치료보다 만족스럽지 못하다. 혈소판 풍부 혈장 (platelet-rich plasma; PRP)의 경우 레이저에 의한 치료 효과를 올려줄 수 있는 많은 종류의 성장인자를 가지고 있다. 더구나 혈소판 풍부 혈장에 의해서 유발되는 빠른 피부 장벽의 회복은 레이저 치료와 관련하여 나타날 수 있는 부작용의 가능성을 줄여준다.

이 연구에서는 25명의 중등도 이상의 여드름 흉터를 가진 환자를 모집하여 4주 간격으로 2회 치료를 실시하였다. 치료는 전체적으로 탄산가스 프락셀 레이저 치료를 시행한 뒤 얼굴을 오른쪽과 왼쪽으로 나누고 한쪽은 혈소판 풍부 혈장을 다른 한쪽은 대조군으로 생리식염수를 진피 내에 주사하였다. 치료 전과 치료를 한 후 1일, 3일 7일, 4주 뒤 효과와 부작용 평가를 시행하였으며, 분자생물학적 변화를 확인하기 위해서 치료 전과 치료 후 1일, 3일,

7일에 조직을 얻었다. 탄산가스 프락셀 레이저와 혈소판 풍부 혈장 치료의 생물학적 기전을 연구하기 위해서 섬유아세포 세포주를 이용하여 시간에 따른 콜라젠과 성장인자들의 발현을 평가하는 실험을 시행하였다.

탄산가스 프락셀 레이저는 여드름 흉터를 유의하게 호전시켰다. 하지만 혈소판 풍부 혈장 치료를 병합하였을 때 여드름 흉터가 더 호전되었다. 연구자 평가에 의해서 혈소판 혈장 치료를 병합한 부위는 약 75%, 생리식염수를 이용하여 대조 치료를 한 부위는 50% 정도의 호전을 보였다. 홍반, 부종, 진물 같은 부작용은 혈소판 풍부 혈장 치료를 한 부위에서 크게 줄어들었다. 자원자들의 주관적 평가에서도 만족도 및 치료 효과에서 혈소판 풍부 혈장 치료를 병합한 부위의 점수가 대조치료 부위보다 유의하게 증가하였다. 피부조직에서는 탄산가스 프락셀 레이저에 의한 피부 반응을 관찰하였고 혈소판 풍부 혈장 치료를 한 부위의 유두진피의 콜라젠 섬유가 더 두껍고 조밀해진 것을 관찰하였다. 면역화학염색을 통해서 혈소판 풍부 혈장 치료를 한 부위가 대조부위보다 TGFβ1과 TGFβ3의 단백질 발현이 증가한 것을 확인하였으며, quantitative PCR을 통해서 TGFβ1과 TGFβ3, 1형 콜라겐의 mRNA 발현이 증가한 것을 확인하였다. 또한 TGFβ의 섬유화와 관련된 신호전달 물질인 β-catenin과 섬유아세포 활성화의 표지자인 αSMA가 혈소판 풍부 혈장 치료한 부위에서 발현이 증가하였다. 세포실험에서 탄산가스

프락셀 레이저를 조사 한 뒤 혈소판 풍부 혈장을 처리하였을 때 p-Akt, TGFβ1, TGFβ3, β-catenin, 1형과 3형의 콜라겐 섬유의 단백질 발현이 농도 및 시간에 따라 증가하였다. PI3K/Akt 저해제를 처리하여 p-Akt를 억제하였을 때 β-catenin의 발현은 억제되었지만, TGFβ1은 억제되지 않았다.

탄산가스 프락셀 레이저를 시행한 뒤 혈소판 풍부 혈장을 병합하여 치료하는 경우 여드름 흉터 치료 효과를 높이고 부작용은 줄일 수 있다. TGFβ1과 TGFβ3의 발현이 증가하는 것은 탄산가스 프락셀 레이저와 혈소판 풍부 혈장 병합 치료에서 여드름 흉터 호전의 기전 중 하나로 생각된다. 세포 시험 결과를 고려할 때 혈소판 풍부 혈장에 의해서 유발된 TGFβ의 증가는 p-Akt 세포신호 전달 경로를 통해서 β-catenin을 활성화하고 이에 따라 섬유아세포가 활성화되어 αSMA 발현이 증가하고 이에 따라 콜라겐 섬유 생산이 증가하는 것으로 생각된다. 이러한 세포 신호 전달은 탄산가스 프락셀 레이저와 혈소판 풍부 혈장에 의해서 여드름 흉터가 호전되는 기전으로 추측된다.

주요어: 여드름 흉터, fractional CO_2 laser, platelet-rich plasma, TGFβ, β-catenin, collagen

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