Combination of Platelet Rich Plasma in Fractional Carbon Dioxide Laser Treatment Increased Clinical Efficacy of for Acne Scar by Enhancement of Collagen Production and Modulation of Laser-Induced Inflammation

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Background: Platelet-rich plasma (PRP) which contains large amounts of growth factors has been tried to enhance therapeutic efficacy of laser treatment for acne scar with unknown underlying mechanism.

Objectives: The present study was conducted to investigate the molecular mechanism of increased clinical efficacy of PRP when combined with fractional laser treatment for treating acne scars.

Methods: Subjects with mild to moderate acne scars were treated with two sessions of fractional CO2 laser therapy given with and without co-administration of PRP. Skin biopsy specimens were obtained at baseline, 1, 3, 7, and 28 days for investigation of molecular profiles associated with skin changes produced by laser plus PRP treatment.

Results: The PRP treatment increased clinical efficacy with decreased severity of adverse effects such as erythema, swelling and oozing. Productions of TGFβ1 and TGFβ3 proteins were more highly elevated on the PRP-treated side of the face compared to the control side at day 28. Furthermore, PRP-treated side showed significant increase of c-myc, TIMP, and HGF expression. Experimental fibroblast culture model was also used. PRP administration after laser irradiation increased expressions of p-Akt, TGFβ1, TGFβ3, β-catenin, collagen 1, and collagen 3 in both dose-dependent and time dependent manners in fibroblast. Moreover, we acquired clinical and histological data through randomized control clinical trial.

Conclusion: Taken together with human study results combined with the data from cell experiments we suggest that PRP treatment increased fibrogenetic molecules induced by fractional CO2 laser, which have association with clinical effect. Lasers Surg. Med. © 2017 Wiley Periodicals, Inc.

Key words: acne scar; fractional CO2 laser; platelet-rich plasma; TGFβ; collagen

INTRODUCTION

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 [1]. Additionally, platelets can also induce the synthesis of various growth factors which promote tissue regeneration [2,3]. PRP was demonstrated to accelerate epithelization and reduce inflammation after wounding in rabbits [4], and has been suggested as a supplement to diabetic foot surgery due to its regenerative capabilities [5]. With regards to the skin, PRP has been reported to induce rapid skin barrier recovery and reduce erythema after carbon dioxide laser therapy [6]. 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.

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 [7]. Therefore, most acne scar treatments work by inducing regeneration and remodeling of the dermal matrix. Until now, ablative laser resurfacing has been regarded as the gold standard treating atrophic acne scars [8]. However, resurfacing provided by conventional ablative laser treatment produces additional cutaneous maladies such as hypertrophic scarring or permanent hypopigmentation. With development of technology, fractional laser therapy was developed to reduce these adverse effects, even safe for dark skin and within a
month after taking isotretinoin [9–12]. Although the remaining viable epidermis and formation of rapid fibrin plug often accelerate postoperative recovery, such therapeutic effects are often limited [13]. While high-energy laser treatment can increase therapeutic efficacy, it can also increase the chance that a patient will experience adverse side effects [14].

To the best of our knowledge, there was no study revealing the molecular mechanism of the effect of PRP on combining with laser treatment. Thereby, we conducted the current study to suggest the molecular mechanism of PRP when combined with laser treatment through cell experimental model. The clinical efficacy was evaluated by randomized control study. Additionally, suggested mechanism of this combined treatment was assessed using human skin samples acquired by clinical trial.

MATERIALS AND METHODS

Preparation of PRP

PRP for treating acne scars was prepared from the autologous blood of each individual subject. PRP for cell experiment was obtained from a healthy donor (age 25, platelet count: 270,000/mm³). None of the subjects used medication influencing platelet function. PRP was prepared and activated as in the published article [6]. Platelet depleted plasma (PDP) was prepared from the supernatant without addition of the pellet.

Clinical Study Design

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. 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. Specific protocol of the current study was registered in clinicaltrials.gov (NCT02396290). The whole scar of both faces was irradiated with a fractional CO₂ 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 1–1.5 cm intervals (0.02 ml at each site). Fractional CO₂ 10600-nm laser irradiation was applied at a fluence of 30–70 mJ/cm² with a 150 μm thermal treatment zone (MTZ) and 12 mm spot size via a 1 ms pulse duration in a non-overlapping manner, and using a single pass. Volunteers were subjected to two identical sessions of treatment, with a 4-week interval between treatments.

Acquisition of Skin Tissue and Assessments of Clinical Outcome

Skin biopsy specimens (2 mm) for the molecular evaluation were obtained on days 0, 1, 3, 7, and 28 after the first treatment session. Subjects were followed-up on days 1, 3, 7, and 28 after each session and at 1 and 2 months after the final session. Efficacy was assessed using the 5-point Investigator’s Global Assessment (IGA) (grade 0; 0%, 1; 1–25%, 2; 26–50%, 3; 51–75%, 4; 76–100% improvement); Echelle d’évaluation clinique des cicatrices d’acné (ECCA) scores, and a subtype (icepick, boxcar, rolling scar) analysis [15]. Skin recovery was assessed using Epithelization Scale composed of erythema, oozing, and swelling [16]. Degrees of erythema were measured using two photometric devices (Spectrophotometer CM-2002®, Konica Minolta, Tokyo, Japan; Derma-spectrometer®, Cortex Technology, Hadsund, Denmark).

Immunohistochemistry (IHC)

Samples were processed for immunohistochemical staining for transforming growth factor (TGF)β1, TGFβ3, c-myc, TIMP, HGF, collagen 1, and collagen 3 (Abcam, Cambridge, MA). The intensity of immunohistochemical staining was evaluated using an image analysis program (Leica QWin version 3.5.1, Leica Microsystems, Wetzlar, Germany).

Cell Treatment With PRP and Laser Irradiation

Fibroblast cells (CCD-986Sk) and keratinocyte (HaCaT) 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 CO₂ laser at a fluence of 10 mJ/cm² in 150 MTZs. The dose of laser irradiation was selected based on results in a previous pilot study. 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 three sets of cells at 30 minutes, 24 hours, and 48 hours, respectively.

Quantitative PCR and Western Blot

The mRNA expression of collagen 1, collagen 3, and TGF1 of fibroblast model at 30 minutes was investigated through qPCR. The qPCR was conducted with the same method as described in the published article [17]. Primer sequences are shown in the supplementary table. To determine the amounts of collagen 1 and collagen 3 protein secreted into culture media, equal aliquots of conditioned culture media obtained from cultures with equal numbers of fibroblast (8 × 10⁴ cells/cc) were fractionated. To investigate changes in expression of cell signaling proteins, total protein was extracted using a cell lysis buffer (Cell Signaling, Beverly, MA). Western blot was done as described in the published article [17].

The blots were primarily probed with t-Akt and Phospho-Akt (Ser473), β-actin mouse antibody, EGFR, and keratin
16 rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), collagen 1, collagen 3, KGF, TGFβ1, and TGFβ3 rabbit antibodies (Abcam). Films of blots were analyzed and quantified using NIH's ImageJ software (Version 1.48).

**Statistical Analysis**

The paired *t*-test and Wilcoxon signed-rank test were used to compare differences of clinical results, staining intensity and quantitative PCR 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). *P*-values <0.05 were considered statistically significant.

**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.

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 CO2 laser/PRP treatment and fractional CO2 laser/NS treatment resulted in ~75% and 50% improvements in acne scarring, respectively (*P* < 0.001). Additionally, ECCA scores indicated a significant improvement following treatment with either regimen (combined fractional CO2 laser/PRP, *P* < 0.001; combined fractional CO2 laser/NS, *P* < 0.001) (Fig. 1A). However, treatment with combined fractional CO2 laser/PRP was superior to treatment with...
combined fractional CO₂ laser/NS for improving all scar subtypes (Fig. 1B).

Skin recovery rates after treatment as assessed using the epithelization scale showed a significant difference between the two treatment modalities on day 1 ($P = 0.01$) (Fig. 1C). 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. All patients experienced erythema and hyperpigmentation after the treatment. However, both the erythema index and colorimetric measurements revealed consistently less erythema of the PRP-treated side compared with the control side (Fig. 1D). At day 84, erythema and hyperpigmentation remained on four PRP-treated sides and five control sides. The mean values of 3-degree visual analogue scale (VAS) 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.

Clinical photographs also showed greater improvement of acne scars on the side treated by combined fractional CO₂ laser/PRP therapy (Fig. 2).

The subjects reported similar satisfaction scores for their two treatment regimens both immediately after treatment and on day 1 after treatment. 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 ($P = 0.02$).

**Investigation With Skin Tissue**

Image analyses of IHC results on day 28 revealed significantly higher TGFβ1 expression after combined fractional CO₂ laser/PRP treatment compared to expression following the control treatment (Fig. 3A, $P = 0.02$). On the PRP-treated side, TGFβ1 expression were at the lowest point on day 1 and then increased on the following days. On days 7 and 28, significantly greater TGFβ3 expression was found on the PRP-treated side compared with the control side (Fig. 3B, $P = 0.03$ and 0.004).

C-myc expression of PRP-treated side continued to increase with time. IHC revealed significantly higher c-myc expression of the PRP-treated side compared to the NS-treated side on study days 7 and 28 (Fig. 3C, both $P$-values = 0.004). The overall pattern of changes between TGFβ1 and c-myc expression were similar. Higher TIMP was observed on the PRP-treated side at day 28 with significance (Fig. 3D, $P = 0.01$). The expression pattern of TIMP was similar to that of TGF β3. HGF were more prominent on the PRP-treated side compared with the NS-treated side day 3 onward. On days 3 and 28, expression of HGF was significantly higher on the PRP-treated side than
on the control side (Fig. 3E, day 3 and 28; both $P$ values = 0.03).

Both collagen 1 and collagen 3 increased with after treatment. Collagen 1 expression was more prominent on the PRP-treated side than on the NS-treated side; On day 28, collagen 1 expression was significantly higher on the PRP-treated side compared to the NS-treated side (Fig. 3F, $P = 0.03$). Collagen 3 expression was higher on the PRP-treated side than on the NS-treated side. The difference was statistically significant on days 7 and 28 (Fig. 3G, both $P$-values = 0.03).

Effects of Laser Irradiation, PDP and PRP on Cell

Morphological examinations were conducted 24 hours later. Treatment with either PDP or PRP 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 (Fig. 4).

We looked for evidence of quantitative changes in cell signaling molecules related to fibrogenesis. Cell-lines (HaCaT and CCD-986sk) were treated with either PDP or PRP in time and dose-dependent manners following laser irradiation.

Messenger RNA expression of collagen and TGF β1 were higher after treatment of 20% PRP compared with single fractional laser treatment (Fig. 5A). The difference of collagen mRNA expression was not statistically significant. There was significant difference of TGF β1 mRNA expression between single treatment of fractional laser and fractional laser followed by 20% PRP treatment ($P = 0.015$).

The amounts of phospho-Akt, TGFβ1, TGFβ3, collagen 1, and collagen 3 were quantified in the cell lysates of fibroblasts. Both PDP and PRP increased cellular expressions of phospho-Akt, TGFβ1, TGFβ3, collagen 1, and collagen 3 compared to cells without PDP or PRP treatment at 30 minutes (Fig. 5B). 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 or 48 hours after treatment (Fig. 5C). PRP treatment appeared to induce higher expressions of secreted collagen 1 and collagen 3 than PDP treatment. The expression patterns of TGFβ1 and TGFβ3 shown after 24 hours were not as clear as those shown at 30 minutes. With PDP treatment, expression of secreted collagen 1 peaked at 24 hours, but did not increase until 48 hours with PRP treatment. Expression of secreted collagen 3 peaked at 24 hours, and then decreased with either PDP or PRP treatment.

PRP treatment produced increased EGFR expression and decreased keratin 16 in HaCaT cell at 48 hours, strongly suggesting that PRP may accelerate epithelization and decrease laser-induced skin damage (Fig. 6).

**DISCUSSION**

Application of growth factors enhances tissue regenerative properties and increases 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 [18], and is often used in the post-operative care of problematic wounds in diabetic patients [2,3,19–22]. 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 two sessions of fractional CO2 laser therapy produced a significant improvement (~50%) in scars compared with baseline evaluations.

PRP is known to elevate levels of TGFβ, which is thought to be an important modulator of fibrosis [27–29]. 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 [29]. Especially, fibroblast proliferation positively correlated with TGFβ1 expression during the early stages after fractional CO2 laser [30]. The in vivo data from our current study suggest that the effects of PRP may be mediated by TGFβ3.

PRP-treatment induced elevated expressions TGFβ1 and TGFβ3 as determined by both IHC studies, and also led to dense collagen depositions. TGFβ3 has been attempted to decrease the scarring response [31]. Therefore, increase of collagen deposition may be induced by comprehensive effect of changes of TGFβ1 and TGFβ3.

C-myc has been regarded as an important regulator of cellular growth control [32]. Increase of c-myc expression resulted in cell cycle progression [33]. Presumably, c-myc may act as a mediator between signals from growth factors released by PRP treatment and cell proliferation. Cooperation of c-myc and TGFβ may play a positive role in some conditions such as epithelial–mesenchymal transition which is common in wound healing process [34]. In our study, expressions of TGFβ and cmyc were higher on the PRP-treated side of faces compared to control sides. This result suggests that both TGFβ and cmyc may contribute to increasing collagen deposition following PRP injection.
Clinical efficacy may have association with total collagen deposition. Increase of TIMP expression by PRP treatment can contribute to increasing collagen deposition.

Our in vitro experiments were conducted to clarify the molecular mechanism for the efficacy shown by combined fractional CO₂ 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 [35,36].

The overall expression patterns of TGFβ, collagen, 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 [37], and a recent study revealed its role in wound healing through matrix regulation [38].

PRP has been reported to reduce the incidence of adverse effects produced by fractional CO₂ laser therapy [6,23]. In the current study, erythema and edema were less severe on the PRP-treated side of each face compared to the NS-treated side. Reduction of inflammation by PRP can be suggested as possible mechanisms for reduced erythema and edema. Upregulation of TGFβ and HGF supports suppressive role of PRP against laser-induced inflammation [39,40]. With the supplementary data, anti-inflammatory effect of PRP may
CO2 laser/PRP treatment. Improvement shown when using combined fractional can be suggested as a mechanism for the clinical treatment for acne scarring. Increased levels of TGF injections increased the efficacy of fractional CO2 laser including TGF and assessment of changes in various growth factors other scar types such as hypertrophic scar with PRP of PRP action, future study should focus on treating are difficult to predict. Thus, to specify the mechanism Clinical effects of laser plus PRP on hypertrophic scar has its distinctive characteristics. There are a few limitations in our study. Wound healing is a dynamic process over a long period. It is beneficial to observe the process over a long span of time rather than a short period. PRP is a complex material containing various growth factors. Thereby, it is ideal to investigate changes in various growth factors contained in PRP. Unlike atrophic scar, hypertrophic scar has its distinctive characteristics. Clinical effects of laser plus PRP on hypertrophic scar are difficult to predict. Thus, to specify the mechanism of PRP action, future study should focus on treating other scar types such as hypertrophic scar with PRP and assessment of changes in various growth factors including TGFβ by PRP treatment. In summary, PRP injections increased the efficacy of fractional CO2 laser treatment for acne scarring. Increased levels of TGFβ can be suggested as a mechanism for the clinical improvement shown when using combined fractional CO2 laser/PRP treatment.

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**Fig. 6.** HaCaT cell treatment with 1% and 20% PDP, PRP. PRP treatment increased EGFR and decreased keratin 16 expression in HaCaT cells at 48 hours.

have association with decreased levels of C-FOS and AP-1 pathway, thus increasing clinical efficacy.

U precautions of EGFR and downregulation of keratin 16 in keratinocytes suggest benefit of PRP in laser-induced wound healing. Increase of EGFR expression implies that PRP may affect epithelial proliferation and migration in wound healing process [41]. Downregulation of keratin 16 indicates that protective effect of PRP against laser-induced damage in keratinocyte.

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**REFERENCE**


SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.