Expression of transforming growth factor-β after different non-invasive facial rejuvenation modalities

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Abstract
Background Transforming growth factor-β (TGF-β) is a major regulator of the synthesis of extracellular matrix (ECM) proteins in human skin as it stimulates fibroblast proliferation and collagen production. Perturbed TGF-β expression may play a key role in the pathogenesis of skin aging.

Objectives This study was conducted to objectively evaluate the effects of different modalities of non-invasive facial rejuvenation on TGF-β expression and to correlate its level with that of newly synthesized collagen.

Methods A total of 36 patients with Fitzpatrick skin types III and IV were divided into six groups. Each group of six patients was subjected to a different non-invasive modality for the treatment of skin aging, including radiofrequency (RF), Nd:YAG 1320-nm laser and Er:YAG 2940-nm laser mini-peels, intense pulsed light (IPL), mesotherapy injection, and electro-optical synergy (ELOS). Skin biopsies were obtained before treatment, at the end of treatment, and at three months post-treatment. In addition, biopsies were obtained from 30 control subjects. Levels of TGF-β were quantitatively evaluated using computerized image analysis of immunostained sections.

Results The expression of TGF-β was statistically significantly increased (P < 0.05) at the end of Nd:YAG 1320-nm and Er:YAG 2940-nm mini-peel treatments compared with baseline levels, and at three months post-treatment with RF and ELOS compared with pretreatment and end-of-treatment levels. However, no significant differences (P > 0.05) were observed in TGF-β level in response to IPL or mesotherapy treatments in comparison with baseline. The level of TGF-β was positively correlated (P < 0.05) to that of newly synthesized collagen at the end of Nd:YAG 1320-nm laser and Er:YAG 2940-nm laser mini-peels, as well as at three months after RF and ELOS treatments.

Conclusions Radiofrequency, ELOS, and Nd:YAG 1320-nm laser and Er:YAG 2940-nm laser mini-peels resulted in an increase in TGF-β expression, which may mediate the effects of these modalities in enhancing dermal collagen expression through the activation of fibroblasts and thereby reverse the photoaging of skin.

Introduction
The desire of human beings to look younger than their chronological age is reflected in the array of different modalities available to help them do so, which include medical preparations, dermabrasion, chemical peels, and laser treatments. Many of these modalities have been well studied, but others have not. Recently, non-invasive rejuvenation was reported to be an effective esthetic treatment that reverses the signs of photo-damaged skin. Photoaged skin was first described in 1986 by Kligman and Kligman as the result of repeated exposure to environmental elements, primarily ultraviolet (UV) radiation, which enhance the breakdown of collagen through the production of reactive oxygen species (ROS) and activator protein-1 (AP-1), which upregulates expression of matrix metalloproteinases (MMP). Additionally, sun exposure decreases the level of transforming growth factor-β (TGF-β) with a resultant decrease in collagen production and will eventually manifest in wrinkles and other signs of photoaged skin.

Together with its known isoforms (TGF-β₁₋₃), TGF-β is a multifunctional cytokine that acts as an inhibitor of cell proliferation and facilitates cellular differentiation in...
a wide variety of cells. TGF-β also plays an important role in tissue remodeling and repair as well as in increasing dermal collagen content. Impaired TGF-β, caused by UV irradiation, may play a role in the pathology of skin aging.

The main constituent of dermal extracellular matrix (ECM) proteins is collagen, primarily type I (80–85%) and type III (10–15%), which provides strength and resilience to skin. We recently reported an increase in collagen production after the use of some non-invasive modalities for facial rejuvenation.

Therefore, the present study focuses primarily on investigating the potential effects of non-invasive treatments for skin rejuvenation by quantifying the histological changes of TGF-β in response to six different non-invasive modalities: radiofrequency (RF); Nd:YAG (neodymium:yttrium–aluminium–garnet) 1320-nm laser; Er:YAG (erbium:yttrium–aluminium–garnet) 2940-nm laser mini-peel; intense pulsed light (IPL); mesotherapy injection and an electro-optical synergy (ELOS) technique. Changes in TGF-β were then investigated for their correlations with the production of newly synthesized collagen.

**Materials and methods**

**Study population**

The present study was conducted on 36 patients (30 women and six men) with Fitzpatrick skin types III and IV who attended the dermatology outpatient clinic at Al-Minya University Hospital, Al-Minya, Egypt, for treatment of signs of photoaging. The mean ± standard deviation (SD) age of the patients was 49 ± 5.4 years (range: 37–72 years). They were divided randomly into six groups of six patients each. The study also included 30 control volunteers (18 females and 12 males) with a mean ± SD age of 46 ± 16.7 years (range: 7–89 years). Control subjects were undergoing dermatosurgical procedures for other reasons. The controls were divided into three groups based on their age: Group A (young; 7–30 years); Group B (middle-aged; 31–60 years); and Group C (older; >60 years).

Treatment and study details were fully explained to all subjects, all of whom signed an informed consent form. Punch biopsies (3 mm) were obtained from the periorbital area in all patients before treatment (baseline), after three months (end of treatment), and six months after the start of treatment (post-treatment). Skin biopsies (30 specimens) were also obtained from the facial skin of the 30 control volunteers. Tissues were fixed in 10% buffered formalin, embedded in paraffin, and sectioned into 5-μm-thick sections. All histological and immunostaining evaluations were carried out in the Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, Philadelphia, PA, USA.

**Device and treatment protocol**

Each group was subjected to a different non-invasive technique for the treatment of photoaging. Patients underwent three months of treatment (six sessions at 2-week intervals). All patients were instructed to avoid sun exposure and to use sunscreens of sun protection factor 30 or more during the daytime.

Volunteers in Group 1 were treated with a monopolar RF skin-tightening device (GSD Biorad; Shenzhen GSD Tech. Co., Shenzhen, Guangdong, China). Two initial passes of 150 J each were performed over the entire face to facilitate the uniform contraction of collagen. Three more additional passes of 200 J each were made over the periorbital, nasolabial, and forehead areas. Patients in Group 2 were treated with Nd:YAG 1320-nm laser (Won Technology Co., Daejeon, South Korea). The device was set to deliver fluencies in the range of 20–25 J/cm² (average: 22.5 J/cm²), with a 30-millisecond (ms) pulse width at a rate of 1.5 Hz. Group 3 was treated with Er:YAG 2940-nm laser (SkinPlus Er:YAG device; Fotona Medical Lasers DD, Ljubljana, Slovenia). The laser was used in a thermal mode and set to deliver a sequence of short pulses, each of a sub-ablation threshold fluence with a total energy of 2–3 J/cm² and pulse duration of 200–250 ms. Patients in Group 4 were treated with an IPL device (IPL Quantum; Lumenis Italy Srl, Formello, Italy). The parameters used were a 575-nm cut-off filter at a fluence of 30–35 J/cm², double pulsing with a 6-ms pulse width, and a delay of 20–30 ms between pulses. Group 5 was treated with mesotherapy injection. The sterile solutions used for injection were composed of a cocktail of multivitamin solution and non-cross-linked high-viscosity hyaluronic acid, provided as two separate vials in a packaging kit (Revitacare® Bio-Revitalisation; Laboratoire Revitacare, Saint Ouen l’Aumône, France).

Group 6 was treated with ELOS technology using an E-light device (Beijing ADSS Development Co., Beijing, China), which produces a combination of IPL and RF energies in the same pulse profile, with the following specifications: 12 × 30-mm² spot size handpiece; 640-nm cut-off filter; 25 J/cm² energy; 30-ms pulse duration; and 30 W RF power. In all treatments except mesotherapy, care was taken in each session to keep the skin cool to prevent the epidermis from being heated.

**Immunohistochemical staining**

The immunoperoxidase (IP) technique was used to evaluate TGF-β. Nonspecific sites were blocked, and the tissues were incubated with antibodies to total TGF-β at 4 °C overnight (1:200; ab66043; Abcam, Inc., Cambridge, MA, USA). Tissues were washed and incubated with biotinylated secondary antibody (1:200; PK-6102; Vector Laboratories, Inc., Burlingame, CA, USA), ABC reagent (Vectastain Elite ABC Peroxidase Kits Mouse; PK-6102; Vector Laboratories, Inc.),...
and signal developed with DAB Chromagen Substrate Kit (K3468; Dako Corp., Carpinteria, CA, USA). All tissue sections were stained under similar conditions to ensure equal staining intensity.

In recent studies, our group used picrosirius red staining to evaluate new collagen production after different non-invasive modalities for skin rejuvenation.

Quantitative evaluation of stained tissues

Quantitative evaluation of picrosirius red-stained and IP-stained tissues was carried out using computer-based software (Image-Pro plus 6.1; Media Cybernetics, Inc., Silver Spring, MD, USA) to measure the percentage of positively stained dermis, as well as to detect the color density of staining; a representative square area of 2.5 × 2.5 cm was used to measure the color density for staining. All values were normalized to baseline. Picosirius red staining was evaluated using a Nikon microscope equipped with filters to provide circularly polarized illumination. When tissues are stained with picrosirius red and viewed under a polarized microscope, large, mature collagen fibers are seen to have stained red, whereas thinner fibers, which represent newly synthesized fibers, are stained yellow to orange.23,24

Statistical analysis

Data were tabulated and analyzed using SPSS Version 16 (SPSS, Inc., Chicago, IL, USA). Statistical analysis was performed using Wilcoxon matched-pairs signed ranks and one-way analysis of variance (ANOVA) tests. Correlations were studied using Pearson’s test to assess the correlation coefficient (r-value) and its significance. Data were expressed as the mean ± SD value. A P-value of <0.05 was considered to indicate statistical significance.

Results

Evaluation of TGF-β expression in response to treatment in all groups

Immunohistochemical staining for TGF-β revealed a statistically significant decrease in level from 9.5 ± 2.7% before treatment to 6.3 ± 1.9% at the end of RF treatment Group 1 (P < 0.001), which was followed by a significant increase in expression to 13.1 ± 3.2% (P < 0.0001) at three months post-treatment (Table 1, Figs. 1 and 2).

Assessment of immunohistochemically stained tissues for TGF-β showed a statistically significant increase in level from 8.8 ± 3.1% before treatment to 14.4 ± 2.7% at the end of Nd:YAG 1320-nm treatment Group 2; (P = 0.025); this was followed by a slight decrease in TGF-β level to 12.5 ± 1.9% (P = 0.186) at three months post-treatment, yet the difference was still statistically significant when compared with baseline values P = 0.031; (Table 1, Figs. 1 and 2).

Levels of TGF-β showed a statistically significant increase from 10.1 ± 1.4% before the Er:YAG 2940-nm mini-peel (Group 3) to 15.9 ± 2.3% at the end of treatment (P = 0.012); this was followed by a significant decrease in level to 12.6 ± 2.9% (P = 0.041) at three months post-treatment compared with the end-of-treatment value (Table 1, Figs. 1 and 2).

The level of TGF-β in volunteers treated with IPL (Group 4) did not show any significant difference at the end of treatment (8.2 ± 3.5%) or at three months post-treatment (7.6 ± 1.7%) compared with the pretreatment level 6.4 ± 2.6%; P = 0.316 and P = 0.534, respectively; (Table 1, Figs. 1 and 2).

Likewise, TGF-β expression showed no significant differences in levels prior to treatment (11.4 ± 2.2%), at

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<tr>
<th>Table 1 Quantitative evaluation of transforming growth factor-β (TGF-β) level in all groups (n = 36, n = 6 per group)</th>
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<td><strong>Level of TGF-β expression, %, mean ± SD</strong></td>
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<tr>
<td><strong>Baseline</strong></td>
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</tr>
<tr>
<td>Radiofrequency</td>
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<td>Nd:YAG 1320</td>
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<td>ELOS</td>
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SD, standard deviation; ELOS, electro-optical synergy.

*Baseline vs. end of treatment, P < 0.05.

bEnd of treatment vs. 3 months post-treatment, P < 0.05.

*Baseline vs. 3 months post-treatment, P < 0.05.
Report

TGF-β expression after non-invasive rejuvenation

El-Domyati et al.
the end of treatment (12.8 ± 2.6%; \( P = 0.159 \)), and at three months post-treatment (10.5 ± 1.6%; \( P = 0.371 \)) in the mesotherapy injection group Group 5; (Table 1, Figs. 1 and 2).

Analysis of immunohistochemical staining for TGF-β showed a statistically significant decrease in level from 11.1 ± 3.4% at baseline to 8.2 ± 2.1% at the end of ELOS treatment Group 6 (\( P = 0.039 \)). A significant increase in TGF-β expression to 15.3 ± 2.0% was noticed at three months post-treatment in comparison with baseline and at the end of treatment \( P < 0.001 \) and \( P = 0.0001 \), respectively (Table 1, Figs. 1 and 2).

Further evaluation of the changes in TGF-β expression between different treatment groups, at the end of Nd: YAG 1320-nm and Er:YAG 2940-nm mini-peel treatments, were statistically significantly higher than those in other groups (\( P < 0.001 \)). However, at three months post-treatment, the changes in TGF-β expression showed statistically significantly higher levels in response to RF and ELOS treatments in comparison with the other treatments \( P < 0.001 \); (Table 1).

**Correlation between TGF-β expression and collagen formation**

One aim of the present study was to investigate the effect of TGF-β expression on collagen formation. In recent studies,\(^2\)\(^-\)\(^9\)\(^-\)\(^11\)\(^-\)\(^21\) our group showed statistically significant, clinically objective improvements in signs of photoaging in the context of alterations in ECM proteins after the use of some modalities for the treatment of skin aging. Specifically, newly synthesized collagen production was reported to be significantly increased (\( P < 0.05 \)) at the end of RF, Nd:YAG 1320-nm and Er:YAG 2940-nm mini-peels and ELOS treatments\(^2\)\(^-\)\(^9\)\(^-\)\(^11\)\(^-\)\(^19\) but not after IPL or mesotherapy treatments\(^2\)\(^0\)\(^-\)\(^21\) (Table 2). Consequently, the correlation between the level of TGF-β expression and newly formed collagen was examined in the present study. TGF-β showed significant positive and linear correlation with newly synthesized collagen at the end of Nd:YAG 1320-nm (\( P < 0.001, r = 0.894 \)) and Er:YAG 2940-nm (\( P < 0.01, r = 0.825 \)) mini-peel treatments. Meanwhile, RF and ELOS treatments showed significant positive linear correlations between collagen and TGF-β expression at three months post-treatment (\( P < 0.001, r = 0.849 \) and \( r = 0.723 \); respectively). By contrast, IPL and mesotherapy did not show statistically significant correlations (\( P > 0.05 \)).

**Comparison of TGF-β expression between treatment and control groups**

Analysis of TGF-β in control subjects showed a statistically significant decrease in the level of TGF-β in facial skin from 15.2 ± 3.1% in Group A (7–30 years) to 10.9 ± 2.8% in Group B (31–60 years) and 6.4 ± 3.3% in Group C aged >60 years; (\( P < 0.05 \); Table 3 and Fig. 3).

The expression of TGF-β at the end of Nd:YAG 1320-nm and Er:YAG 2940-nm mini-peels showed statistically significantly (\( P < 0.05 \)) higher levels in comparison with those in control Groups B and C (aged 31–60 years and > 60 years, respectively), whereas no statistically significant differences were observed in comparison with

<table>
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<th>Table 2 Mean levels of newly synthesized collagen before and after treatment in all groups (( n = 36, n = 6 ) per group)</th>
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<td><strong>Dermal positive newly synthesized collagen, %, mean ± SD</strong></td>
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<td><strong>Baseline</strong></td>
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SD, standard deviation; ELOS, electro-optical synergy.

\(^a\)Baseline vs. end of treatment, \( P < 0.05 \).

\(^b\)End of treatment vs. 3 months post-treatment, \( P < 0.05 \).

\(^c\)Baseline vs. 3 months post-treatment, \( P < 0.05 \).
Group A controls (aged 7–30 years). The expression of TGF-β at three months post-treatment showed statistically significantly ($P < 0.05$) higher levels in response to RF and ELOS treatments in comparison with Group B (31–60 years) and Group C (>60 years) controls, whereas Nd:YAG 1320-nm and Er:YAG 2940-nm mini-peels showed statistically significantly ($P < 0.05$) lower levels at this time-point. With the exception of ELOS ($P = 0.736$), the levels of TGF-β after treatment in all groups were statistically significantly lower ($P < 0.05$) than those in Group A controls (7–30 years).

Nonablative as well as noninvasive techniques are new modalities used for skin rejuvenation, tightening, and scar remodeling. They promote the production of dermal ECM proteins without disruption of the epidermis, thus limiting adverse effects and minimizing downtime.

Previously published studies discussed the efficacy of noninvasive modalities; however, most of these were based on subjective evaluations. Their authors evaluated results with reference to improvements in the symptoms of skin aging (wrinkles, telangiectasia, and pigmented lesions) before and after treatment, or by comparing clinical outcomes, side effects, and patient downtime.

Signaling by TGF-β is important for ECM remodeling as it modulates re-epithelialization and deposition of new collagen, and stimulates expression of ECM in mesenchymal cells. Macrophages, endothelial cells, and fibroblasts are all sources of elevated TGF-β in injured skin. At the wound site, TGF-β signaling is thought to be important for ECM deposition and remodeling and thus to accelerate wound healing. In wound-healing processes, the isoforms of TGF-β (TGF-β₁,₂,₃) play a key role in all phases, and any absence or increase may disturb the wound-healing process.

To the best of our knowledge, no previous work has reported the quantitative assessment of changes in TGF-β expression after noninvasive rejuvenation. 

<table>
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<th>Level of expression, %, mean ± SD</th>
<th>TGF-β</th>
<th>Newly formed collagen</th>
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<tr>
<td>Group A control (7–30 years)</td>
<td>15.2 ± 3.1</td>
<td>25.2 ± 2.7</td>
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<tr>
<td>Group B control (31–60 years)</td>
<td>10.9 ± 2.8</td>
<td>14.6 ± 5.2</td>
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<tr>
<td>Group C control (&gt;60 years)</td>
<td>6.4 ± 3.3</td>
<td>11.4 ± 3.5</td>
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SD, standard deviation.

Table 3 Mean levels of transforming growth factor-β (TGF-β) and newly formed collagen in control groups ($n = 30$, $n = 10$ per group).

Figure 3 Expression of transforming growth factor-β (TGF-β) and newly formed collagen in control groups of different ages. Representative TGF-β immunostaining (top row) and picrosirius red staining (bottom row) show a decrease in TGF-β and in newly synthesized collagen formation with age. Levels of TGF-β (brown color in the dermis) and newly synthesized collagen (orange–yellow color) are lower in Group C (aged >60 years) than in Groups A (aged 7–30 years) and B (aged 31–60 years). (Original magnification: IP, ×300; picrosirius red, polarized light, ×200)
after different non-invasive rejuvenation modalities for ameliorating the effects of photoaging. The aim of the present study was to improve on subjective evaluations by using objective means to quantitatively highlight the role of TGF-β in collagen synthesis. Additionally, we aim to strengthen the findings of the present work by correlating TGF-β expression with the levels of newly synthesized collagen reported in our recent studies.

It has been reported that heat generated by RF affects the structural molecule of the collagen triple helix, with successive breakage of intramolecular hydrogen bonds, leading to collagen fibril denaturation with immediate contraction. Over time, as a thermally mediated healing response, fibroblasts are stimulated to increase new collagen deposition and remodeling, resulting in more collagen tightening and an overall increase in collagen content.

The mechanism of ELOS technology is based on two combined approaches: (i) a photothermal mechanism (IPL), in which the pulsed optical energy targets separate chromophores, resulting in differences in temperature between the target and the surrounding tissue, and (ii) the creation of stress waves at the skin surface (RF), leading to uniform heat at a controlled depth in the dermal layers. Thus, both energies generate a thermal wound at the targeted area with consequent remodeling and reorientation of collagen fibers and the synthesis of newly formed collagen over months.

In the present work, we show that TGF-β was significantly decreased or inhibited at the end of RF (P < 0.001) or ELOS (P < 0.05) treatments in comparison with baseline values. However, TGF-β started to increase significantly (P < 0.001) at three months post-treatment, reflecting the long-term effects of RF and ELOS on collagen deposition and remodeling after the cessation of treatment.

Unlike in other treatment groups (Nd:YAG 1320-nm and Er:YAG 2940-nm mini-peel, IPL and mesotherapy), our results showed a transient decrease in TGF-β at the end of RF and ELOS treatments. This unique response of TGF-β expression to RF and ELOS (combined RF and IPL) treatments may be attributable to the effect of electrical energy produced by RF, which creates a thermal wound in the dermis, and to the difference in the mechanism of action between RF and other treatments. These findings agree with those of Schultzze-Mosgau et al. and Helfrich et al., who reported that sun exposure decreases the level of TGF-β and causes a resultant decrease in procollagen production in photoaged skin. Consequently, increased TGF-β expression in response to non-invasive rejuvenation supports the beneficial effect of these modalities in promoting collagen production and remodeling.

Conclusions

Recent studies by our group using different non-invasive modalities for the treatment of photoaged facial skin reveal clinical improvements in wrinkles, as well as in ECM proteins after treatment with monopolar RF, ELOS, Nd:YAG 1320-nm laser, and Er:YAG 2940-nm laser but not with IPL and mesotherapy treatments.

The results of the present work provide quantitative histological evidence supporting the beneficial effects of non-invasive treatments for skin rejuvenation and elucidate a plausible mechanism of action of these modalities. As TGF-β is significantly (P < 0.05) correlated to newly synthesized collagen, dermal remodeling may reflect an initial stimulation of TGF-β production, which subsequently stimulates dermal fibroblasts to synthesize qualita-
tively normal collagen. These findings may suggest that TGF-β, in concert with other mediators, acts in harmony to regulate and maintain ECM remodeling and tissue repair.

References


