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Effect of platelet-rich plasma on ultraviolet b-induced skin wrinkles in nude mice

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KEYWORDS

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Summary *Background:* Platelet-rich plasma (PRP) is researched and used in many clinical fields as it contains an abundance of various growth factors. Recently, a topical injection of PRP has been clinically tried for treatment of photoageing-related skin wrinkles. Nevertheless, there have been only a few studies including objective data or explaining the mechanisms of PRP. Therefore, the authors performed animal experiments to collect laboratory data and to infer the basal mechanism of the effect of PRP on skin rejuvenation.

Methods: Mice photoaged by ultraviolet B (UVB) irradiation for 8 weeks were divided into three groups (no-treatment group, saline injected group and PRP-injected group) with 10 mice in each group. After 4 weeks, the degree of wrinkle formation was compared among three groups by replica analysis, and skin biopsies were performed. An additional *in vitro* assay with growth-factor-neutralising antibodies was performed to evaluate whether growth factors contained in PRP could accelerate fibroblast proliferation and collagen production, which may play a major role in skin rejuvenation.

Results: The wrinkles in the PRP-injected group were significantly reduced than in the other groups. Biopsy results indicated that the dermal layer was remarkably thicker in the PRP-injection group. In *in vitro* assay, fibroblast proliferation and collagen production were increased in the experimental group through growth factors in the PRP.

Conclusion: Although more *in vivo* studies and research about the mechanism of PRP are required, the results of this study indicate that PRP is effective in the rejuvenation of photoaged skin.

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Skin ageing consists of intrinsic and extrinsic ageing. Extrinsic ageing progresses due to environmental factors, which could be attributable to ultraviolet rays in most cases, and hence, extrinsic ageing is also called 'photo-ageing'.^{1–3} Extrinsic ageing is characterised by small and fine wrinkles, roughness, dryness, laxity and pigmentation. The characteristics of extrinsic ageing are the result of epidermal thinning, atypia of keratinocytes and collagen degradation in the dermal layer due to the increased synthesis of collagenase. Collagen production is decreased with the reduction of skin elasticity, and wrinkles are formed by the collapse of fibroblasts.⁴

Recently, platelet-rich plasma (PRP) is being widely researched in many clinical fields. In the field of plastic surgery, it is used to stimulate bone adhesion and wound healing, and to minimise bleeding during surgery by enhancing coagulation.^{5–12} Such functions can be attributable to the presence of rich growth factors in PRP, especially epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF β) and vascular endothelial growth factor (VEGF). It has been reported that the concentrations of these growth factors are several times higher than in normal plasma.^{13,14}

There have been many studies regarding the role of growth factors in the skin, and several growth factors have been reported to have positive effects on skin rejuvenation through stimulative effects on the proliferation of collagen and fibroblasts, and on the growth of keratinocytes.^{15–17} However, the production, transportation and storage of growth factors require high costs, and unintended complications can develop during the processes. Therefore, if growth factors can be derived from the patients themselves, superfluous costs could be saved and concerns of complications could be set aside to a great extent. Recently, various clinical trials for anti-wrinkle treatments have been conducted by using topical injections of PRP, based on this background knowledge. However, so far, studies conducted by analysing the effects of PRP on wrinkles with comparable control groups or experimental data concerning the mechanism of PRP function are very limited. It is questionable whether the favourable clinical effects are the result of PRP or if they are transient effects caused by oedema.

Therefore, we devised this current study to confirm the effects of PRP on wrinkles formed by photoageing through animal experiments. Moreover, *in vitro* studies were performed to confirm whether PRP stimulates fibroblast proliferation and collagen production, which are considered to have important roles in skin rejuvenation. By using various neutralising antibodies of growth factors, we tried to confirm whether or not growth factors in PRP could play major roles in the stimulation of the skin rejuvenation process to provide fundamental knowledge concerning the mechanism of the anti-wrinkling effects of PRP.

Materials and methods

Animals for experiments

A total of 30 female nude mice, aged 6 weeks, were purchased from Sankyo Laboservice Corporation Inc. (SLC, Tokyo, Japan). The experiment was approved by the Seoul

National University's Institutional Animal Care and Use Committee (IACUC), and the Guideline for the Care and Use of Laboratory Animals was observed.

Preparation of PRP

Under the approval of Institutional Review Board (IRB) of the Seoul National University Bundang Hospital, a 32-ml sample of venous blood was collected from a 28-year-old healthy male under informed consent. PRP was manufactured by using the commercialised materials of Regen PRP (Regen Laboratory, Mollens, Switzerland) and formulated by following the instructions of the manufacturer. After distributing 8 ml of peripheral venous blood into four sets of Anticoagulant Citrate Dextrose Solution A (ACD-A) tubes (8 ml per tube, each containing 0.9 ml of ACD-A solution; final blood:ACD ratio 10:1), blood plasma samples were collected by conducting 10 min of centrifugation by using a Regen bench centrifugation system. To analyse platelet count, the Sysmex XE-200 system (TOA Medical Electronics Co., Kobe, Japan) was used.

Fibroblast isolation

Skin biopsy was done at the dorsum of the nude mouse (1 cm \times 1 cm). Biopsy samples were washed 7–8 times with 5% antibiotics/antimycotics-added phosphate-buffered saline (PBS). After slicing the samples into small pieces, they were immersed in 0.92 unit dispase solution and incubated overnight at 4 °C. After separating the tissue into epidermal and dermal parts, the separated dermal layer was sliced into pieces as small as possible, and treated with 0.35% collagenase type I solution and incubated for 2 h in an incubator to carry out the reaction. The reaction solution was retrieved for centrifugation at 12 000 rpm for 5 min. After discarding the supernatants, the pellet was suspended in Dulbecco's Modified Eagle Medium (DMEM) and transferred to a culture dish. As the preparation reached about 80% confluence in a 37 °C CO₂ incubator, subculture was performed. The following experiments were conducted by using fibroblasts acquired from the third subculture.

Fibroblast proliferation

To instigate the proliferation of fibroblasts, the water-soluble tetrazolium salt (WST) technique was used. Initially, fibroblasts were washed twice with PBS, and treated with trypsin-ethylenediamine tetraacetic acid (EDTA) to detach cells. The detached cells were collected by adding DMEM and centrifuged for the seeding of 3×10^4 count of cells. After incubating the cells for 24 h by using DMEM media, the media was refreshed with serum-free

Table 1 Primer sequence

	Sequence
Collagen type 1	(F) CATCTCCCCTTCGTTTTTGA
	(R) CTGTGGAGGAGGGTTTCAGA
β -actin	(F) CCAAGGCCAACC GCGAGAAGATGAC
	(R) AGGGTACATGGTGGTGCCGCCAGAC

Table 2 Parameters used in assessment of wrinkles

Parameters	Description
Rt	Skin roughness
Rm	Maximum roughness
Rz	Average roughness
Rp	Smoothness roughness
Ra	Arithmetic average roughness

media. The preparation was divided into a total of six groups, including an untreated control group, a PRP-only treated group and four other PRP-treated groups that were prepared by adding only a single neutralising antibody among the selections of anti-PDGF-BB neutralising antibodies (Sigma, St. Louis, MO, USA), anti-EGF neutralising antibodies (Sigma, St. Louis, MO, USA), anti-VEGF neutralising antibodies (Sigma, St. Louis, MO, USA) and anti-TGF β neutralising antibodies (Sigma, St. Louis, MO, USA) ($n = 10$). On the 7th day, after adding PRP and selected neutralising antibody into each media, the absorbance was measured. Before the measurement, 200 μ l of fresh media was additionally added. After adding 10% of WST-8 (Dojindo, Kumamoto, Japan), the preparation was vortexed and placed in a 37 °C CO₂ incubator for 1–4 h. Afterwards, the absorbance was measured at 450 nm by using an enzyme-linked immunosorbent assay (ELISA) reader.

Collagen production

After seeding a 3×10^4 count of fibroblasts and incubating with DMEM media for 24 h, the media was replaced with a new fresh serum-free media. For the total of six test groups, PRP and preselected neutralising antibody ($n = 10$) were added to each media preparation. On the 7th day of incubation, 1 ml of the collagen assay kit (Biocolor Ltd., Belfast, UK) reagent was added with 100 μ l of culture soap. The preparation was well vortexed at the room temperature for 30 min and centrifuged at 10 000 rpm for 10 min. After centrifugation, the supernatant was discarded and the remaining pellet was thoroughly dried without moisture. For each pellet, 1 ml of alkali reagent was added and well dissolved, and 200 μ l of the preparation was transferred into a 96-well plate to measure the absorbance at 540 nm by using an ELISA reader.

Real-time polymerase chain reaction (RT-PCR)

The fibroblasts were detached and subsequently seeded in 1×10^6 culture media. After dividing the preparation into six groups as done in the other experiments, PRP and preselected neutralising antibody were added to each group. RNA was extracted on the 7th day. They were washed twice

with PBS and fibroblasts were lysed by adding lysis buffer. After adding 70% EtOH into the soup, the preparation was filled in a column and centrifuged at 10 000 rpm for 15 s and centrifuged again for 15 s at 10 000 rpm after adding buffer RW (washing buffer from RNeasy kit, Qiagen, Hilden, Germany). The samples were centrifuged for 15 s at 10 000 rpm after adding RPE buffer (washing buffer from RNeasy kit, Qiagen, Hilden, Germany), which was followed by centrifugation for 2 min at 10 000 rpm after adding RPE buffer. Subsequently, the preparations were centrifuged again for 1 min at 10 000 rpm. After adding 30 μ l of RNase-free water to the media, the isolated RNA fraction was collected into an Eppendorf (EP)-tube to measure RNA concentration. The measured total 1 μ g RNA was collected and added to an reverse transcriptase (RT) premix (Intron Biotechnology, Seoul, Korea), and 1–2 μ l of template DNA and 10 μ g primer (Table 1) were added, and distilled water (DW) was added to make a final volume of 20 μ l. After preparing 1.5% agarose gel, electrophoresis was performed and photographed after staining with ethidium bromide (EtBr).

Animal experiment

All mice were housed at a temperature of 24 °C and 50% humidity, and a 12/12 h light/dark cycle was maintained at a specific pathogen-free (SPF) animal laboratory facility at the medical science department of the Seoul National University. Mice were provided with sterilised water and fed without limitation, and monitored daily. The mice were irradiated with ultraviolet B (UVB) on the back 5 times a week for 8 weeks using an UVB-emitting system of Biolink BLX-312 UV crosslinker (Vilbert Lourmat, Marne-LaVallee, France). By using Toshiba SE lamps, the emission peak was achieved at a wavelength of 312 nm without filtering UVB, and 55% of the total UVB volume was controlled to be generated between the wavelengths of 290 nm and 320 nm. During exposure, the mice were allowed to move freely within the cage. The irradiation intensity represented as the minimal erythral dose (MED) was set at 1 MED during the first 2 weeks (60 mJ cm⁻²), and was elevated to 2 MED (120 mJ cm⁻²) in the 3rd week, to 3 MED (180 mJ cm⁻²) in the 4th week and to 4 MED (240 mJ cm⁻²) during the 5th–8th weeks of the experiment. The total irradiated UVB volume was approximately 115 MED (6.9 J cm⁻²).

After the generation of wrinkles, the mice were divided into three groups with 10 mice in each group. As the negative control, no special treatment was carried out for the first group. The second group was set as the positive control and 1 ml of subcutaneous injection of physiological saline was given to their backs. The mice of the third group were used as the experimental group, and were treated with 1 ml of subcutaneous injection of manufactured PRP on their backs.

Table 3 Fibroblast proliferation and collagen production ($n = 10$)

	Control	PRP	- PDGF-BB	- EGF	- VEGF	- TGF β
Fibroblast proliferation	3.145 \pm 0.27	3.391 \pm 0.11	3.228 \pm 0.25	3.199 \pm 0.16	3.256 \pm 0.13	3.202 \pm 0.09
Collagen production	0.084 \pm 0.01	2.25 \pm 0.23	1.821 \pm 0.12	1.474 \pm 0.37	1.840 \pm 0.32	1.166 \pm 0.18

- PDGF-BB: cultured with PRP and neutralising anti-PDGF-BB antibody, - EGF: cultured with PRP and neutralising anti-EGF antibody, - VEGF: cultured with PRP and neutralising anti-VEGF antibody, -TGF: cultured with PRP and neutralising anti-TGF β antibody.

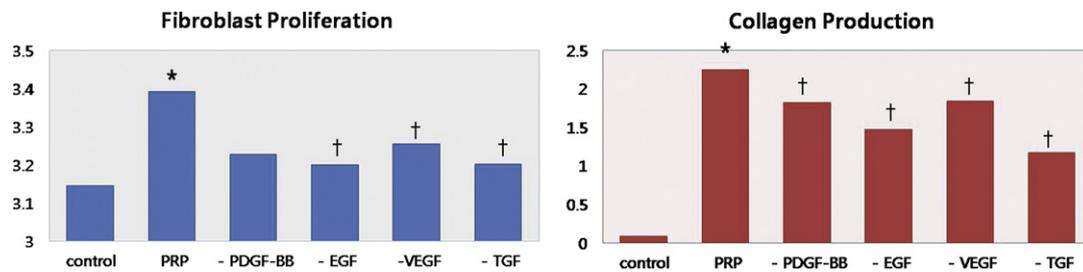


Figure 1 The amount of fibroblast proliferation (left) and collagen production (right) in each group. * $P < 0.05$ Compared with control group, † $P < 0.05$ Compared with PRP group, $n = 10$. - PDGF-BB: cultured with PRP and neutralising anti-PDGF-BB antibody, - EGF: cultured with PRP and neutralising anti-EGF antibody, - VEGF: cultured with PRP and neutralising anti-VEGF antibody, - TGF: cultured with PRP and neutralising anti-TGF β antibody.

Skin replica and wrinkle analysis

After 8 weeks of UVB irradiation and on the 4th week after injection, the back skin of the mice was replicated by using a silicone product of Flextime (Heraeus Kulzer, NY, USA). To simplify measurement, all replicas were trimmed into a circular form with a 1-cm diameter and images were analysed using the SV600 Skin visiometer wrinkle analysis system (Courage & Khaskasa, Cologne, Germany). The parameters used in the assessment of skin wrinkles are listed in Table 2.

Histology

At the 4th week after injection, the tissues were evaluated. Each 1 cm \times 1 cm piece of back skin was fixed with 10% formalin neutral buffered solution. After treatment with polyester wax, the skin samples were sliced into 6- μ m thicknesses. The sliced sections were treated with haematoxylin and eosin (H&E) and Masson's trichrome staining solutions. Through tissue evaluations, the thickness of the dermal layer and presence of collagen fibres were observed. The thickness of the dermal layer was calculated by measuring at five different sites from each section, and the mean value of the thickness of the dermal layer for each group was used for the comparison.

Statistical analysis

The results were expressed as mean \pm SD. Statistical analysis was performed by using an Statistical Package for Social Sciences (SPSS) program (SPSS, Inc., USA), and analysis of variance (ANOVA) and *post hoc* multiple

comparison tests were performed. When the p -value was found to be less than 0.05, the result was considered statistically significant.

Results

Platelet-rich plasma

The platelet count of the manufactured PRP was $1.341 \times 10^6 \text{ ml}^{-1}$, which was 4.6 times higher than that of normal whole blood ($130\text{--}400 \times 10^6 \text{ ml}^{-1}$).

Fibroblast proliferation and collagen production

Compared with the untreated control group, the PRP-treated group showed an increase of fibroblast proliferation and collagen production ($p < 0.05$) (Table 3). In the fibroblast proliferation assay, the PRP- and neutralising antibody-treated group showed a decrease in fibroblast proliferation in the order of VEGF, PDGF-BB, TGF β and EGF antibody. Excluding the PDGF-BB-treated group, the other antibody-treated groups showed statistically significant reduction compared with the PRP-treated group. In the collagen production assay, the decrease of the production was significant in the order of VEGF, PDGF-BB, EGF and TGF β . The reductions observed from all groups were found to be statistically significant compared with the PRP group (Figure 1).

Real-time polymerase chain reaction (RT-PCR)

The intensity of the Collagen I RNA band was found to be reduced in the order of PRP group, VEGF-added group and

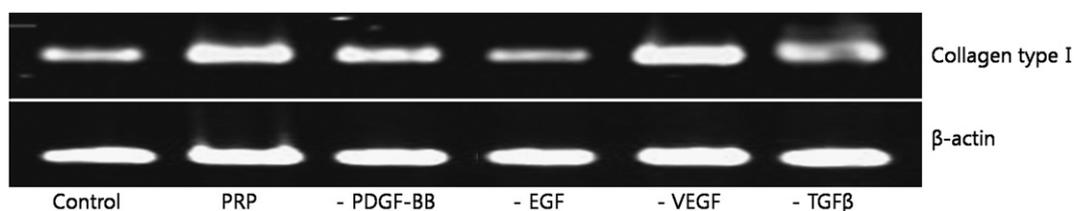


Figure 2 Effect of PRP with each neutralising antibody on the expression of collagen type I RNA.

Table 4 Wrinkle analysis (n = 10)

	T0				T4			
	NT	NS	PRP	P	NT	NS	PRP	P
Rt	2.29 ± 0.5	2.31 ± 0.55	2.04 ± 0.42	0.423	2.12 ± 0.72	2.23 ± 0.53	1.22 ± 0.37	0.001
Rm	1.9 ± 0.34	1.88 ± 0.3	1.8 ± 0.36	0.719	1.85 ± 0.62	1.91 ± 0.37	1.08 ± 0.3	<0.001
Rz	1.41 ± 0.27	1.32 ± 0.2	1.3 ± 0.26	0.588	1.33 ± 0.45	1.42 ± 0.23	0.76 ± 0.22	<0.001
Rp	0.94 ± 0.3	0.86 ± 0.2	0.79 ± 0.21	0.355	0.78 ± 0.3	0.88 ± 0.2	0.44 ± 0.15	<0.001
Ra	0.45 ± 0.13	0.45 ± 0.14	0.39 ± 0.1	0.418	0.37 ± 0.14	0.41 ± 0.1	0.2 ± 0.08	0.001

T0: initial value.

T4: value after 4 weeks.

The unit of R is arbitrary unit.

PDGF-BB-added group, EGF-added group and TGFβ-added group and control group (Figure 2). This result was consistent with the results observed from the collagen production assay.

Wrinkle analysis

The five sets of wrinkle parameters that were measured after 8 weeks of photoageing did not show a statistically

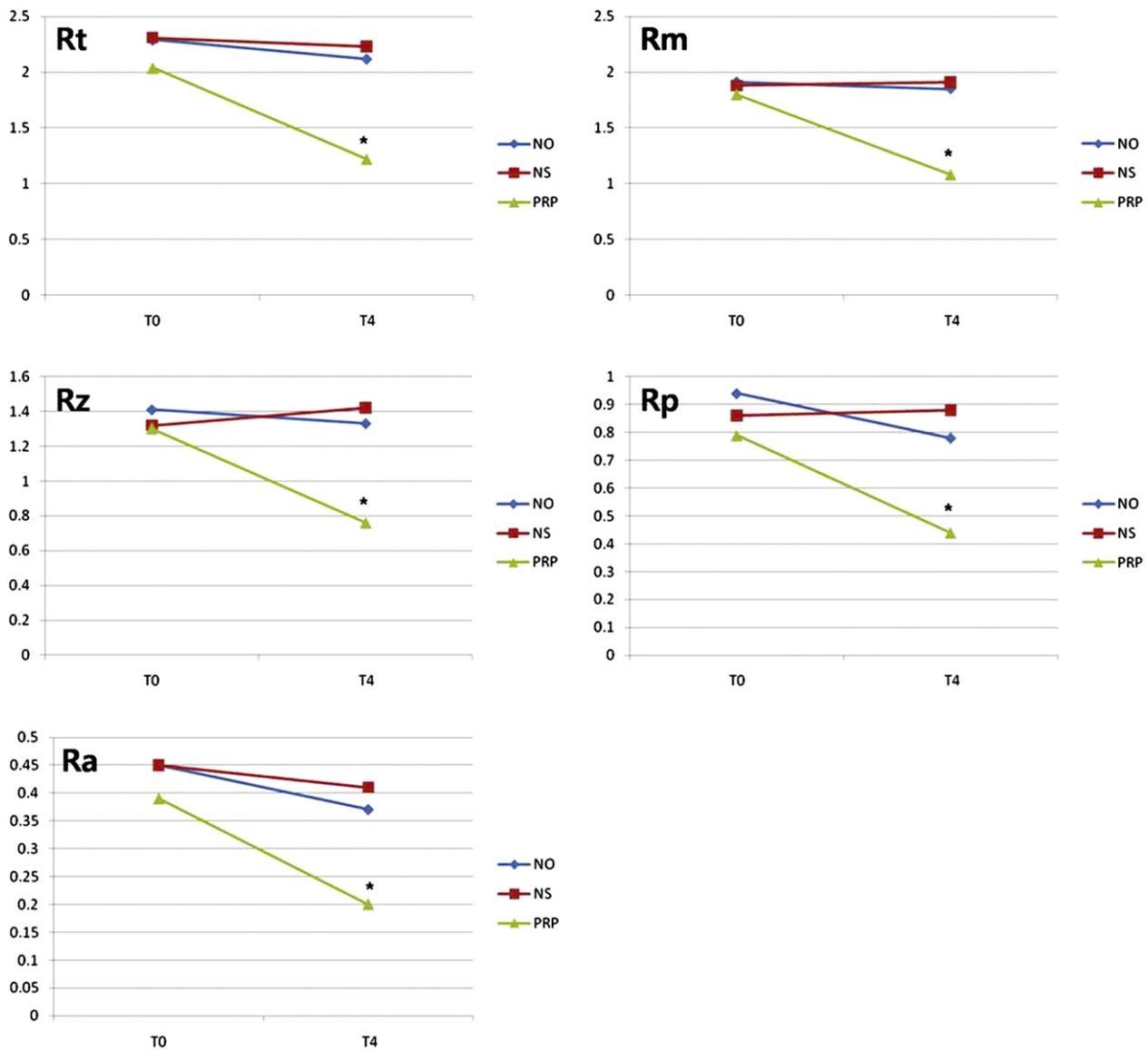


Figure 3 The changes of wrinkle parameter in each group. NO: no-treatment, NS: normal saline, PRP: platelet-rich plasma, T0: initial value, T4: value after 4 weeks, * $P < 0.05$ Compared with other group.

significant difference between groups (Table 4). The wrinkle parameters that were measured after 4 weeks of experiment revealed that all parameters of the PRP-injected group were significantly lower than the no-treatment and the saline-injected groups ($p < 0.05$). No statistically significant difference was observed between the no-treatment group and the saline-injected group (Figures 3 and 4).

Histological observation

The dermal thickness of the PRP-injected group was 284 ± 16.2 , which was significantly increased compared with the non-treated group (196 ± 14.2) and the saline-injected positive control group (212 ± 17.4) ($p < 0.05$) (Figures 5 and 6). Although the dermal thickness of the physiological saline-injected group was thicker than the non-treated negative control group, it was not statistically significant.

Discussion

The platelet count of the PRP produced in this current experiment was 4.6 times higher than the basal value. This increase satisfies the condition that the platelet count of PRP be 3–7 times that of the basal value, which has been suggested by many researchers.^{6,18–20} Comparing the platelet concentration level of results reported in many researches to the current experiment, it was possible to confirm indirectly that the current experiment produced PRP-containing rich growth factor.^{21–23}

Lorraine H. Kligman, while studying photoageing in an animal model, exposed the mice to UVB rays and subsequently performed histological studies. After UV exposure, initial histological analysis revealed that an increase in metabolism and in fibroblasts led to an increase in collagen synthesis; however, over the course of time, severe damage in mature collagen was observed under electronmicroscopy and through Gieson's staining. The results were similar to

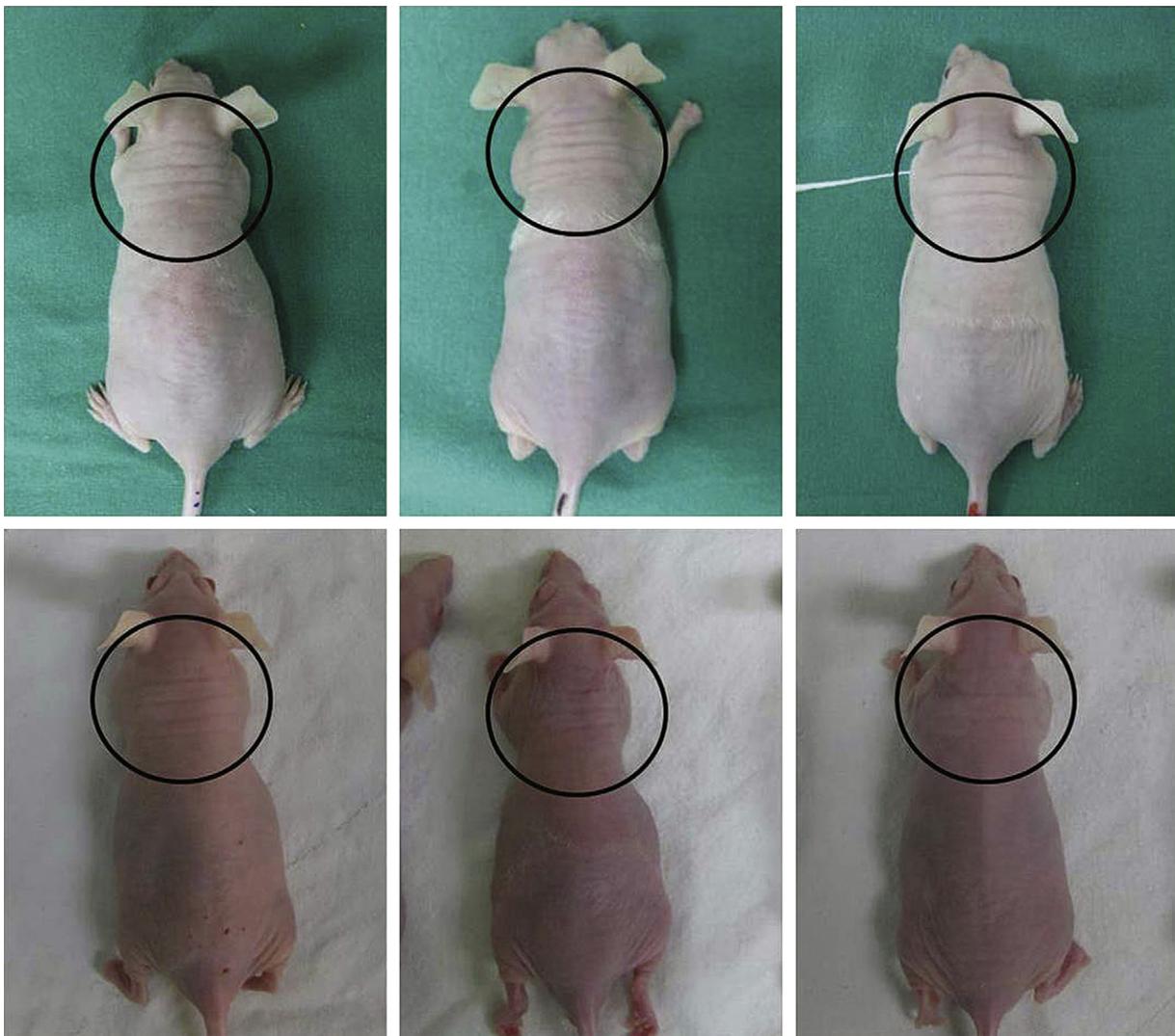


Figure 4 Wrinkles in (above, left) initial non-treatment group, (above, centre) initial normal saline injected group, and (above, right) initial PRP injected group. Wrinkles changes in (below, left) no-treatment group after 4 weeks, (below, centre) normal saline injected group after 4 weeks, and (below, right) PRP injected group after 4 weeks.

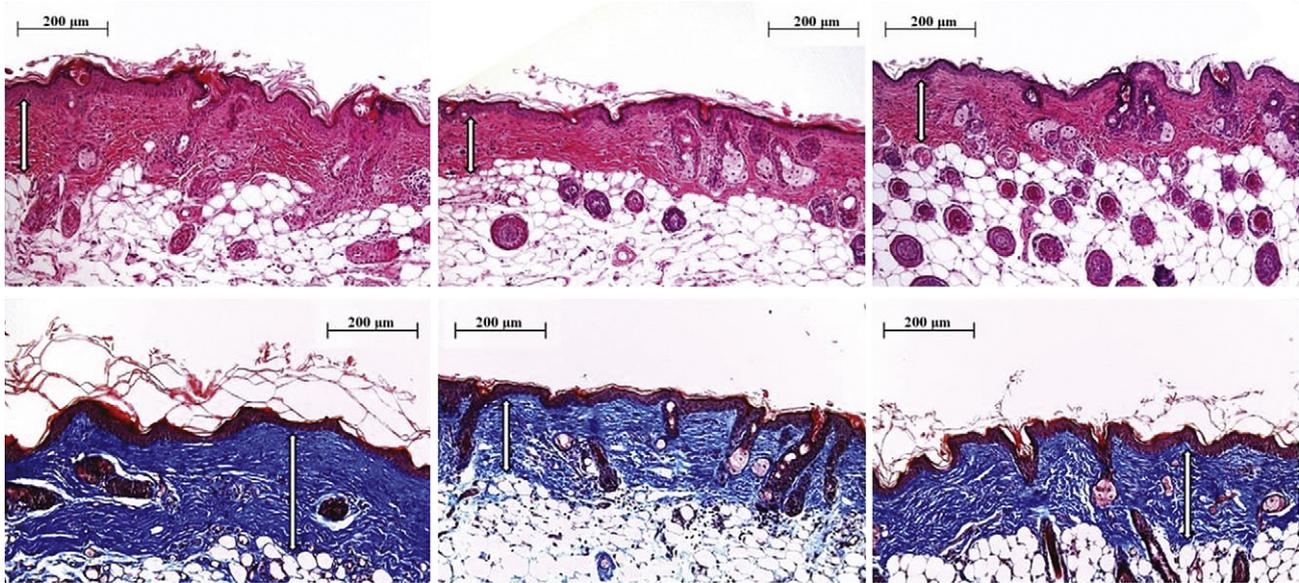


Figure 5 H&E staining shows that dermal thickness in the PRP injected group (above, left) was thicker than those in the non-treatment group (above, centre) and the saline injection group (above, right). In the Masson's trichrome staining, collagen fibres were stained blue, and collagen contents were increased in the PRP injected group (below, left) compared to those in the no-treatment group (below, centre) and the saline injected group (below, right). Scale bars are 200 µm.

histological changes in human skin after exposure to natural sunlight.²⁴

Much time is required to induce photoageing through natural exposure to UV light. The amount of UVB exposure in experimental studies does not usually equate to average sun exposure. Therefore, Cho et al. used a fixed protocol to induce photoageing while studying the anti-wrinkling effects of vitamin C, vitamin E, pycnogenol and evening primrose oil on UVB-induced skin wrinkles. The authors applied this protocol in the preparation of this study.²⁵

Rejuvenation of skin injury caused by UV light is a complex process that organically involves several cytokines and growth factors such as in a wound-healing process. The involved cytokines and growth factors tend to function by interacting with other several factors and control proteins rather than function independently. Five major growth factors such as TGF, insulin-like growth factor (IGF), PDGF, EGF and VEGF have been known to be related to the wound-healing processes. The growth factors released from platelets initiate the interaction, and the release is continuously stimulated by inflammatory cells, fibroblasts and epithelial cells to maintain the wound-healing process.¹⁵ The most important rejuvenation process for photoaged skin is the collagen remodelling process, and dermal fibroblasts are known to have the most important function. The production of collagen of fibroblasts is stimulated by many growth factors including IGF, EGF, interleukin-1 (IL-1) and tumour necrosis factor (TNF)- α . *In vivo* studies report TGF β to be the most stimulative growth factor.^{15,26} The reported results are consistent with the results of the current experiment.

Fibroblast proliferation and collagen production were increased in the PRP-treated group than in other groups. On the other hand, the amount of increase was reduced in each neutralising antibody-treated group than in the PRP-

only treated group. In this study, we can assume that PRP could rejuvenate skin through the action of growth factors.

The significance of wrinkle parameters used in the result analysis can be summarised below. Rt is the distance from the highest wrinkle height to the lowest wrinkle height among wrinkle valleys, which indicates skin roughness. Rm is the highest Rt value that was observed from five equally dissected portions of a wrinkle valley, which indicates maximum roughness. Rz is the mean Rt value that was calculated from Rt values observed from five equally dissected portions of a wrinkle bent, which indicates average roughness. The reading error caused by replica shape is small in the case of Rz than Rt. Rp is the distance of wrinkle height from the parallel line made at the highest wrinkle height to the middle line located below, which indicates smoothness or roughness. Ra is the distance of a wrinkle after drawing two parallel lines at the top and bottom of a middle line. The wrinkle distance meets the

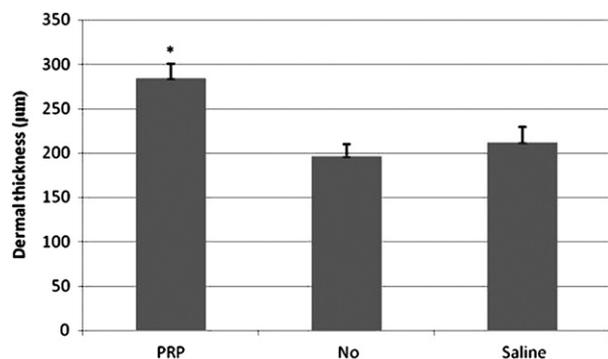


Figure 6 Dermal thickness in H&E staining. PRP injection increased dermal thickness. * $P < 0.05$ compared to no-treatment group and saline injected group.

parallel lines and represents the average deviation of Rp values, which indicates the arithmetic average roughness. Rt, Rm and Rz are the values that represent wrinkle depth. Rp and Ra represent skin roughness and shallow wrinkle depth. Therefore, a smaller R value indicates shallow depth of wrinkles and more regularity of the skin surface.

The results acquired from the current study showed that all the R-values measured 4 weeks after the PRP injection were higher than in the non-treated group or the saline-injected group. In other words, wrinkles in the PRP-injected group showed statistically significant reduction than in other groups. In addition, no statistically significant difference was observed between the saline-injected group and the non-treated group.

According to Coleman et al., the duration of oedema after filler injection is less than 1 week. Although a 4-week experiment is insufficient to evaluate the long-term effects of PRP injection, the effects on photoageing cannot be explained only by a transient volume effect.²⁷

Based upon these results, the PRP effect is not considered to be a transient volume effect, and the effect caused by oedema disappeared within 4 weeks.

Photoageing is a complex process that shares many pathological features in common with skin wounds.^{28,29} Deformation of the extracellular matrix is one of the histological characteristics of photoaged skin, and alterations and reductions in collagen components cause reductions in skin strength and elasticity.^{30,31} During the course of rejuvenation of skin injuries, dermal fibroblasts have been known to play key roles through the interaction of keratinocytes, adipocytes and mast cells. In addition, fibroblasts produce extracellular matrix, glycoprotein, adhesive molecules and various cytokines.³² By providing these molecules and maintaining the interactions between the cells, dermal fibroblasts stimulate wound healing and play essential roles in maintaining skin youth. By using lasers or localised medications, many anti-ageing treatments have focussed on the activation of fibroblasts to stimulate interactions between cells and increase the production of extracellular matrix.

Histological results and *in vitro* assay results suggest an approximate mechanism of PRP regarding skin rejuvenation. Through organic and complex interactions between growth factors, such as TGF, EGF and PDGF, present in PRP, the proliferation of fibroblasts is stimulated. Fibroblasts activate the skin rejuvenation process by helping the migration and proliferation of other cells, and produce collagen and extracellular matrix that are key factors in skin rejuvenation.

PRP is used for dermal augmentation, used in combination with free fat injection and in many other clinical applications. Sclafani used PRP for dermal augmentation and observed aesthetic improvements of the nasolabial fold in less than 2 weeks and the results lasted for up to 3 months.³³ Further, Cervelli added PRP to fat grafts and obtained aesthetic results in facial contouring and in the soft tissue of the skin surface, which lasted for up to 18 months.³⁴ Controlled animal studies of soft and hard tissues have suggested that the application of autogenous PRP can enhance wound healing. Nevertheless, healing of hard and soft tissue is mediated by a complex array of intracellular and extracellular events that are regulated by many signalling proteins, a process that is, at present, incompletely understood.³⁵ Especially, further studies are

needed to investigate the mechanism of the aesthetic effect of PRP including the long-term effect in human skin wrinkles. Although future studies would be required, the results of this study confirm that PRP injection is effective to relieve skin wrinkles caused by photoageing and suggest the basic mechanism of skin rejuvenation.

Conflict of interest

None.

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Disclosure

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