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# Fluid platelet-rich fibrin stimulates greater dermal skin fibroblast cell migration, proliferation, and collagen synthesis when compared to platelet-rich plasma

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# Abstract

**Background:** Regenerative therapies in the field of facial aesthetics have become a growing field of interest with many recent advancements made over the past decade to meet the growing worldwide demand. While first versions of platelet-derived concentrates were formulated with anticoagulants (PRP), recent modifications to centrifugation speeds and times have permitted the development of a liquid platelet-rich fibrin (fluid-PRF) without use of anticoagulants.

**Objective:** To compare this entirely natural platelet concentrate (fluid-PRF) to formally utilized PRP on skin cell behavior and regeneration.

**Methods:** Dermal skin fibroblast was cultivated with either fluid-PRF or PRP and investigated for their ability to promote/influence cell viability, migration, spreading, proliferation, and mRNA levels of known mediators of dermal biology including PDGF, TGF-beta, and fibronectin.

**Results:** All platelet concentrates were nontoxic to cells demonstrating high cell survival. Skin fibroblasts migrated over 350% more in fluid-PRF when compared to control and PRP (200% increase). Fluid-PRF also significantly induced greater cell proliferation at 5 days. While both PRP and fluid-PRF induced significantly elevated cell mRNA levels of PDGF, it was observed that TGF-beta, collagen 1, and fibronectin mRNA levels were all significantly highest in the fluid-PRF group. Lastly, fluid-PRF demonstrated a significantly greater ability to induce collagen matrix synthesis when compared to PRP.

**Conclusion:** The findings from the present study demonstrate greater regenerative potential of fluid-PRF on human skin fibroblasts. Future clinical use of fluid-PRF in the field of facial aesthetics is necessary to further evaluate the potential advantages of anticoagulant removal from platelet concentrates.

#### KEYWORDS

facial aesthetics, injectable PRF, i-PRF, liquid PRF, platelet-rich fibrin, PRF, skin rejuvenation

Wang and Yang have contributed equally to this work.

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The aging process of skin is an inevitable progression that occurs as we gradually age.<sup>1</sup> This may be characterized by a decline in regenerative properties and a disruption in facial remodeling resulting in a net loss of tissue described in the literature as resorption.<sup>2</sup> Over time, total collagen content within the skin also decreases resulting in a qualitative degeneration of tissue components including dermal collagen and elastin.<sup>3</sup> These processes result in three-dimensional alterations in facial shape and contour; skin laxity, folds, and rhytides; and surface changes, including skin roughness and xerosis.<sup>3</sup>

Due to the ever-growing demand for facial rejuvenation and aesthetics, regeneration of aging tissues will remain a high priority with new technologies and devices constantly evolving. One such technology associated with positive treatment outcomes has been autologous mesotherapy with blood derivatives.<sup>4,5</sup> Platelet-rich plasma (PRP) has been utilized routinely over the past two decades as an effective adjunct to various medical procedures.<sup>6</sup> Platelet-rich plasma (PRP) has seen widespread use owing to its autologous consistency derived from blood enriched in platelets, cytokines, and growth factors following centrifugation at high g-forces using anticoagulants.<sup>7</sup> Since its introduction, PRP has been used in many areas of medicine, for example, for the management of wounds, for soft tissue injuries, in the field of maxillofacial surgery, for oral and periodontal surgery, and in orthopaedics, gastrointestinal surgery, and burns. In more recent years, PRP has been utilized for a wide range of dermatological indications including scar revision, facial wound healing, fat grafting, alopecia, increasing dermal volume, and skin rejuvenation.<sup>6,8</sup> In the field of facial aesthetics, PRP is utilized as a minimally invasive injectable material to fill tissue volume voids and augment lost or missing dermal tissues. Several growth factors are released from  $\alpha$ -granules of platelets including TGF, PDGF, VEGF, IGF-1, EGF, and FGF, secreted after the activation of platelets by aggregation initiators.<sup>9</sup> Several growth factors work by stimulating the process of fibroblast collagen synthesis.<sup>10</sup> Despite the numerous reported indications and benefits of PRP, concerns have been raised regarding their incorporation of anticoagulants, known suppressors of tissue regeneration.<sup>11,12</sup>

Platelet-rich fibrin (PRF) is a second-generation platelet concentrate consisting of platelets and growth factors harvested blood, however without the use of anticoagulants.<sup>13,14</sup> PRF is achieved with a simplified preparation, with no biochemical manipulation of blood, and follows only one centrifugation cycle. PRF retains various growth factors in a fibrin mesh structure that, during the tissue remodeling process, will gradually release growth factors and cytokines capable of further enhancing wound healing.<sup>13,14</sup> Therefore, PRF not only supports hemostasis and wound healing but also favors the natural wound healing process. Over the past decade, PRF has gained tremendous momentum having been utilized for a variety of dental and medical procedures.<sup>15</sup> In medical aesthetics, PRF has been utilized for the management of facial soft tissue defects, facial defects, deep nasolabial folds, superficial rhytids, lipostructure surgical procedures, and acne scars.<sup>15</sup> Reported advantages for combining medical

procedure with PRF include faster revascularization of the defect. faster wound healing, and complete immune biocompatibility.<sup>15</sup> One of the reported difficulties reported with utilizing PRF as opposed to PRP was that PRF is a three-dimensional scaffold, whereas PRP can be injected directly into host tissues due to its fluid consistency. Interestingly, very recently, a fluid formulation of PRF (termed fluid-PRF) contains fibrinogen and thrombin prior to its conversion to solid fibrin.<sup>13</sup> While no anticoagulants are used, fluid-PRF remains fluid for 15 minutes and may therefore be utilized as an injectable material where it rapidly coagulates and adds fibrin volume shortly thereafter. These properties make PRP ideal for a number of medical uses, for example, in the field of facial aesthetics where it can be used to promote revascularization of host tissues and is utilized as a minimally invasive 100% natural injectable material. The aim of the present preclinical study was therefore to investigate the regenerative potential of fluid-PRF in comparison with traditional standard PRP. Human skin fibroblasts were therefore assessed for their ability to promote dermal skin cell migration, proliferation, cellular mRNA levels of known mediators and constituents of dermal biology, and collagen synthesis in vitro.

### 2 | MATERIALS AND METHODS

#### 2.1 | Preparation of PRP and fluid-PRF

Blood was collected from laboratory members who volunteered to donate whole blood samples with an ethical approval by our University's Internal Review Board (IRB). PRP was prepared as previously described.<sup>16</sup> First, PRP and platelet-poor plasma (PPP) portions were separated from the red blood cell (RBC) fraction by centrifuging 10ml of whole blood with anticoagulant (EDTA) at 900 g for 5 min, and then, PRP was separated from PPP by centrifuging second time at 2000 g for 15 minutes. Finally, approximately 1 mL of PRP was collected. Fluid-PRF was produced using 10 mL of whole blood without anticoagulant, and centrifugation was carried out at 60 g for 3 minutes. The upper layer consisting of approximately 1 mL of plasma was designated as fluid-PRF. We then transferred the PRP and fluid-PRF to six-well culture dishes and added 5 mL culture media (DMEM; HyClone, Thermo Fisher Scientific Inc) and processed as further described.

#### 2.2 | Isolation of human dermal fibroblasts

Normal facial skin was harvested from three human donors undergoing cleft lip repair surgery. The fibroblast cells were obtained from above the lip on the skin surface. Signed ethical approval and consent were obtained from the donor's parent following approval by the IRB. Collected samples were rinsed three times with phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM sodium phosphate, pH 7.2) that contained 1% antibiotics (100 U/mL penicillin G, 100 µg/mL streptomycin, HyClone) and fractionated into small pieces using sterile surgical scissors. The skin tissue pieces were then transferred into T25 culture flasks with DMEM containing 20% fetal bovine serum (FBS; Gibco, Life Technologies Corporation) with 1% antibiotics in an incubator at 37°C under 5% CO<sub>2</sub>. On the 7th day, cells were seen around the

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skin tissue pieces. Once cell confluency was reached, skin fibroblasts were trypsinized and further cultured in DMEM with 10% FBS. For all experiments, cells from passage three through seven were used.

# 2.3 | Conditioned media from PRP/fluid-PRF and cell culture

PRP and fluid-PRF were incubated for 3 days at 37°C in a 5% CO<sub>2</sub> atmosphere, and thereafter, conditioned media were harvested and utilized in future experiments as 20% of the total volume as previously described.<sup>16</sup> Human skin fibroblasts were cultured in growth medium containing DMEM, 10% FBS, and 1% antibiotics with/without 20% conditioned media from fluid-PRF or PRP at a density of 10 000 cells per well for cell viability, cell migration, cell morphology, and proliferation experiments and a density of 50 000 cells per well for real-time PCR and collagen immunofluorescent staining in 24-well plates. The medium was changed two times per week for experiments >5 days in length.

## 2.4 | Cell viability

At 24 hours post-cell seeding, human skin fibroblasts were costained with 2  $\mu$ mol/L membrane-permeable dye calcein AM (Dojindo) and 4  $\mu$ mol/L propidium iodide (PI, Sigma) for 15 minutes at 37°C. Live cells were stained in green with calcein AM, and dead cells were stained in red with PI. Fluorescent images were captured with an Olympus DP71 fluorescent microscope (Olympus Co.). Thereafter, percentages of live versus dead cells with PRP and fluid-PRF were reported.

#### 2.5 | Cell migration assay

For the cell migration assay, 24-well plates and polyethylene terephthalate cell culture inserts with a pore size of 8  $\mu$ m were utilized (Costar, Corning Inc). Briefly, the lower compartment of the wells was filled with 20% PRP or with 20% fluid-PRF. After starving the cells in DMEM containing 0.5% FBS for 12 hours, 10 000 cells were resuspended and seeded in the upper compartment. After 24 hours, 4% formaldehyde was utilized to fix cells for 15 minutes and stained with 0.1% crystal violet solution for 10 minutes (GoodBio Technology Co., Ltd). The upper side of the filter membrane was rinsed and gently wiped by a cotton swab to remove the cell debris. Images on the lower side of the filter were taken under an Olympus DP72 microscope (Olympus Co).

### 2.6 | Cell morphology

For cell morphology, human skin fibroblasts were seeded at a density of 10 000 cells with/without PRP or fluid-PRF (20% concentration) in 24-well plates. At 2, 4, 8, and 24 hours, cells were fixed with 4% formaldehyde followed by rinsing with PBS for 5 minutes. Then, cells were stained with 5  $\mu$ g/mL phalloidin-FITC (green fluorescence, Sigma-Aldrich) for 1 hour in dark conditions at 37°C as previously described.<sup>16</sup> Nuclei were stained with DAPI (blue fluorescence). Fluorescent images were captured using an Olympus DP71 fluorescence microscope (Olympus Co) and compared for morphological differences. Analysis of cell surface area was performed by ImageJ software.

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# 2.7 | Cell proliferation assays

The Cell Counting Kit-8 (Dojindo) was utilized for proliferation assays of human skin fibroblasts. Briefly, human skin fibroblasts were seeded in 24-well plates at a density of 10 000 cells per well with 20% culture medium from PRP or fluid-PRF. At 1 day, 3 days, and 5 days, cell numbers were determined using the Cell Counting Kit-8 and measured at 450 nm using a microplate reader (PowerWave XS2, BioTek, Winooski) as previously described.<sup>17</sup> The experiment was performed in triplicate with three independent experiments.

#### 2.8 | Real-time PCR analysis

To investigate the expression of regeneration-related and ECM-related genes, platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF- $\beta$ ), collagen type I alpha1 (COL1a1), and fibronectin (FN1) were analyzed by real-time PCR. All the specific primers and GAPDH genes of human are listed in Table 1.

The human skin fibroblasts were cultured with/without PRP or fluid-PRF (20% concentration) in 24-well plate at a density of 50 000 cells per well. After 3 and 7 days of culture, total RNA was extracted from cells using AxyPrep<sup>TM</sup> Multisource Total RNA Miniprep Kit (AXYGEN) according to the manufacturer's protocol. The RNA concentration was determined by a NanoDrop 2000 UV-Vis Spectrophotometer as previously described. A total of 1 µg RNA solution was used as template for the synthesis of cDNA with First Strand cDNA Synthesis Kit (GeneCopoeia), and the final volume is 100 µL. The real-time PCR amplification reaction utilizing All-in-One<sup>TM</sup> qPCR Mix Kit (GeneCopoeia) was performed on a CFX Connect<sup>™</sup> Real-Time PCR Detection System. Gene expression levels relative to house-keeping gene GAPDH were normalized to the control group. Data were log-transformed using the delta-delta CT method prior to one-way ANOVA with the Bonferroni test using GraphPad software v. 6 (GraphPad Software). The experiments were performed in triplicate with three independent experiments.

ТΑ	BLE	1	List of	primer	sequences	for rea	l-time	PCF
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Genes	Primer sequence
hPDGF F	CACACCTCCTCGCTGTAGTATTTA
hPDGF R	GTTATCGGTGTAAATGTCATCCAA
hTGF-β F	ACTACTACGCCAAGGAGGTCAC
hTGF-β R	TGCTTGAACTTGTCATAGATTTCG
hCOL1A1 F	TCTAGACATGTTCAGCTTTGTGGAC
hCOL1A1 R	TCTGTACGCAGGTGATTGGTG
hFN1 F	ACCTACGGATGACTCGTGCTTTGA
hFN1 R	CAAAGCCTAAGCACTGGCACAACA
hGAPDH F	GCACCGTCAAGGCTGAGAAC
hGAPDH R	TGGTGAAGACGCCAGTGGA

#### 2.9 | Collagen type I staining

Human skin fibroblasts were cultured in a 24-well plate at a density of 50 000 cells per well for 7 days. Cells were rinsed with PBS and fixed with 4% formaldehyde for 10 minutes. Cells were then permeabilized with 0.5% Triton X-100 (Merck) in PBS for 3 minutes at room temperature. Subsequently, cells were incubated with polyclonal rabbit to collagen type I (1:100, Boster Biological Technology Ltd) diluted in PBS containing 2% bovine serum albumin (BSA) for 1 hour, followed by incubation with FITC-conjugated goat anti-rabbit (1:200, Invitrogen) diluted in PBS for 1 hour. Finally, nuclei were stained by DAPI (blue fluorescence). After each step, the cells were washed with PBS three times. Images were taken using an Olympus DP71 fluorescence microscope (Olympus Co.). Quantitative analysis of staining intensity was performed by ImageJ software.

# 2.10 | Statistical analysis

Statistical analysis was performed by one-way ANOVA with the Bonferroni test using GraphPad software v. 6, and statistical significance was considered at P < 0.05. All data are expressed as the mean ± SE.

# 3 | RESULTS

#### 3.1 | Cell viability

First, the effects of fluid-PRF and PRP on skin dermal cell viability were studied. Human skin fibroblasts were costained by calcein AM (green fluorescence depicting live cells) and propidium iodide (red fluorescence depicting dead cells) with/without PRP or fluid-PRF. After 24 hours, >95% of the cells remained viable, with no significant differences observed between groups demonstrating that both PRP and fluid-PRF exhibited no toxicity with respect to cell survival (Figure 1).

#### 3.2 | Cell migration

Since the recruitment and migration of human skin fibroblasts play an important role during early wound healing, the effects of fluid-PRF and PRP on early cell migration were examined using a transwell assay. As shown in Figure 2, both PRP and fluid-PRF significantly stimulated the migration of human skin fibroblasts at 24 hours. PRP significantly induced a 200% upregulation in migration when compared to the control group, whereas fluid-PRF significantly induced a >300% increase in migration when compared to the control and significantly higher than PRP (Figure 2).

#### 3.3 | Cell morphology and proliferation

Human skin fibroblasts were then investigated for morphological differences with/without PRP and fluid-PRF at 2, 4, 8, and 24 hours postseeding. Both PRP and fluid-PRF promoted skin fibroblast



**FIGURE 1** Live/dead assay of human skin fibroblasts treated with PRP or fluid-PRF at 24 hours (A) Merged fluorescent images of cells costained with calcein AM (green fluorescence represents live cells) and PI (red fluorescence represents dead cells; scale bars =  $100 \ \mu$ m); (B) the percentage of living cells quantified with live/dead assay. No significant difference was observed among the three groups. Assay was performed in triplicate with three independent experiments



**FIGURE 2** (A, B) Migration assay of human skin fibroblasts cultured with fluid-PRF and PRP after 24 hours (scale bars =  $100 \mu m$ ; \* denotes significant difference between two groups, P < 0.05, and \*\* denotes significantly higher than all other treatment groups, P < 0.05.) Assay was performed in triplicate with three independent experiments

spreading at 2, 4, and 8 hours, while no significant difference was observed at 24 hours (Figure 3). Analysis of cell surface area confirmed these trends (Figure 3).

Then, the effects of PRP and fluid-PRF on skin cell proliferation were investigated. While differences at 1 day between groups were

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not significant, both PRP and fluid-PRF significantly increased cell proliferation of human skin fibroblasts at 3 and 5 days postseeding. Fluid-PRF further demonstrated significantly highest cell numbers at 5 days (Figure 3).

# 3.4 | Expression of human skin fibroblast regeneration-related and ECM-related genes

Next, the effect of PRP and fluid-PRF on the expression of regeneration-related genes including TGF- $\beta$  and PDGF was evaluated by realtime PCR (Figure 4, 4). Both PRP and fluid-PRF induced an increase in PDGF and TGF- $\beta$  expression at 3 and 7 days, while fluid-PRF demonstrated the significantly highest PDGF mRNA levels at 3 days and TGF- $\beta$  mRNA levels at 3 and 7 days postseeding (Figure 4, 4). Thereafter, ECM-related genes including collagen 1 (COL1) and fibronectin (FN1) expression of human skin fibroblast were evaluated (Figure 4, 4). While PRP was able to significantly increase the expression of COL1 at 7 days when compared to control group, fluid-PRF significantly increased COL1 mRNA levels at 3 and 7 days when compared to all other groups (Figure 4). Both PRP and fluid-PRF significantly induced mRNA expression of FN1, with fluid-PRF demonstrating the significantly highest levels when compared to all other groups (Figure 4).

# 3.5 | Collagen type I staining

To evaluate the effect of PRP and fluid-PRF on collagen synthesis by human skin fibroblasts, collagen type 1 immunofluorescent staining was performed. As presented in Figure 5, PRP showed a slight significant increase in intracellular staining of collagen type 1, whereas fluid-PRF induced a roughly twofold increase in collagen type 1 staining among the three groups and significantly higher than all other modalities (Figure 5).

# 4 | DISCUSSION

In the present study, the regenerative potential of an injectable platelet concentrate formulated without anticoagulants (fluid-PRF) was investigated on skin fibroblast activity when compared to standard PRP. To the best of the authors' knowledge, this is the first regenerative study to date investigating the use of fluid-PRF in the field of facial aesthetics either in a preclinical model or in a clinical setting. The purpose was to first test and characterize the regenerative potential of fluid-PRF in vitro on dermal skin fibroblasts when compared to the current blood-derived standard (PRP) in the field.

One of the main advantages of using PRF as a regenerative modality in various fields of regenerative medicine is its low cost in comparison with other regenerative modalities or recombinant growth factors/hormones.<sup>15</sup> Since blood is collected without the use of anticoagulants, this therapy is entirely autologous forming a fibrin clot either during centrifugation or slightly afterward depending on the centrifugation technique utilized. Original PRF scaffolds were originally centrifuged for 12 minutes at 2700 RPM (roughly 700 g-force at the RCF-max).<sup>18</sup> While PRF as a scaffold material has gained tremendous popularity in various fields of medicine and dentistry, it has been shown that high centrifugation



**FIGURE 3** Effects of PRP and fluid-PRF on the morphology and proliferation of human skin fibroblasts (A) Cell morphology at 2, 4, 8, and 24 hours; (B) average surface planar area of cells (C) Cell proliferation at 1, 3, and 5 days (\* denotes significant difference between two groups, P < 0.05, and \*\* denotes significantly higher than all other treatment groups, P < 0.05.) Assay was performed in triplicate with three independent experiments



**FIGURE 4** Expression of regenerationrelated and ECM-related genes of gingival fibroblasts cultured with PRP and fluid-PRF at 3 and 7 days (A) PDGF, (B) TGF- $\beta$ , (C) COL1, and (D) FN1 (\* denotes significant difference between two groups, P < 0.05, and \*\* denotes significantly higher than all other treatment groups, P < 0.05.) Assay was performed in triplicate with three independent experiments



**FIGURE 5** Immunofluorescent collagen type 1 (COL1) staining of skin fibroblasts cultured with PRP and fluid-PRF at 7 days (A) COL1 staining (green) merged with DAPI staining (blue; scale bars =  $100 \ \mu$ m) (B) COL1 staining quantification (\* denotes significant difference between two groups, *P* < 0.05, and \*\* denotes significantly higher than all other treatment groups, *P* < 0.05.) Assay was performed in triplicate with three independent experiments

speeds result in a net loss of leukocytes and other growth factors, which are driven down the centrifugation tubes as a result of excessive g-force.<sup>19</sup> For these reasons, it was recently proposed to utilize lower centrifugation speeds to improve the quantity of leukocytes and growth factors contained within PRF scaffolds.<sup>19,20</sup> During this development, one interesting finding was observed: The plasma layer could be separated in a fluid formulation prior to

the formation of a fibrin clot consisting of fibrinogen and thrombin. This fluid state of PRF can be utilized for roughly 15 minutes and has therefore been utilized as an injectable of various fields of medicine, for example, in dental medicine to combine with various biomaterials, orthopedic injections in osteoarthritic knees, and very recently in the field of facial aesthetics.

This study investigated in a cell culture model the effects of either PRP or fluid-PRF on skin fibroblast behavior. Previously, PRP has been utilized in various fields of regenerative medicine, dentistry, and facial aesthetics due to its perceived ability to increase new blood flow to defective/damaged tissues.<sup>21</sup> Not surprisingly, both platelet formulations were deemed nontoxic to cells and did not affect cell death/apoptosis after 24 hours of culture with either PRP or fluid-PRF (Figure 1). Thereafter, the migration of cells was investigated using a transwell assay where it was found that fluid-PRF had the ability to significantly increase the migration of fibroblasts when compared to PRP (Figure 2). In a clinical context, this means that following facial aesthetics with fluid-PRF, more local regenerative cells may be recruited to the defective tissues following injection. Furthermore, it was also found that cells tended to proliferate at higher levels when compared to PRP, both necessary actions for tissue regeneration.

mRNA levels of growth factors and extracellular matrix proteins important to dermal fibroblast behavior were thereafter compared by real-time PCR (Figure 4). It was found that in general, fluid-PRF increased mRNA levels of these associated genes. Interestingly, our group recently showed that fluid-PRF contained higher levels of growth factors within its PRF scaffolds when compared to PRP. The present findings further show that not only certain growth factors are found at higher levels within fluid-PRF scaffolds, but also cells exposed to fluid-PRF produce higher mRNA levels of growth factors.

What remains of interest is to fully characterize the specific function of leukocytes contained within PRF scaffolds. Our research group recently showed that lower centrifugation speeds produced a higher leukocyte count within fluid-PRF<sup>13,14</sup>; however, the specific role of leukocytes during tissue regeneration and wound healing remains somewhat unclear. Interestingly, leukocytes have the added advantages that they are immune cells responsible for host defense and resistance against incoming pathogens during the healing process.<sup>22</sup> They also secrete a wide variety of growth factors associated with tissue regeneration.<sup>22</sup> Lastly (and potentially most importantly), three separate studies by three independent groups recently showed that when leukocytes were added to standard PRP preparations (PRP typically contains low concentrations of leukocytes), wound healing was drastically improved.<sup>23-25</sup> Therefore, despite their exact role remaining somewhat unclear, various previous studies suggest they play a vastly important role during wound healing.

While the present study remains a preclinical study, the data show that fluid-PRF supports skin fibroblast migration and proliferation, increases cellular mRNA levels of potent regenerative growth factors and extracellular matrix molecules, and further improves collagen synthesis. Future animal research is necessary to further determine the cell types recruited following local injection of fluid-PRF in vivo. Further study is warranted to evaluate its clinical performance in the field of facial aesthetic as well as long-term stability when utilized for aesthetic reasons.

#### CONFLICT OF INTEREST

All other authors declare no conflict of interest.

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