

## Research Article

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# The Addition of Hyaluronic Acid into Platelet-Rich Fibrin Lysate in Restoration of Senescent Human Dermal Fibroblasts Activities

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## ABSTRACT

Senescent human dermal fibroblasts had reduced capacity in proliferation and collagen synthesis. It is due to unresponsiveness against transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) stimulation. Either platelet-rich fibrin (PRF)-lysate or hyaluronic acid (HA) can restore TGF- $\beta$ 1 signaling pathway.

To determine whether HA addition to PRF lysate has a better activity than PRF-lysate alone in restoring senescent human dermal fibroblasts (HDFs) activities.

HDF isolated from six different human skins was divided into normal HDFs and senescent HDFs which are induced by serum starvation. The senescent groups were then given 50% PRF-lysate and various levels of HA. Amelioration of TGF- $\beta$ 1 signaling was measured by cellular proliferation index and collagen deposition.

Addition of HA into PRF-lysate resulted in a significant increase in proliferation index and collagen deposition index than PRF-lysate alone. The best level of HA for this mixture ranged from 20.83 mM to 41.67 mM. HA in PRF lysate is an excellent candidate material for treating clinical signs related to senescent human dermal fibroblasts.

**Key words:** Collagen deposition, hyaluronic acid, platelet-rich fibrin lysate, proliferation index, senescent human dermal fibroblasts

**Ethical permission:** This experiment had gain approval from the local ethical committee, Ref: KE/FK/471/EC/2016 dated 17-05-2016.

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## INTRODUCTION

Failure of chronic ulcer healing (Harding *et al.*, 2005; Clark, 2008) and wrinkle lines in the photoaged skin (Naru *et al.*, 2005; Yin B *et al.*, 2013) are both resulting from the presence of senescent fibroblasts. Senescent fibroblasts either isolated from chronic ulcers (Cowin *et al.*, 2001; Kim *et al.*, 2003) or from photo-aged skin (Quan *et al.*, 2004) are unresponsive towards transforming growth factor (TGF)- $\beta$ 1 stimulation due to down-regulation of membrane-TGF- $\beta$ 1 receptor gene expressions. Impaired TGF- $\beta$ 1 signaling in human dermal fibroblasts (HDFs) leads to reduced proliferation and collagen synthesis (Czuwara-Ladykowska *et al.*, 2001). Decreasing of dermal collagen content effect on fibroblasts microenvironment mechanical forces to shrink and to cause further hiding of the remaining TGF- $\beta$ 1 receptors (Quan *et al.*, 2013).

Previous studies showed that the administration of platelet-derived growth factor (PDGF) restores gene expression of TGF- $\beta$ 1 receptors (Czuwara-Ladykowska *et al.*, 2001; Pan *et al.*, 2010) where high levels of PDGF can be obtained from the secretion factors of platelets (Anitua *et al.*, 2009). Moreover, the high concentration of platelets can be obtained from platelet-rich fibrin (Dohan *et al.*, 2009) and a previous study showed that 50% PRF lysate (PRF lysate)

can restore the amelioration of chronically UVA-irradiated human dermal fibroblasts as a model of UV-induced senescent HDFs (Wirohadidjojo *et al.*, 2016).

Furthermore, hyaluronic acid (HA) has the capability to restore fibroblasts microenvironment mechanical forces indicated by fibroblasts elongation, and this event is associated with up-regulation of TGF- $\beta$  signaling pathway. Structural properties of the dermal extracellular matrix play a significant role in modulating fibroblasts function (Quan *et al.*, 2013). In this study, the effect of HA addition into PRF-lysate in proliferation index and collagen deposition of senescent HDFs is reported.

## MATERIAL AND METHODS

### Isolation and culture of human dermal fibroblasts

Human dermal fibroblasts were obtained from human skin from 6 different individuals with informed consent. After mechanical removal of epidermal tissue from the skin, dermal parts were cut in 2-4 mm<sup>3</sup> size, placed into a culture flask, immersed in a small amount of growth medium consisting of high glucose Dulbecco's minimal essential medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS-Gibco) and 1% penicillin/streptomycin (Gibco), then incubated at 37°C until tissues were attached on the bottom of the flask. The medium was then exchanged with ten mL of fresh medium every 72 hours until 60% of the HDFs were outgrowing. The human dermal fibroblasts were then harvested and replicated until tertiary passage.

### Induction of cellular senescence

Senescence of HDFs were artificially driven by culturing HDFs from these individuals in DMEM containing 1% fetal bovine serum for 48 hours. This treatment resembled previous studies (Alcorta *et al.*, 1996 and Ramirez *et al.*, 2001). Two hundreds  $\mu$ L of  $5 \times 10^3$  HDFs/mL suspension in DMEM+10% FBS was placed in all 96 well plates, incubated for 24 hours in 37°C and 5% CO<sub>2</sub>. The medium was then aspirated, rinsed with PBS and filled with DMEM containing 1% FBS-Gibco for 48 hours.

### Collection of platelet-rich fibrin (PRF) lysate

PRF was isolated from peripheral blood of a volunteer by a standard protocol (Dohan *et al.*, 2009). Isolation of PRF lysate was done by incubating PRF for 24 hours with 4°C temperature, using applied method from the study by He *et al.*, 2009.

### Hyaluronic acid

Hyaluronic acid (Z fill deep®, New-Ulm, Germany) which consisted of hyaluronic acid 23 mg/ml (Molecular weight 3 Mio. Daltons). Based on previous study, the best level of HA in stimulation of senescent HDFs is 20.83 mM (Sansan *et al.*, 2015). Therefore, this experiment started to add 20.83 mM HA into 50 % PRF-lysate.

## Experiments

### Treatments

Senescent HDFs were treated with 200  $\mu$ L of 50% PRF lysate, 20.83 mM HA; 41.67 mM HA; 83.33 mM HA and addition of those levels of HA into 50% of PRF lysate (50% PRF lysate+20.83mM HA; 50% PRF lysate+41.67 mM HA and 50% PRF lysate+83.33 mM HA) diluted in DMEM supplemented with 1% FBS. The negative control consisted of DMEM plus 1% FBS. As a control, normal fibroblasts were cultured in growth medium (DMEM plus 10% FBS). All of the cells were cultivate for 72 hours in 37°C in 5% CO<sub>2</sub>.

### Measurement

#### Proliferation Index

Cellular viability was measured using a MTT (-(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The medium was removed, and the cells were washed with PBS. 200  $\mu$ L complete medium plus 50  $\mu$ L of (5 mg/mL) MTT (MP biomedical-France) was added to each well. The plates were wrapped with aluminum foil and incubated at 37°C in 5% CO<sub>2</sub> for 4 hours. The medium and MTT then removed and the remaining MTT-formazan was dissolved by adding 200  $\mu$ L DMSO (Dimethyl sulfoxide). The optical density resulting from the formazan in each well was measured at 570 nm using a spectrophotometer.

$$\text{Determination of proliferation rate} = \frac{\text{The OD of experimental groups}}{\text{The OD of paired normal Fibroblasts}} \times 100\%$$

### Cellular collagen deposition rate

The collagen deposition assay was based on an insoluble collagen of Sirius red assay following Taskiran *et al.*, 1999. After treatment, those well were briefly washed with PBS, and the cells were fixated with Bouin solution for one hour. The wells were then washed off with tap water and allowed to dry at room temperature overnight. Two

hundred microliters of 0.1 % Sirius red in saturated picric acid (Sigma-Aldrich, USA) was added to each well for one hour. The un-bound Sirius red was washed for four times, using 200  $\mu$ L 0,1 N HCL. The Sirius red bound to collagen was dissolved using 200  $\mu$ L of 0.5 N NaOH, and the optical density was read using a spectrometer at 570 nm. Collagen deposition rate among experimental groups was calculated based on following formula:

$$\text{Collagen deposition} = \frac{\text{The OD of experimental groups}}{\text{The OD of Normal Fibroblasts}} \times 100\%$$

### Statistics

All the results are shown as average  $\pm$  standard error. Analysis and comparison among groups was conducted with ANOVA test followed by LSD for post hoc test or Friedman depending on the normality of data distributions.  $P < 0.05$  was considered significant.

## RESULTS

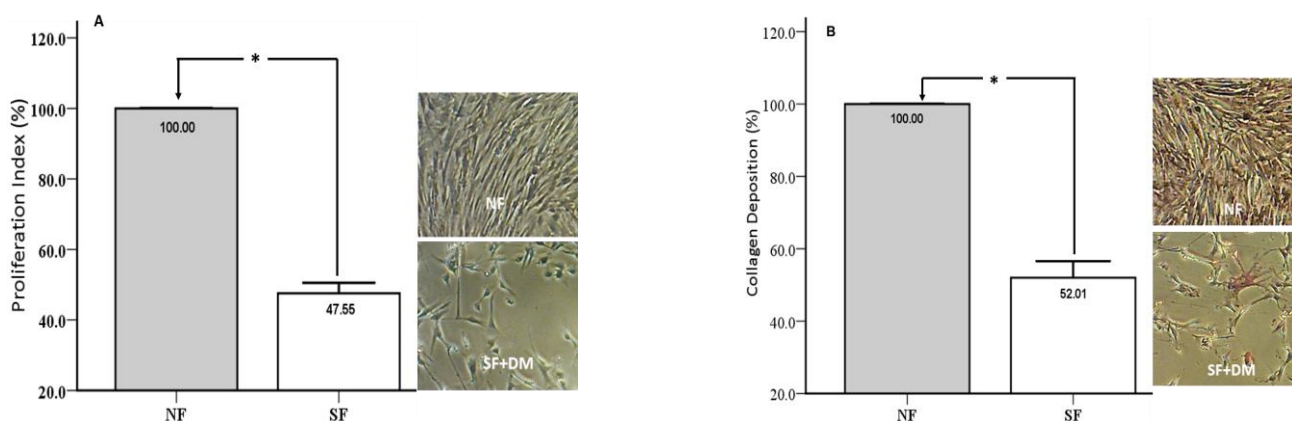


Fig.1: Characteristic of senescent HDFs induced by serum starvation

(A) Proliferation Index, (B). Collagen Deposition; NF= normal fibroblasts; SF=starved fibroblasts; DM=dilution medium; \* $p < 0.05$

On this figure, serum starvation of HDFs in dilution medium for 48 hours significantly inhibits ( $p < 0.05$ ) the cellular proliferation index and collagen deposition. They also presented larger and more polygonal cells morphology.

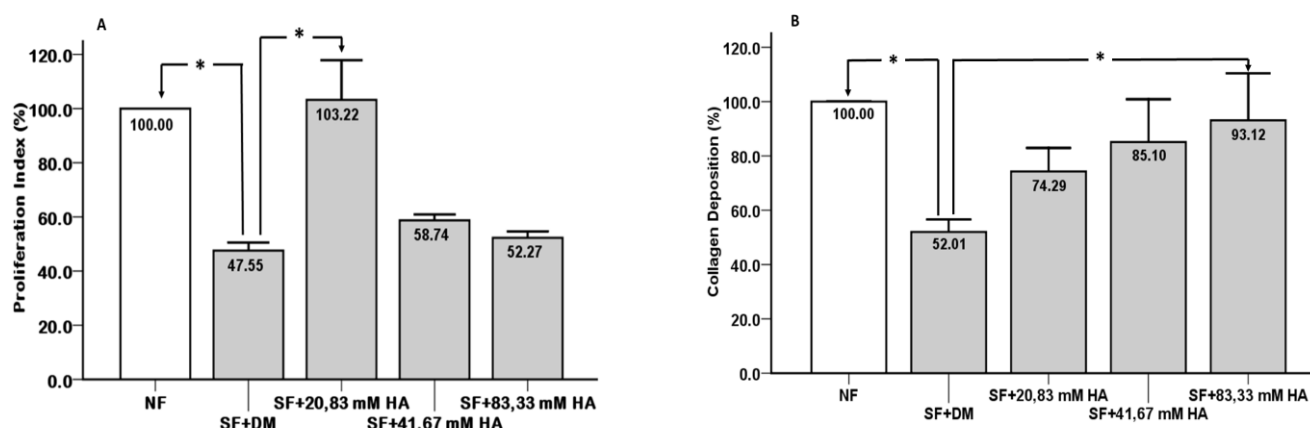


Fig. 2: The effect of hyaluronic acid on senescent HDFs (A) proliferation index and (B) collagen deposition.

NF=normal fibroblasts; SF=starved fibroblasts; DM=dilution medium; HA=hyaluronic acid;

\* $p < 0.05$ .

This figure shows that HA could elevate proliferation index and collagen deposition of senescent HDFs significantly ( $p < 0.05$ ) compared to dilution medium (negative control). The highest proliferation was observed in medium containing 20.83 mM HA (Fig. 2A). On the other hand, the most significant fibroblasts collagen deposition was observed in medium containing 83.33 mM HA (Fig. 2B).

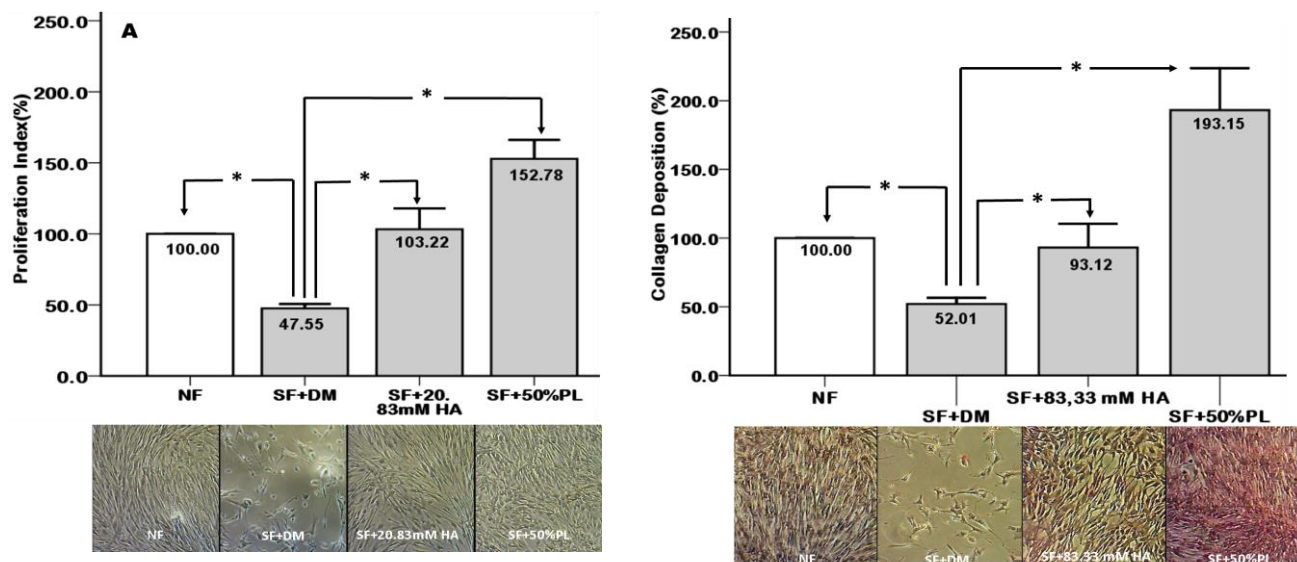


Fig. 3: The effect of hyaluronic acid and 50%PRF lysate on senescent HDFs

(A) Proliferation index and (B) collagen deposition

NF= normal fibroblasts; SF= starved fibroblasts; DM= dilution medium; HA= hyaluronic acid; PL= platelet lysate;

\*p<0.05

In Figure 3, there is a higher elevation of senescent HDFs proliferation index and collagen deposition cultured in 50% PRF lysate compared to those cultured in HA. However, this difference is not statistically significant ( $P>0.05$ ). Senescent HDFs cultured in 50% PRF lysate appeared to be more compact and denser compared to those cultivated in HA.

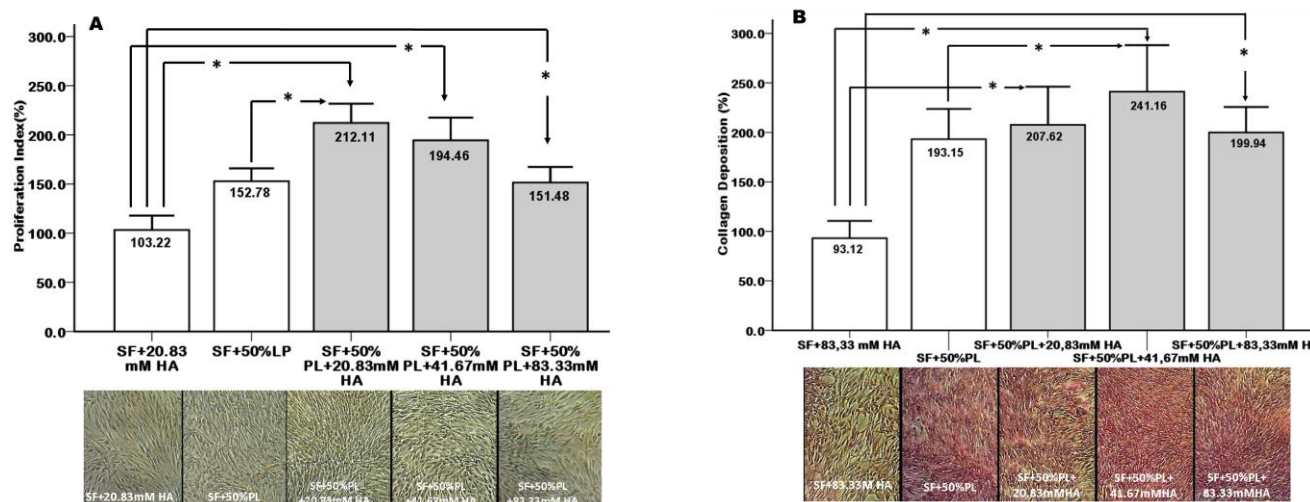


Fig. 4: The effect of addition of HA into 50% PRF lysate on senescent HDFs

(A) proliferation index and (B) collagen deposition,

SF= Starved fibroblasts; HA=hyaluronic acid; PL= PRF lysate;

\*p<0.05

The figure above shows that addition of HA into PRF-lysate gave a significantly better proliferation index and collagen deposition index than PRF-lysate alone. The highest proliferation index appeared on senescent HDFs cultured in 50% PRF lysate + 20.83 mM HA (Fig. 4A). However, the highest collagen deposition was observed in senescent HDFs cultured in 50% PRF lysate +41.67 mM HA (Fig. 4B).

The figure below shows that the best medium to restore senescent HDFs activities (proliferation index and collagen deposition) were the combination of 50% PRF lysate + HA. This medium had significantly different values for both normal fibroblasts and starved fibroblasts cultured in dilution medium (negative control), 20.83 mM HA, or 50% PRF lysate. We can also find the best and densest fibroblasts morphology in this medium combination.



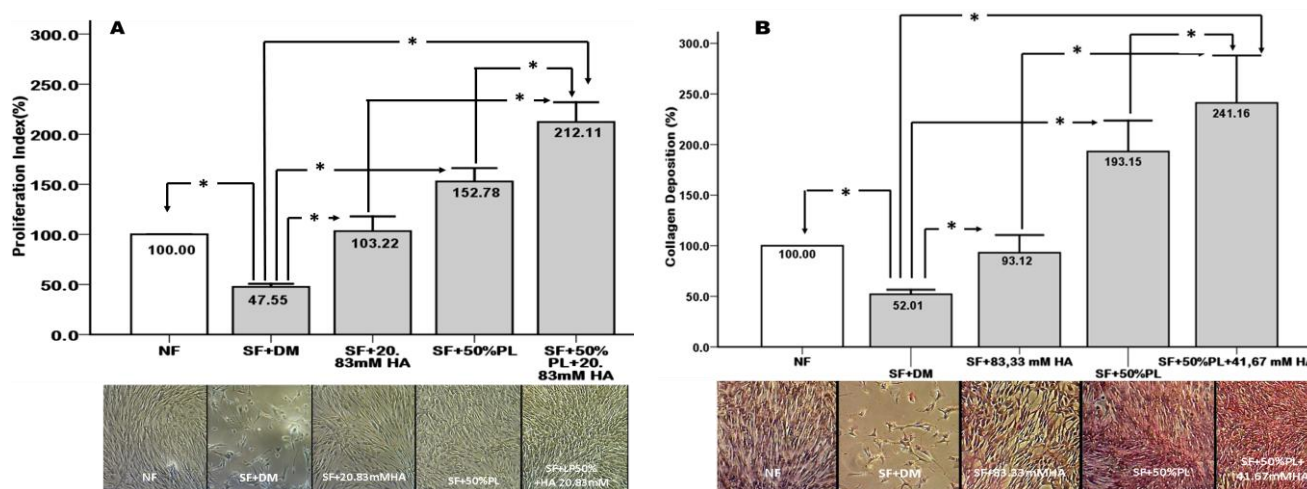


Fig. 5: Effect of HA, 50% PRF lysate and addition of HA into 50%PRF lysate on senescent HDFs

(A) proliferation index and (B) collagen deposition;

NF=normal fibroblasts; SF=starved fibroblasts; DM= dilution medium; HA= hyaluronic acid; PL= PRF lysate;

\* $p < 0.05$ .

## DISCUSSION

Serum starvation can produce artificially senescent HDFs (Alcorta *et al.*, 1996; Wang *et al.*, 2007). A similar event was also found in this study (Fig.1), specifically that serum starvation of HDFs in dilution medium can significantly inhibit ( $p < 0.05$ ) the cellular proliferation index and collagen deposition, possibly due to the dilution medium ability to decrease overall cellular function. Senescent HDFs are described as larger and polygonal in shaped compared with compact, spindle shape of normal skin fibroblasts (Agren *et al.*, 1999). A similar event was also found in this study. Senescent HDFs showed a larger and more polygonal morphology (Fig. 1).

Senescent HDFs displayed a decreased proliferative response to growth factors due to impaired intracellular signaling. Recently, increasing evidence suggests that senescent fibroblasts are unresponsive to the stimulatory action of transforming growth factor beta 1 (TGF- $\beta$ 1). It has been showed that some of the mechanisms that might be responsible for HDFs unresponsiveness to TGF- $\beta$ 1 included the decrease of TGF- $\beta$  type II receptor expression and abnormalities in the downstream signaling pathway involving MAPK and the early Smad pathway (Kim *et al.*, 2003). TGF- $\beta$ - Smad pathway is the fibroblast's main regulator for the proliferation as well as synthesis of type I procollagen (Capelo *et al.*, 2005). In senescent HDFs, reduced TGF- $\beta$  signaling and CTGF/CCN2 expression contribute to decreased collagen production (Quan *et al.*, 2006; Quan *et al.*, 2010).

Incubation of platelet concentrate at 4°C for 24 hours produces the high concentration of PDGF-BB, epidermal growth factor and TGF- $\beta$ 1 (He *et al.*, 2009). Those growth factors effectively stimulate both collagen and hyaluronic acid production by dermal fibroblasts (Kim *et al.*, 2014). Growth factors, such as PDGF-BB and TGF- $\beta$  are key regulators of cellular functions, including proliferation, migration, and differentiation. Growth factor signaling is modulated by context-dependent cross-talk between different signaling pathways. PDGF-BB- mediated Smad 2 phosphorylation was dependent on the kinase activities of both TGF- $\beta$  type I receptor (T $\beta$ RI) and PDGF  $\beta$ -receptor (PDGFR $\beta$ ) (Porsch *et al.*, 2014). TGF- $\beta$  is a ubiquitous, multifunctional cytokines that play a crucial role in regulating pre-collagen synthesis. TGF- $\beta$  initiates its cellular action by binding to specific cell surface receptor complexes typically composed of TGF- $\beta$  type I (T $\beta$ RI) and TGF- $\beta$  typeII (T $\beta$ RII). Binding of TGF- $\beta$  to T $\beta$ RII activates the intrinsic serine/threonine kinase activity of T $\beta$ RI, which phosphorylated transcription factors Smad 2 and Smad 3 (Quan *et al.*, 2004). On the surface of dermal fibroblasts membrane, the receptors for PDGF-BB and TGF- $\beta$ 1 both physically interact and ameliorate each other's signaling and stability (Porsch *et al.*, 2014). Other responsible factor for content shortage of collagen in senescent fibroblast is the expression of gene MMP-1 to degrade collagen type I. Study showed that platelets can release tissue inhibitors of MMP (Villeneuve *et al.*, 2009). Improvement of collagen deposition in senescent HDFs can be obtained by inhibition of MMP-1 activity by MMP tissue inhibitors in PRF lysate (Wirohadidjojo *et al.*, 2016).

TGF- $\beta$  signaling pathway is also influenced by mechanical force and pivotal to dermal fibroblast function (Eckes *et al.*, 2006). The addition of exogenous, monomeric hyaluronic acid to cultured fibroblasts is reported to trigger TGF- $\beta$  signaling and collagen production (David-Raoudi *et al.*, 2008; Mast *et al.*, 1993). Some of these responses are mediated by binding of HA to CD44, a cell surface glycoprotein (David-Raoudi *et al.*, 2008). Another study found that injection exogenous hyaluronic acid stimulates proliferation of fibroblasts (Quan *et al.*, 2013). This stimulation is suggested

due to the increase of local mechanical forces, characterized by fibroblast elongation/spreading, furthermore it is also mediated by up-regulation of type II TGF- $\beta$  receptor and connective tissue growth factor (Eckes *et al.*, 2006; Quan *et al.*, 2013). Similar events were also found in this experiment, fibroblasts elongation presented after treatment with combination of 50% PRF lysate with hyaluronic acid (Fig.5). Importantly, fibroblasts elongation is associated with up-regulation of the TGF- $\beta$  signaling pathway (Quan *et al.*, 2013). Thus, this study found that structural properties of fibroblasts played a significant role in modulating senescent fibroblasts function. Fibroblasts proliferation can be driven by numerous mechanisms, including increased mechanical force (Eckes *et al.*, 2006).

HA in PRF lysate is a potent candidate material for senescent human dermal fibroblasts treatment. It is assumed that HA and growth factors from PRF lysate act synergistically to repair proliferation and collagen deposition of senescent human dermal fibroblasts by ameliorating TGF- $\beta$ 1 signaling. Hyaluronic acid was shown to interact with growth factors, thereby protecting them from degradation by proteases (Locci *et al.*, 1995). Therefore, due to hyaluronic acid specific properties correlated with growth factors, HA addition into human growth factors obtained from PRF lysate might be particularly well suited to help improve proliferation index and collagen deposition of senescent HDFs. This combination may be utilized to increase the positive outcome of the restoration of senescent human dermal fibroblasts activities.

## CONCLUSIONS

The addition of HA into PRF lysate is a good candidate material for treating clinical signs related to senescent human dermal fibroblasts.

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