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The effect of platelet rich fibrin (PRF) on serum starved human dermal fibroblast

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ABSTRACT

Healing failure on chronic ulcers was suspected due to the decrease of Growth Factors (GFs) supply caused by either GFs trapped in the fibrin, or degraded by protease, or decreased level due to reduction of GFs gene expression. Administration of various GFs can stimulate healing of chronic ulcers. High level of GFs is available in biologic material called Platelet Rich Fibrin (PRF). This study was conducted to have *in vitro* evidence of PRF effect on GFs-serum starved human dermal fibroblasts as representative cells of chronic ulcers. Human dermal fibroblasts (HDFs) were isolated from foreskin of six boys aged 11- 14 years-old. After 24 hours of serum deprivation, HDFs were treated by 100, 50, and 25% PRF lysate diluted in cultured medium. Cellular migration was measured using scratch assay, while cellular viability was measured using MTT assay and collagen deposition was measured using Sirius Red assay. The HDFs of serum starvation group showed significant impairment activities in terms of cellular migration (25%), cellular proliferation (20%), and collagen deposition (10%) ($p<0.05$). Administration with various levels of PRF lysate could significantly recover those activities ($p < 0.05$). Because cellular activities of serum starved HDFs is similar with fibroblasts isolated from the bottom of chronic ulcers and administration of various levels of PRF lysate was capable to recover those activities, it can be concluded that PRF is a good biologic material to stimulate healing of chronic ulcers. However, in order to get better evidence based medicine, both pre clinical and clinical studies must be performed.

ABSTRAK

Kegagalan sembuh pada ulkus khronis disebabkan oleh rendahnya suplai *growth factor* (GF) akibat GF terjebak dalam fibrin, atau dirusak oleh protease bakteri, atau akibat menurunnya ekspresi gena penyandi GF. Pemberian GF dapat memacu penyembuhan ulkus khronis. Berbagai GF ternyata terdapat dalam material biologis seperti fibrin kaya platelet (FKP). Penelitian ini dilakukan untuk memperoleh bukti *in vitro* bahwa FKP dapat mengembalikan fungsi fibroblast tanpa serum sebagai model fibroblas dari ulkus khronis. Fibroblas dermis manusia (FDM) diisolasi dari kulit kulup 6 anak laki-laki berumur 11-14 tahun. Setelah dibebaskan dari serum selama 24 jam, FDM kemudian diberi 100, 50, 25% lisat FKP dan medium pelarut sebagai kontrol. Selanjutnya, aktivitas selular berupa timbunan kolagen, proliferasi dan migrasi sel diukur berturut-turut dengan metode Sirius Merah, MTT, dan goresan. FDM tanpa serum ternyata secara mengalami penurunan kemampuan menimbun kolagen sebesar 10%, proliferasi sebesar 20% dan penurunan kemampuan migrasi sebesar 25% secara signifikan ($p<0,05$). Penambahan lisat FKP

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ternyata dapat memulihkan aktivitas selular FDM tersebut. Dari eksperimen ini dapat disimpulkan bahwa FKP merupakan material biologis yang dapat dikembangkan untuk memacu penyembuhan ulkus khronis. Namun demikian, untuk memperoleh bukti-bukti klinis yang lebih baik, uji pra klinis dan uji klinis tetap diperlukan.

Keywords: chronic ulcers - human dermal fibroblasts - serum starvation - platelet rich fibrin lysate – growth factors

INTRODUCTION

Chronic ulcers are difficult to heal spontaneously due to their characteristics of low cellular proliferation rates,[1-3] low cellular migration capacities,[3-5] followed by cellular senescence of fibroblasts on their wound bed and edges.[3,6] All of these may be caused by lack of growth factor supplies[7,8] as they are indicated by the decrease of GF-gene expressions among diabetic patients who tend to have chronic ulcers,[9] the entrapment of GFs by fibrin that spill out when capillaries leak due to high venous pressure in patients with venous leg ulcers,[7,10,11] and GFs degradation by protease contained in bio-film on the surface of chronic ulcer.[2,12] The theory of GFs supply deficiency will be strengthened when considering the findings of the clinicians who succeeded in stimulating chronic ulcers healing using either recombinant platelet derived growth factor (r-PDGF),[13] or recombinant epidermal growth factor (r-EGF),[14-16] or recombinant fibroblast growth factor (r-FGF) that were applied topically.[17-19]

Platelets are the source of autologous GFs, which are released when platelets had been activated. Based on the technology of platelet-rich plasma (PRP), Eppley *et al.,*[20] found that the level of PDGF, EGF, and FGF increased almost fourfold compared to the level of human plasma. Various levels of PRP supernatant have been able to restore activity of artificial fibroblasts senescence not due to serum starvation but due to repeated Ultraviolet B exposure.[21] Beside PRP technology, another convenient technology to obtain GFs is PRF technology[22] and although it indicates slower release of GFs,[23,24] but its lysate contains high levels of GFs.[25]

PRF often used by dentists to stimulate extraction socket healing,[26] treatment of maxillary sinus,[27] and as grafting material for intrabony periodontal defects.[28,29] Eventhough the application of PRF in dermatological field has rarely been published, our dermatology experience indicates that it is effective to treat chronic ulcers which failed to heal under conventional PRF treatment. Unfortunately, in the absence of placebo in our cases, we could not make a conclusion that those healings occurred due to PRF administration. In order to obtain *in vitro* evidence about the effect of PRF lysate in chronic ulcers, we performed HDFs cultivation in 24-hour-serum starvation and hope it will show characteristic similar to fibroblast isolated from the bottom of chronic ulcers. After that, we add various levels of PRF lysate and measured the changes in cellular activities compared to control.

MATERIALS AND METHOD

This study used *in vitro* experiment design to compare the proliferation, migration, and accumulation rate of collagen between serum starved HDFs and control group. The schematic review of serum starved HDFs followed by administration of 100, 50 and 25% PRF lysate and diluent medium as control was showed in the FIGURE 1.

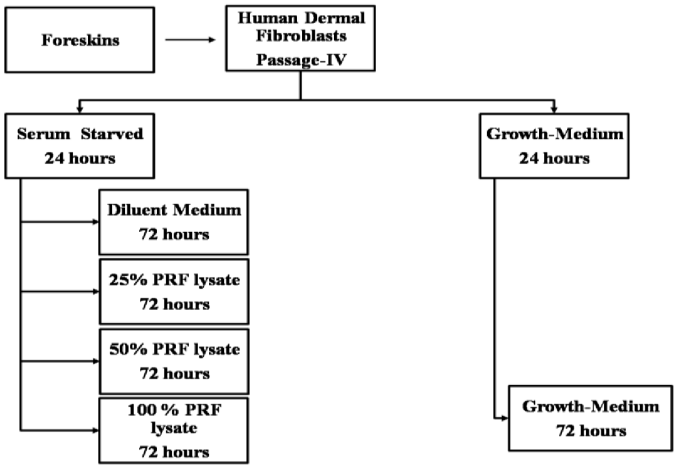

FIGURE 1. Experiment Scheme

Cell cultures.

Human fibroblasts from foreskin of healthy six boys were cultured using the explants technique as described previously.[30] The cells were cultured in Dulbecco Modified Eagle Medium (DMEM) with 4.5 g/L D-glucose, 2mmol/L L-glutamine, 2.5mg/L amphotericin B, 100ug/L streptomycin, 100unit/L penicillin, and 10% fetal bovine serum. All materials were purchased from Gibco-USA. The forth passages or more were used for the experiments.

PRF lysate

PRFs were isolated based on a method by Dohan *et al*.[22] Briefly, 20 mL of blood was taken from volunteers into sterile glass tube, centrifuged 400 g for 10 min, and after 5 min of room temperature incubation, PRF gel was separated from the red blood cells and transferred into new sterile tubes and kept at 4^0 C for 24 hr. Supernatants were then collected and considered as 100 % PRF lysate as previously described by Yao Su *et al*.[24] Additional dilution with DMEM was performed to have final concentration of 50 and 25%.

Experiment

Experiment was carried out with HDF re-seeding in wells of 200 uL cell suspension containing 10^3 cells/mL. After 72 hr of incubation, various treatments carried out in accordance with a written protocol in FIGURE 1. DMEM enriched with 0.5% fetal bovine serum was used for serum starvation medium, diluent medium of PRF, and control.

Collagen deposition

Collagen deposition was calculated from optical density of insoluble collagen based on Sirius Red assay.[31] Briefly, after washing the wells with phosphate buffer saline (PBS), cells were fixed with *Bouin* solution for an hour, and then rinsed with tap water until yellow color was completely removed. The plates were let to dry in room temperature for overnight. Two hundred µL of Sirius Red (Sigma-Aldrich, USA) were diluted in saturated picric acid and were subsequently added for each well for an hour. Unbinding Sirius Red was removed by washing four times with 200µL 0.1 N HCl until supernatants were clear. The binding Sirius Red was diluted in 200µL 0.5 N NaOH and their optical density was read by 550 nm filtered spectrometer.

The collagen deposition of normal HDFs was assumed to be 100% and collagen deposition of various treated groups was accounted as : (Unsoluble collagen of treated group /Unsoluble collagen of paired normal HDFs) x 100 %.

Proliferation index

Proliferation index was calculated from cellular viability that was determined in formasan-blue optical density of 570 nm filtered spectrometer. The proliferation index of normal HDFs was assumed to be 100% and the proliferation index of various treated groups was determined as :(Cellular viabilities of treated group/Cellular viabilities of paired normal HDFs) x 100 %.

For these purposes, all of medium was aspirated and the wells were rinsed using PBS for 3 times, 10 min each. Two hundred μL of fresh medium were filled into each well and 50 μL of 50 mg/mL MTT (MP Biomedicals-USA) were added. The plates were covered by aluminum foil and incubated for 6 hr in 37°C. Medium containing MTT were aspirated and 200 μL DMSO were then filled and followed by 25 μL glycine buffer on the top. Optical density was immediately read by 570 nm filtered spectrometer.

Fibroblast migration assay

Fibroblasts migration assay was performed based on method and computed based on method of Liang, *et al.,*[32] with slight modification. Briefly, after serum starvation, all of wells were linearly scratched with the blunt tip of a 32G sterile needle through the centre of the well bottom. The cells were then cultivated in the experimental medium for 72 hr. The cells were then stained with Meyer's haematoxylin and microscopic photo images were taken using a Moticam350 (China) camera in JPG format. By using Adobe Photoshop, both blue color's pixels of fibroblasts along scratch line and white color's pixels of empty space can be measured.

Fibroblast migration capacity was counted as: (pixel of fibroblasts along scratch line/total pixel along scratch line) x 100%.

Statistical analysis

All datas were presented as mean. Because of the data was not normally distributed, we used Wilcoxon rank sign test for testing the paired data and $p<0.05$ was considered as significant different.

RESULTS

Serum starved HDFs revealed various decreases cellular activity in: collagen deposition (from 100% to 90.4%), proliferation index (from 100% to 80.9%), and in migration capacity (from 53.45% to 28.44%). Administration of various levels of PRF lysate affected on those variables as in following FIGURE 2-4.

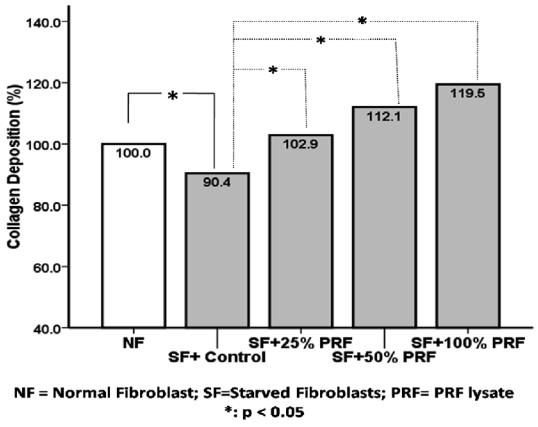

FIGURE 2.Collagen deposition among various groups

It shows that serum starvation caused depression of collagen deposition and various levels of PRF lysate were able to restore starved fibroblasts in collagen deposition and

its response was paralel with lysate's level (FIGURE 2).

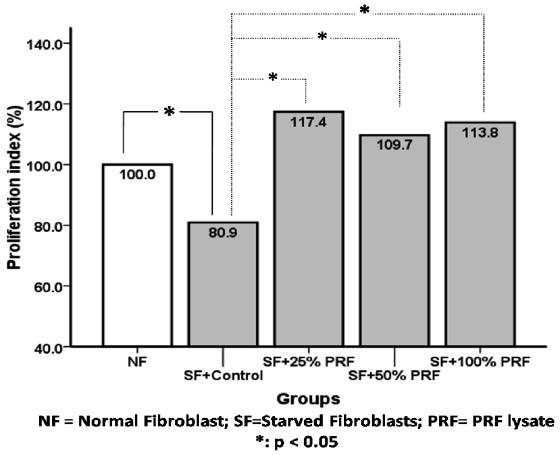

FIGURE 3. Proliferation index among various groups

It appears that serum starvation caused in suppression of proliferation index and administration of PRF lysate in various levels were able to ameliorate this activity significantly ($p<0.05$), even 25% PRF lysate could induce starved fibroblast better than normal fibroblasts in this variable (FIGURE 3).

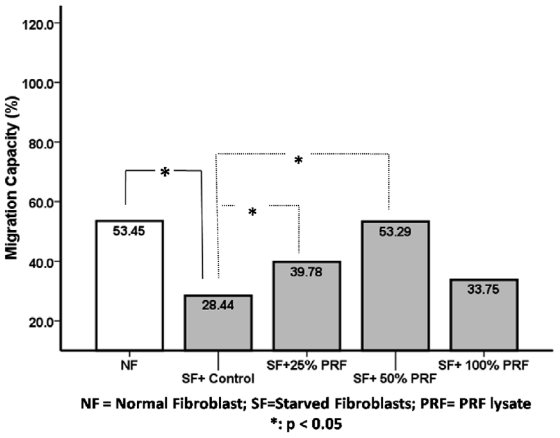

FIGURE 4. Migration capacity among Various Groups

It shows that serum starvation caused depression of migration capacity of fibroblasts and various levels of PRF lysate were able to restore starved fibroblasts in migration capacity, mainly among starved fibroblasts that had administration of 50% PRF lysate (FIGURE 4).

DISCUSSION

As shown on FIGURE 2 to 4, serum starvation for 24 hr was able to suppress HDFs in collagen synthesis (FIGURE 2), proliferation index (FIGURE 3), and migration capacity (FIGURE 4). Those are similar with the characteristic of fibroblasts isolated from the edge of venous ulcers that it is published by Herrick *et al*.[33] On the FIGURE 2, our experiment revealed a restoration of collagen deposition of serum starved HDFs from 90.4% to the highest achievement of 113.8% due to administration of 100% PRF lysate. The lowest level of PRF lysate (25%) was still had improvement effect to 102.9%. These phenomena may be caused by level of GFs in this lysate. A study showed that PRF lysate contains a rich variety of growth factors which required for the synthesis of collagen such as PDGF.[34] This effect may not be found among fresh wound with normal GFs supplies as it is indicated by an experiment where collagen synthesis in tubes inserted from the edge of the new wound incision for ten days has not been shown better compared to albumin as control.[35]

On the FIGURE 3, administration of PRF lysate on serum starved HDFs could ameliorate proliferation index from 80.9 to 113.8% by 100 % PRF lysate. In reverse phenomena, lower level of PRF lysate could improve a better proliferation index than 100% PRF lysate, such as in 25% PRF-lysate. It may be happened due to the better availability of nutrient in addition to GFs. In 100% PRF lysate, there is no additional medium as a source of glucose, amino acid, and other nutrients that are important for mitosis of HDFs. Similar events with proliferation index were also found on

FIGURE 4 of migration capacity. In wound healing processes, fibroblast migrations are crucial events and the researchers have found that it has under responsibility of PDGF,[36] EGF and extracellular matrix component such as collagen type I and fibronectin.[37,38] Naturally, PDGF and EGF are released by platelets immediately after the blood clotting,[39] contained in the serum as important supplement for fibroblast migration and proliferation on regular HDFs cultivation,[40,41] and they have been found in high level of PRF lysate.[25]

These findings also indicate that platelet's products are important for human dermal cells proliferation and migration, such as in newest creation of cultured human dermal substitutes without medium and animal products, but human serum or platelet's product was still required.[42-44] Compare to PRP technology, isolating autologous GFs by PRF technology is considered simpler; it also contains various GF types which required for stimulation of healing processes as shown in our experiment. Similar result has been reported by Jørgensen *et al.*,[45] in their pilot study where PRF has also effective for recalcitrant chronic wound.

The weakness of our study is on angiogenesis variable as an important factor in healing processes. Another study has shown the effect of platelet rich fibrin matrix, another PRP based technology, capable to stimulate angiogenesis via endothelial cell proliferation.[45] Differ with PRP technology which has been shown capable to stimulate the proliferation of normal HDFs[46] as well as UV-induced HDFs senescence,[21] research on PRF application in dermatology field is very limited. However, in order to achieve a better level of evidence based medicine of PRF application, various in-vitro and clinical researches are still needed.

CONCLUSION

PRF as a simple method to isolate autologous GF and it indicates a therapeutic effect in stimulate-healing of chronic ulcers. Research in large scale is needed to get a better level in evidence based medicine.

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