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ORIGINAL ARTICLE

Comparison of Advanced Platelet Rich Fibrin (A-PRF) and Culture Media Conditioned Warton's Jelly (CMCWJ) on Fibroblast Cells Proliferation

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Abstract

Objective: To compare the potency of fibroblast cells proliferation in 12.5% and 25% Culture Media Conditioned Warton's Jelly (CMCWJ) and Advanced Platelet Rich Fibrin (A-PRF) cultured medium. **Material and Methods:** Fibroblast cells were divided into five groups: Group I (Control Group): serum-starved fibroblast without any treatment as a negative control; Group II: fibrolast that supplemented in 12.5% CMCWJ medium; Group III: fibrolast that supplemented 12.5% A-PRF medium; Group IV: fibrolast that supplemented 25% CMCWJ medium, and Group V: fibrolast that supplemented 25% A-PRF medium. The fibroblasts proliferation was counted by an automated cell counter. Statistical analysis was performed using One-way ANOVA and Post hoc Tamhane test was conducted to analyze the potential fibroblast proliferation differences in different concentration of CMCWJ and A-PRF group. **Results:** There were no significant differences in the fibroblast cell proliferation between GI and GIV, GII and GIV, GII and GIII, GII and GV, also GIV and GV. There were significant differences between GI and GII, GI and GIII, GI and GV, also GIII and GIV. **Conclusion:** The 12.5% CMCWJ group, 12.5% A-PRF group and 25% A-PRF group has excellent potential ability of fibroblast cells proliferation, meanwhile 25% CMCWJ group has the lowest mean potency of fibroblast cells proliferation compared to other groups. The 12.5% A-PRF Group has the highest mean of fibroblast cell proliferation amongst other groups.

Keywords: Fibroblasts; Cell Proliferation; Platelet Rich Fibrin; Wharton Jelly.

Introduction

The latest invention of science and technology in biomedical field has brought a breakthrough towards tissue regeneration by applying the concept of tissue engineering. Tissue regeneration concept can be applied to endodontic treatment called regenerative endodontic treatment. One of the main elements in forming the pulp tissue are fibroblasts. It is known that fibroblasts are the most common cells of connective tissue in human and tissue engineering techniques can be used fibroblasts that do not originate from the tissue of origin [1-3]. Lately, fibroblasts that derived from preputium tissues has become popular to be used as standard research material especially in tissue engineering research [4,5].

A changing paradigm of regenerative current treatment concept from cell-based therapy to be non-cell-based therapy using secretome as a source of growth factor. Growth factor is one of the main component in tissue engineering that regulates cell activity [6,7]. Stem cells are able to produce secretome rich in growth factor when cultured in a medium [8-10]. Medium containing secretome of stem cell called a conditioned medium or CMCWJ (Culture Media Conditioned Warton's Jelly), as well as other sources of growth factors can be obtained from platelet concentrates such as A-PRF (Advanced Platelet Rich Fibrin) [11-13].

CMCWJ is a source of stem cells derived from umbilical cord, that has a combination of pluripotent and multipotent properties as included in the extra embryonic stem cells, they have high plasticity compare to adult stem cells. The advantages of CMCWJ is able to secrete many growth factors, especially TGF-β, which plays a role in cell proliferation. Secretome can be produced by Wharton's jelly mesenchymal stem cells containing TGF-β, FGF, VEGF and IGF. These growth factors play a role in the regulation of cell activity through activation of intracellular signaling pathways such as proliferation, differentiation, apoptosis and secretion of the protein as a paracrine effect.

Platelet-rich Fibrin (PRF) is considered as an ideal scaffold for tissue regeneration because it is considered to have all of the attributes required for a scaffold. One properties of the PRF is able to release growth factors in the regulation process of wound healing, immunity, and the promotion of tissue regeneration. PRF is the second-generation platelet concentrate preparation that not requires any additional anticoagulant or materials. Besides the manufacturing process is much simpler with a single centrifugation step is another advantage to minimize contamination [14-16].

In order to optimize the number of growth factors in the PRF, in a study conducted modification speed and time of platelet cells centrifugation (2700 rpm for 10 minutes to 1500 rpm for 14 minutes), can create the new formation of PRF that called Advanced PRF (A-PRF). This condition increase number of granulocytes, which has the ability to release growth factors at the distal gel PRF, will boost the stimulation of healing and tissue regeneration. Meanwhile, on the original PRF, granulocytes wasted in the lining cells between red areas with buffy coat [7,17].

It was reported that the fibroblast cell proliferation significance test with A-PRF application concentration of 50%, 25%, and 12.5% have the ability to initiate cell proliferation of fibroblasts. The

authors did not found significant difference between groups 50% A-PRF with 12.5% A-PRF, but 25% concentration of A-PRF has the highest value. The increase of A-PRF concentration is not directly proportional to the proliferation effects. This study suggested there is an optimal platelet concentration limits for cell proliferation, which is 2.5 times higher from the ideal normal plasma levels [18]. Another authors stated that both Lysates PRF and Advanced-PRF (A-PRF) with a concentration of 25% has the best ability when compared with a concentration of 50% or 12.5% [18,19].

Moreover, it is assumed that 25% PRF is the concentration that produces the best proliferation of fibroblast, but the high concentration of platelet can cause the reduction of pH level that may harm the fibroblast cells proliferation [18]. Other fibroblast proliferation study also reported that there is a difference between groups CMCWJ 25% and 12.5% even though is not statistically significant. It can be assumed that a high concentration did not increase the cells proliferation itself. Treatment with two concentrations indicate a change in cell shape CMCWJ that can be closed to normal, the cells swell with defined border. Even from two groups with different concentrations CMCWJ, the microscopic structure of 25% CMCWJ group showed cell figure that similar with normal fibroblast cells [20].

Research on the potential comparison of CMCWJ and A-PRF on cell proliferation of fibroblasts cells with less concentration than 50% still needs further verification. The aim of this study was to compare the potency of CMCWJ dan A-PRF in 12.5% and 25% level of concentration on fibroblast cells proliferation.

Material and Methods

Fibroblasts preputium cells have been stored in transported medium Dulbecco's Modified Eagle's Medium (DMEM), Penstrep 400 µg/mL, Gentamycin Sulfate 100µg/mL and Amphoterycin B 10µg/mL inside of an icebox. After that, the fibroblasts cells have been stored in cryo-preservation confluently exposed to serum starvation for 48 hours before used as samples. Fibroblasts artificially damaged by culturing fibroblasts in DMEM containing 1% fetal bovine serum (FBS) for 48 hours (serum starvation) [21].

Initially 200 mL suspension of 5 x 103 fibroblasts/mL in DMEM and 10% FBS were placed into each of the wells of a micro-plate containing 24 wells (Iwaki Glass Co., Funahashi, Japan). After 24 hours incubation at 37°C and 5% CO_2, the media aspirated, rinsed with PBS and results triplicate of the culture medium is replaced with DMEM containing FBS 1%, while the triplicate of fibroblasts normal replaced with DMEM + FBS 10%. All cultures of fibroblasts were incubated for 48 hours at 37°C and 5% CO_2.

Fibroblast cells were divided into five groups: Group I (Control Group): serum-starved fibroblast without any treatment as a negative control; Group II: fibrolast that supplemented in 12.5% CMCWJ medium; Group III: fibrolast that supplemented 12.5% A-PRF medium; Group IV: fibrolast that supplemented 25% CMCWJ medium, and Group V: fibrolast that supplemented 25%

A-PRF medium. Serum starvation technique was applied by replacing foetal bovine serum (Gibco Invitrogen Co., New York, NY, USA) in the culture medium from 10% to 1% within 48 hours. The fibroblasts proliferation was counted by an automated cell counter (Scepter Cell Counter Sensors® 60 µm, Merck, Mexico City, Mexico).

Statistical Analysis

Triplicate experiments procedures were performed to ensure accurate analysis (IBM SPSS Statistics for Windows Software, version 21; IBM Corp., Armonk, NY, USA) using One-way ANOVA and Post hoc *Tamhane* test was conducted to analyze the potential fibroblast proliferation differences in CMCWJ and A-PRF.

Results

There were significant differences between the five groups ($p<0.05$) (Table 1). Group III has the highest mean number of fibroblast cells proliferation. The second highest mean number is in the Group V and the lowest mean in the Group IV.

Table 1. Comparison of potential ability of serum-starved fibroblast proliferation between groups after 24h evaluation.

Groups	Mean (SD)	p-value
G I (Control)	157.833,33 (21.160,49)	
G II (12.5% CMCWJ)	233.166, 67 (36.565,92)	
G III (12.5% A-PRF)	312.833,33 (47.822,24)	0.00*
G IV (25% CMCWJ)	221.666,67 (36.565,92)	
G V (25% A-PRF)	303.500,00 (57.892,14)	

*One-way ANOVA; Statistically significant.

In this study serum starvation procedure is used to represent damage of the pulp fibroblasts with the aim to evaluate the proliferative effect by in-vitro evaluation (Figure 1. a1 and a2). Qualitative result from each group showing more flat fibroblast cells and thin layer membrane of the cells after serum starvation for 48 hours of fibroblast cells (Figure 1. a1 and a2). It is shown also fibroblast morphology in different cultured medium (Figure 1. b, c, d and e) with 12.5% and 25% level of concentration. The 12.5% groups (Figure1. b and d) showing more dense of fibroblast cells formation compare to 25% groups (Figure 1. c and e) both in CMCWJ group and A-PRF group.

It is shown from Table 2, the 12.5% and 25% A-PRF groups (GIII and GV) have significant fibroblast cells proliferation compare to control, also 12.5% A-PRF group compare to 25% CMCWJ group (GII and GIV). The 25% of CMCWJ group has the lowest mean of proliferation ability of fibroblast cells compare to 12.5% CMCWJ group, 12.5% A-PRF group, and 25% A-PRF group (Table 2).

Based on the results of a post hoc *Tamhane* analysis (Table 2), it is known that there were there were significant differences between GI and GII, control GI and GIII, GI and GV, also GIII

and GIV. On the other hand, there were no significant differences in the fibroblast cell proliferation between control GI and GIV, GII and GIII, GII and GIV, GIV and GV (Table 2).

Table 2. Comparison of fibroblast cells proliferation between groups after 24h evaluation.

Comparison	Mean	SD	p-value
G I vs G II	75.333,33	15.474,35	0.010*
G I vs G III	155.000,00	21.349,21	0.002*
G I vs G IV	63.833,33	17.247,38	0.059
G I vs G V	145.666,70	25.163,67	0.010*
G II vs G III	79.666,67	23.366,41	0.079
G II vs G IV	11.500,00	19.689,39	1.000
G II vs G V	70.333,33	26.896,30	0.277
G III vs G IV	9.333,33	24.576,52	0.044*
G IV vs G V	81.833,33	27.954,03	0.166

*Post hoc Tamhane; Significant at the 0.05 level.

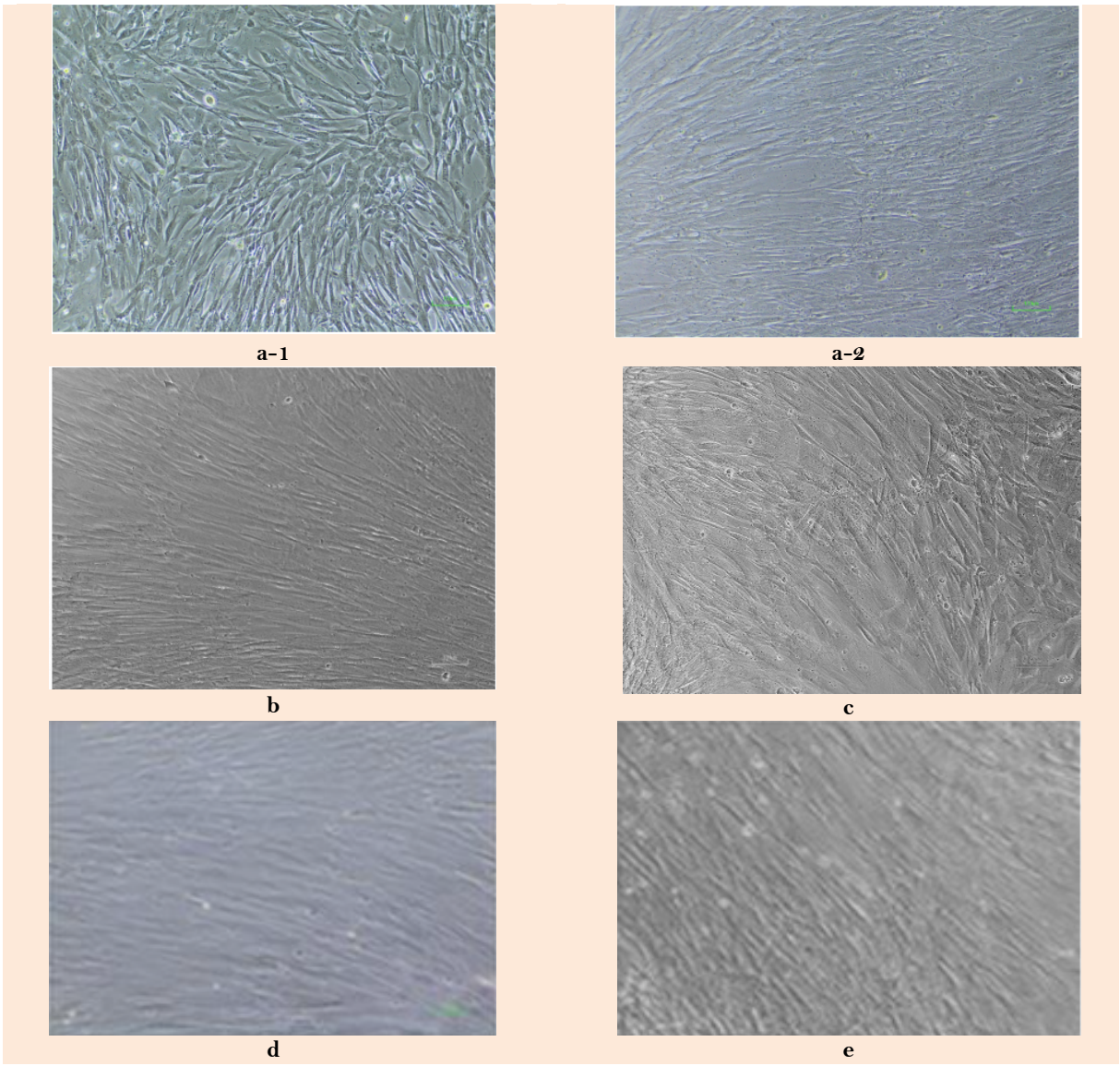

Figure 1. Qualitative result of each group. (a-1): Fibroblast cells before starvation treatment; (a-2): Impaired fibroblasts after starvation treatment (control group); (b): 12,5% CMCWJ group; (c): 25% CMCWJ group;. (d): 12,5% A-PRF group; (e): 25% A-PRF group. (Magnification: x100, scale: 100µm; Inverted Nikon Eclipse TS100™).

Discussion

In this study, each medium came from a different source of stem cell types that can create different reaction in fibroblast cells proliferation. A-PRF is Adult Multi-potent Stem Cells, which are taken from the blood that produces high level of growth factor Platelet Derived Growth Factor (PDGF). Although, CMCWJ is Embryonic Pluripotent Stem Cells which are taken from gelatinous substance within the umbilical cord has been reported previously with excellent potential ability to induce cells proliferation and differentiation [12]. Fibroblast cells showing greater proliferation ability in A-PRF medium compare to CMCWJ. This result can be a basic knowledge that fibroblast, can have a better proliferation and regeneration in A-PRF medium and low concentration of CMCWJ.

The result of this study (Table 1) are also accordance to the previous study reported [18] revealed that the fibroblast cell proliferation significance test with A-PRF application concentration of 50%, 25%, and 12.5% have the ability to initiate cell proliferation of fibroblasts. Although from statistical analysis test, there was no significant difference between groups 50% A-PRF and 12.5% A-PRF, but value proliferation of 25% A-PRF is the highest (statistically significant compared to other groups) [18].

Although on CMCWJ contains lots of TGF-β which has a regulatory function in cell proliferation and differentiation, amount concentration of CMCWJ is also a medium base that will affect the ability of proliferation [22]. This statement is related with the result that 25% CMCWJ group has a lower mean of Fibroblast cells proliferation compare to 12.5% CMCWJ group, 12.5% A-PRF group and 25% A-PRF group. It was also reported that there is a difference between groups CMCWJ 25% and 12.5% even though is not statistically significant. It can be assumed that a higher concentration did not increase the cells proliferation itself. Even from two groups with different concentrations CMCWJ, the microscopic structures of 25% CMCWJ group showed cell figure that resemble with normal fibroblast cells [20]. Similar with that, the result of this research also showing better potential ability of 12.5 CMCWJ compare to 25% CMCWJ. Further research using lower concentration under 12.5% CMCWJ medium need to conduct for verification of this founding.

In the earlier study, fibroblasts were destroyed artificially using UVA irradiation (total dose of 10 Jcm-2). The UVA irradiation may damage the DNA of cells, whereas in this study, fibroblasts were treatment by serum starvation and replacing the medium from FBS 10% to 1% FBS. Serum starvation procedure has been used extensively to study the mechanism involved in cellular stress response, protein degradation, autophagy, apoptosis, and/or to simulate certain pathological conditions. Therefore, in this study serum starvation procedure is used to represent damage of the pulp fibroblasts with the aim to evaluate the proliferative effect of CMCWJ and A-PRF conditioned medium in various concentrations [4,5,21].

Platelet concentrate incubation at 4°C for 24 hours resulted in high level concentration of PDGF-BB and TGF-β1, it is also found in the Advanced PRF (A-PRF). PDGF and TGF-β is a major growth factor that can trigger the healing process of soft tissue and bone through stimulation

of collagen production, in order to improve the closure of the wound and initiate the bone formation. PDGF can coordinate migration, proliferation and resistance of mesenchymal cells. From all cytokine, TGF-β contain fibrous is the most powerful agent. These cytokines stimulate high synthesis of matrix molecules, such as fibronectin and collagen I by the fibroblasts and osteoblast [7,14,16]. Accordance with the result of this study, that 12.5% A-PRF has the highest potential ability of fibroblast cells proliferation, A-PRF is multi-potent platelet derived stem cells that has a great number of growth factors like PDGF and TGF-β1.

In the previous study, decreased expression of TGF-β1 receptor genes in fibroblasts with artificial damage procedures due to TGF-β1 signaling and cell proliferation, collagen synthesis, and migration. It is assumed that the proliferation index had improved due to the presence of TGF-β1 signaling improvements, based on the results of previous research on dermal fibroblasts. Dermal fibroblasts on the surface of the membrane, the receptors for PDGF-BB and TGF-β1 interact and improve the signal and stability of each other [4,5]. Based on this research, we assumed that the use of starvation Fibroblast cells with combination of culture medium, might also doubled induce the proliferation ability of fibroblast cells itself. It is showed from the result, that the mean of each culture medium (CMCWJ and A-PRF) groups can elevate almost two times higher from the negative control group.

During the healing process, the PRF fibrin matrix can act as a three-dimensional scaffold, where platelet and cytokines are released to be trapped in it during the period of time [23]. On the other hand, this gel forms can inhibit release of growth factor. Therefore, gel PRF incubated at 4°C for 24 hours will deflate and the supernatant on top of it will contain a higher level of growth factors. This finding related to the result of this research that 12.5% A-PRF and 25% A-PRF has a good potential ability as a medium culture of fibroblast proliferation.

On the contrary, the formation of Lysate PRF (L-PRF) and A-PRF as low density concentrate culture medium that contains Growth Factors (GFs), it is assumed that the role of fibrin matrix as a scaffold in tissue engineering are almost disappeared. This suggests that changing formation of PRF to A-PRF can produce a positive influence on the tissue healing process base on the source of GFs of its medium. This statement is in accordance with the paradigm changing of regenerative current treatment concept from cell-based therapy to be non-cell-based therapy using secretome or concentrate media as a source of GFs. Growth factor is the main component in tissue engineering that regulates cell activity and promote molecule signaling pathways on its niche environment [18,19,23].

It is shown from Table 2, the 12.5% and 25% A-PRF groups have significant fibroblast cells proliferation compare to control, also 12.5% A-PRF group compare to 25% CMCWJ group. The 25% of CMCWJ group has the lowest mean of proliferation ability of fibroblast cells compare to 12.5% CMCWJ group, 12.5% A-PRF group, and 25% A-PRF group (Table 1). It can be assumed that lower concentration of CMCWJ and A-PRF more suitable for fibroblast cells proliferation.

It is stated that CMCWJ may appear to promote healing by paracrine signaling, but direct cell-cell contact effect of CMCWJ on human fibroblasts may down regulate fibroblast proliferation [12]. From the previous study it is reported that the inhibitory mechanism as result of some growth factors act as an endogenous inhibitors of cell growth that may be down regulated in certain condition [13]. The result of this study showing a decrease number of fibroblast cells proliferation on 25% CMCWJ group compare to control, we assumed that this outcome related to direct cell-cell contact effect of CMCWJ on human fibroblasts may down regulate cells fibroblast proliferation as mention from the previous research.

From the previous research it is demonstrated that a concentration of 50% CMCWJ has the best result for premature-aged fibroblast cell proliferation. A previous study reported that 50% concentration has the lowest increasing number after CMCWJ treatment, and significant level is reached at 25% until 12.5% CMCWJ group. We might assume that there are some inhibitory molecules that avoid proliferative activity occurred in the level of 50% or more CMCWJ. The result of this study in accordance with the assumption above, that the higher concentration of CMCWJ also has the lowest fibroblast proliferation result compare to other groups.

Conclusion

Regeneration potency of CMCWJ and A-PRF as alternative sources of growth factors that needed in fibroblast cells proliferation had been proven from this experiment. Somehow, the result of this study showed that the A-PRF group has excellent potential ability in cells proliferation compare to CMCWJ. It can be concluded that A-PRF in 12.5% and 25% concentration has superior potential ability compare to 12.5% and 25% CMCWJ groups. The 25% CMCWJ group showing the least potential ability compare to other cultures medium. Lower concentration of CMCWJ and A-PRF showing a better potential ability of fibroblast cells proliferation compare to higher concentration, so this can be a basic knowledge for further research ahead or clinician in regenerative endodontic fields.

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