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Comparison of fibroblast cell regeneration in three different concentrations of Wharton's Jelly mesenchymal stem cells conditioned medium (WJMSCs-CM)

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Abstract. Wharton's Jelly-derived mesenchymal stem cells (WJMSCs) have gained interest as an alternative source of stem cells for regenerative medicine. Although many studies have characterized Wharton's Jelly biologically, the effects of different concentrations in a cultured medium have not yet been compared. Damaged fibroblasts, the primary components of irreversible dental pulpitis, irreversibly impair the ability to regenerate and lead to the disruption of extracellular matrix. This study was performed to evaluate the potency of three WJMSCs- CM concentrations in improving serum-starved fibroblasts. Fibroblasts were cultivated in five passages, and divided into four groups. The first group (the control group) consisted of fibroblast cells that had been treated using starvation methods. The other groups (the treatment groups) were treated with various concentration of WJMSCs- CM (50%, 25% and 12.5%). Proliferative ability was evaluated using a cell count method and analyzed with a one-way ANOVA. Cultivation of serum-starved fibroblasts produced significantly higher cell counts in 12.5% WJMSCs-CM compared to the 50% group. It can be concluded that 12.5% WJMSCs-CM is the most efficient concentration for fibroblast proliferation.

1. Introduction

Biology-based therapy that regenerates pulpodentinal tissues to maintain pulpal vitality and advances in tissue engineering and biotechnology have opened new avenues for designing biological methods for pulp treatment. The aim of the methods are regenerating partial pulp tissue for irreversible pulpitis or replacement via synthesis of the total pulp in pulp necrosis. In order to regenerate the pulp, several approaches can be taken, including an effort to induce the pulp's own regenerative capacity. This idea of pulp regeneration began as early as 1963, when Ostby demonstrated tissue ingrowth in necrotic pulp using blood clots from the apical region. Growth factors and their platelets derived from blood clots induce new pulp formation. Pulp regeneration is based on a tissue engineering concept known as triad engineering factors: stem cells, a scaffold and a growth factor or signaling molecule [1,2].

The dental pulp consists of various cells. Fibroblasts, the most numerous cells, are widely distributed throughout the connective tissue of the pulp [3]. Inflammation of dental pulp downregulates all the living cells in the dental pulp, thus decreasing its proliferation capacity. Notably, the locally derived growth factors, neuropeptides, cytokines, and chemokines, that are released from the dentin matrix by pulpal cells modulate defense and repair processes within the tissue. Nevertheless, this mechanism is weak, since irritation continuously damages the cells and low-

compliance characteristics of dental pulp chamber restrain the healing process. Therefore, another source of growth factor is needed to stimulate cell proliferation [4-6].

Extra-embryonic perinatal MSCs have been a popular and promising source of stem cells recently; they represent an intermediate stem cell type that combines some of the pluripotent properties of embryonic stem cells (ECSs) with some multipotent properties of adult postnatal MSCs. Due to their close ontogenetic relationship with embryonic stem cells, extra-embryonic tissue-derived MSCs have immune privilege characteristics, possess a broader multipotent plasticity, and proliferate faster than adult postnatal MSCs. Moreover, because extra-embryonic tissues are normally discharged after birth, these cells can be isolated while effectively avoiding ethical concerns [7,8]. Stem cells are cultured in the medium. The secreted factors, ormolecules secreted from the cells in the medium, are referred to as secretome, micro vesicles or exosome, and can be found in the medium where the stem cells are cultured; thus the medium is called a conditioned medium [9]. A medium conditioned with Wharton's jelly mesenchymal stem cells has been used in clinical research and in various applications but is found less often in dentistry research.

2. Materials and Methods

Human fibroblast cells in cryop reservation were thawed and passaged at regular intervals using 0.25% trypsin. Impaired fibroblasts were induced by the starvation method; the medium was changed from DMEM (Dulbecco Minimal Essential Medium) with 10% FBS (Fetal Bovine Serum) into one with 1% FBS and incubated for 48 hours. The serum-starved fibroblasts were counted using an automated cell counter (Luna-II™, Logos Biosystems). The Luna-II™ automated cell counter is an image-based cell counting device that features an autofocusing liquid lens. It avoids the subjectivity and time expenditure associated with manual cell counting.

Commercially available WJMSCs-CM (Dermama Biotechnology, Solo, Indonesia: HWJ 1609B) was used as a source of signaling molecules to induce proliferation of impaired fibroblasts. Three different concentrations of WJMSCs-CM were prepared by adding DMEM-high glucose, 1% FBS, 1% penicillin, 1% streptomycin, 1% amphothericin B mixtures to 100% WJMSCs-CM. Afterward, 50%, 25% and 12.5% concentrations of WJMSCs-CM were prepared. The first group was the control group, that is, serum-starved fibroblasts without treatment; the second group was treated with 50% WJMSCs-CM; the third and fourth groups used 25% and 12.5% WJMSCs-CM, respectively.

Serum-starved fibroblasts were cultured in 50%, 25% and 12.5% WJMSCs-CM for 48 hours and incubated at 37 °C and in 5% CO_2. All of the experiments were performed in triplicate. The proliferative potency was measured using an automatic cell counter before and after treatments. Cell counts were conducted twice, with the second count using a new field of view. A one-way ANOVA was performed to analyze these values and to obtain the level of significance for the difference across all groups. Posthoc Bonferroni tests revealed the significant differences between each pair of groups.

3. Results and Discussion

3.1 Results

Fibroblasts showed typical fibroblast morphology, with large flat and spindle-shape cells. After treatment with serum starvation, the fibroblast cells became flattened and sparse. This study showed that applying WJMSCs-CM improved the ability of serum-starved fibroblasts to proliferate. The highest fibroblast cell proliferation was obtained in the 12.5% WJMSCs-CM group, with a statistically

Table 1. Comparison of cell count between serum-starved fibroblast proliferation groups

	Mean (SD)	p-value
Control	218750 (26133.312)	
WJMSCs-CM 50%	155817 (61702.201)	
WJMSCs-CM 25%	221667 (36565.922)	
WJMSCs-CM 12.5%	233167 (31447.840)	0.017

One-way ANOVA; $p<0.05$ = significant

Table 2. Post hoc analysis between groups comparison of serum-starved fibroblast proliferation

	Mean Difference	CI 95%		p-value
		Minimum	Maximum	
Control vs 50% Group	62933.333	-6828.71	132695.38	0.094
Control vs 25% Group	-2916.667	-72678.71	66845.38	1.000
Control vs 12.5% Group	-14416.667	-84178.71	55345.38	1.000
50% Group vs 25% Group	-65850.000	-135612.04	3912.04	0.072
50% Group vs 12.5% Group	-77350.000*	-147112.04	-7587.96	0.024
25% Group vs 12.5% Group	-11500.000	-81262.04	58262.04	1.000

* The mean difference is significant at the 0.05 level.

Post hoc Bonferroni; $p < 0.05$ = significant

significant difference compared to the 50% group ($p < 0.05$) (Table 1 and 2). The highest proliferation was obtained in the 12.5% group and the lowest in the 50% group. The ANOVA and post hoc Bonferroni test results are shown in Tables 1 and 2.

3.2 Discussion

Stem cells are very promising for regenerative medicine due to their self renewal and differentiation potentials, it should be fully characterized to be used for patient [10]. Previously, sources of stem cell tissues were classified into two general categories depending on the time of ontogenesis: embryonic and postnatal tissues; the latter were also called adult tissues [11]. In the last decade, the list of putative human stem cell sources has been amended to include human perinatal extra-embryonic tissue. Generally, human extra-embryonic tissues are represented by different parts of the placenta, fetal membranes (amnion and chorion), and umbilical cord. Furthermore, it has been shown that extra-embryonic MSCs can be isolated from umbilical cord blood and amniotic fluid [12].

Wharton's Jelly Mesenchymal Stem Cells (WJMSCs) may appear to promote healing by paracrine signaling, but direct cell -cell contact of WJMSCs to human fibroblasts may downregulate fibroblast proliferation. Wharton's Jelly stem cells, as human umbilical cord perivascular cells (HUCPVCs), shows that these stem cells preferentially express factors related to neuroprotection, neurogenesis, and angiogenesis, and they do demonstrably contain paracrine factors from the IL6 superfamily and a significant level of pro-angiogenic bioactive molecules, such as VEGF. Stem cells secrete various growth factors that have been studied by proteomic methods, which revealed the presence of various growth factors and other cytokines in the conditioned medium [13]. According to ELISA interpretation, TGF-β1 and VEGFare present in WJMSCs-CM. However, IGF and bFGF are also available in lower concentrations [14]. Furthermore, the vitality of the pulp was induced by revascularization; VEGF is one of the growth factors that play an important role in the revascularization mechanism. [15-17]. In UVB-induced prematurely aged fibroblast cells, maximum proliferation was observed in a 50% concentration of WJMSCs- CM, yet a 100% concentration has failed to produce the greatest proliferation [18]. In contrast, in this experiment, a 50% concentration of WJMSCs-CM produced the lowest proliferation.

Based on the present study, it can be stated that there are some reasons why that lowest concentration of WJMSCs-CM has the maximum proliferative potency. First, it can be assumed that fibroblast cells differentiate faster than they proliferate. Despite studies over many years, it is still not clear to what extent cells control proliferation and differentiation. The responses of cells to treatment with differentiating agents such as TGF-β suggested that exit from the cell cycle into G1/G0 occurs quite quickly [19]. Moreover, TGF-β has an important role in regulating fibroblast proliferation and differentiation. Therefore, there was the possibility that fibroblast cells differentiated after being seeded in WJMSCs-CM [20]. Secondly, as a result of some growth factors, inhibitory mechanisms act as endogenous inhibitors of cell growth that might be downregulated in certain conditions, such as TGF-β [2,21,22]. This assumption could explain the decreasing proliferation capacity in high concentrations of WJMSCs-CM. Another assumption is that the contact-inhibited and serum-starved

methods both induce pathways that are expressed at a higher level in quiescent than in proliferating fibroblasts [23].

4. Conclusion

WJMSCs-CM in a 12.5% concentration demonstrated the maximum potential for fibroblast proliferation. Therefore, the umbilical cord tissues that remain as biomedical waste have great potential in dental pulp regeneration. However, further experiments with other methods related to dental pulp regeneration are required to analyze the potency of WJMSCs-CM.

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