Influence of Different Concentrations of Human Platelet Rich Plasma Versus Fetal Bovine Serum on Periodontal Ligament Derived Stem Cells

Original Article

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ABSTRACT

Periodontitis is a complex immune-inflammatory disease, characterized by the destruction of the periodontium. Periodontal regeneration implicates the use of mesenchymal stem cell populations for cell-based therapies. Periodontal ligament stem cells (PLSCs) isolated from the periodontal (PDL) tissue of human teeth, are the most extensively studied and applied for regeneration of the periodontal tissues. Platelet rich plasma (PRP) is widely used for various tissue engineering applications, because of its several advantages over fetal bovine serum (FBS). In this study, we isolated PLSCs from four extracted sound wisdom teeth. Venous blood was drawn from healthy male and activated PRP was prepared. The effect of various concentrations of human PRP (hPRP) in comparison with 10% FBS on PLSCs proliferation and osteogenic differentiation have been detected. Statistical analysis was done using a One-way analysis of variance (ANOVA). Our study showed that hPRP can effectively induce hPLSC's proliferation as well as stimulate osteogenic differentiation of hPLSC's. The 10% hPRP had the highest proliferation rate than 5% hPRP, 15% hPRP as well as 10% FBS with statistical significance at 3 and 5 days' groups. The RT-PCR showed that osteopontin gene in 10% FBS expressed higher than all PRP groups with no statistical significance and the osteopontin gene expression in the 10% hPRP group was higher than the 5% PRP and 15% PRP with no statistical significance.

Key Words: Fetal bovine serum, periodontitis, periodontal ligament stem cells, platelet rich plasma.

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INTRODUCTION

Periodontitis is a multifactorial genetic disease, caused by synergistic interaction of allelic variants of multiple genes accompanied by environmental and bacterial factors. Some genetic disorders show periodontitis as a manifestation, such as Papillon–Lefèvre syndrome, Chediak Higashi and Haim Munk syndrome, besides metabolic bone disorders as hypophosphatasia and hypophosphatemic rickets (**Abdelfattah and Hassib, 2015**).

Periodontal ligament stem cells (PLSCs) were first isolated by **Shi *et al*. (2002)**. These cells have the ability to multi-differentiate into osteogenic, chondrogenic, adipogenic, and neurogenic lineages. PLSCs are involved in regenerative therapy, they can stimulate bone, tooth and periodontal regeneration. On behalf of therapeutic purposes, we need a sufficient number of cells, and so in-vitro cell proliferation is essential. Fetal bovine serum (FBS) is commonly used for this aim in different cell types in vitro. Nevertheless, FBS introduces foreign proteins into the cultural system and more outstandingly has the hazard of transmitting infectious agents. Accordingly, FBS has limited clinical use. Hence, many researches have been done to find an appropriate alternative for FBS (**Tavakolinejad *et al*., 2014**).

The human platelet rich plasma (hPRP) might be a noble contestant to replace FBS, as well as, autologous hPRP can be simply produced at low cost. Moreover, its heterologous supplies prompt a poor immune response. Many studies have confirmed that hPRP improves proliferation of mesenchymal stem cells (MSCs) (**Tavakolinejad *et al*., 2014**).

Platelet-rich plasma (PRP) is the blood fraction enriched by platelets. Upon platelets activation, granular contents were secreted comprising growth factors and regulatory proteins, as platelet derived growth factor (PDGF), transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF). Such growth factors are involved in cell proliferation, migration, as well as differentiation (**Lai *et al*., 2018**).

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In the current research, the effect of various concentrations of hPRP in comparison with 10% FBS on PLSCs proliferation and osteogenic differentiation have been detected.

PATIENTS AND METHODS:

Isolation and Cell Culture (Liu et al., 2014; Gay et al., 2007)

Four sound impacted molars were extracted from healthy patients, aged from 18-25 years. The teeth were extracted for patients attending the Clinic of the Oral Surgery Department, National Research Centre, Egypt and informed consent was obtained before extraction. The periodontal tissue was dissected into small pieces and digested in a solution of 2 mg/ml Collagenase NB 4 (SERVA, USA) for 30 minutes at 37°C in a water bath. Single cell suspension was seeded into a T-25 flask (Costar, USA) with DMEM supplemented with 10% fetal bovine serum (FBS) (Equitech-Bio Inc. , USA), 100 U/ml penicillin, 100 µg/ml streptomycin and 1% Fungizone (Lonza Bioproducts, Belgium), then incubated in 5% carbon dioxide incubator at 37°C. Once the cells became 70%-80% confluent they were passaged by using Trypsin/EDTA to get the next passage of cells. Harvested passage 3 cells were taken for the experimental groups.

Experimental groups

There were four experimental groups: 1) (positive control) 10% FBS, 2) 5% hRPR, 3) 10% hPRP and 4) 15% hPRP. We had evaluated the proliferation and osteogenic differentiation capability for these groups.

Preparation of activated PRP

A total of 50 ml of venous blood was drawn from healthy male aged 31 years. Briefly, 50 ml of withdrawn blood was initially centrifuged at 2000 rpm for 7 min to separate PRP and platelet-poor plasma portions from the red blood cell fraction. The PRP and platelet-poor plasma portions were again centrifuged at 2900 rpm for 10 min to separate PRP from platelet-poor plasma. The PRP samples were stored in Eppendorf tubes at -70°C. After three freeze–thaw cycles to release the growth factors contained in platelets, the PRP preparations were ready to be applied to each culture. The final concentrations of PRP (5%, 10% and 15%) were calculated as volume percentage of added PRP to the total volume of culture media (**Tavakolinejad S *et al*., 2014, Wen *et al*., 2016**).

Cell proliferation assay

Cells of passage 3 were cultivated in 24-well culture plate. Cells of different groups were trypsinized after 1, 3 and 5 days of culture, the cells were harvested and the cell number was determined by counting using the trypan blue dye-exclusion assay, where 100 micro liter of trypan blue was mixed to 100 micro liter of treated cellular suspension and left for 10 minutes, then 10 µl of the mixture was spread into both chambers of the hemocytometer. Afterwards, the hemacytometer was observed under an inverted light microscope using the 20 x objective lens. Total count of cells in 1ml = (count in 1ml/n) x df x cf (**Abd El Latif *et al*., 2019**).

Osteogenic Differentiation

PLSCs were seeded onto six-well plates; cultured to 70% confluence; and incubated in osteoinduction medium composed of DMEM supplemented with 10% FBS, 1% Pen-Strept, 10 nM dexamethasone, 10 mM β-glycerophosphate and 50 mg/mL L-ascorbic acid 2-phosphate (all purchased from Sigma, Aldrich, USA) (**Perry *et al*., 2008**).

Evaluation of osteogenic Differentiation

Alkaline phosphatase activity

After 7 days (early stage of osteoblast differentiation), the samples were stained for alkaline phosphatase (ALP) activity using ALP kit. According to the manufacturer's instructions (Sigma– Aldrich, United States. Positive reaction is determined by the appearance of blue color.

Alizarin Red Stain

After 14 days (late stage of osteogenic differentiation), the samples were fixed with 70% ethanol for 20 minutes and stained with alizarin red stain to reveal calcium accumulation in vitro. Positive reaction was determined by the appearance of orange–red nodules.

Real Time Polymerase Chain Reaction

The total RNA was extracted from the 4 groups and Real Time PCR was performed to measure the mRNA level of osteogenic differentiation marker gene, osteopontin (SPP1) to prove the osteoblast phenotype. Total RNA isolation was obtained by using RNeasy Mini Kit (Qiagen, catalog no 74104, Crawley, UK) according to the manufacturer's instruction. Reverse transcription of RNA into cDNA was carried out by using High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction.

Real-time polymerase chain reactions were performed in StepOne Real-Time PCR System (Applied Biosystems, USA) using Power SYBR Green Master Mix. Real Time RT-PCR amplifications were performed to analyze the mRNA level of osteopontin. The Forward (F) and reverse (R) primer purchased from Invitrogen. For data analysis, the StepOne software v2.0.2 (Applied Biosystems, Carlsbad, CA) calculated the levels of target gene

expression in samples. The expression level of osteopontin gene was stabilized to a house keeping gene Beta actin. The quantification was performed using the comparative Ct method also called 2 –ΔΔCt method (**Livak & Schmittgen, 2001**).

STATISTICAL ANALYSIS:

All experiments were done in triplicate. The data are expressed as the mean standard deviation ± and were analyzed using a One-way analysis of variance (ANOVA). If any significance were detected by the ANOVA it was followed by the Tukey's Post hoc test to determine the group(s) responsible for the significance. Results were expressed in the form of P-values. The significance level was set at $P \leq 0.05$.

RESULTS:

Periodontal ligament stem cells were successfully isolated from periodontal tissue of impacted molars (**Figure 1**).

Cell proliferation assay (Figure 2)

Our results revealed that 10% hPRP has the highest proliferation rate than 5% hPRP, 15% hPRP as well as 10% FBS with statistical significance at 3 and 5 days' groups.

Osteogenic Differentiation

Alkaline phosphatase activity (Figure 3)

In all experimental groups, after 7 days of osteogenic induction, PLSCs revealed positive blue stain for ALP activity.

Alizarin Red S Staining (Figure 4)

In all experimental groups, PLSCs revealed orange red nodules indicating calcium crystals deposition and successful osteogenic differentiation of PLSCs after 14 days of osteogenic induction.

RT-PCR for Osteopontin Gene (Figure 5)

By using Oneway Anova, Osteopontin (SPP1) in 10% FBS group was higher than all PRP groups with no statistical significance and the SPP1 gene expression in the 10% PRP group was higher than the 5% h PRP and 15% PRP with no statistical significance.

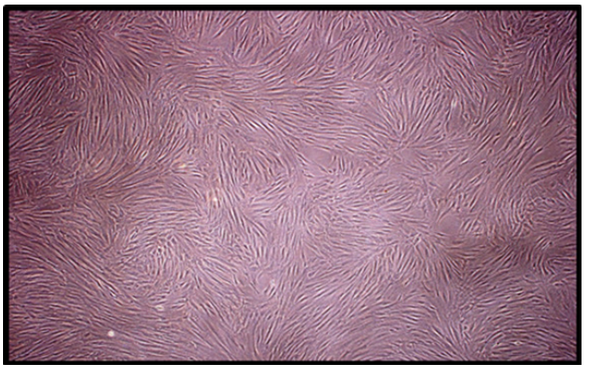

Fig. 1: Micrograph (4X) presenting isolated PLSCs when reached about 80% confluence.

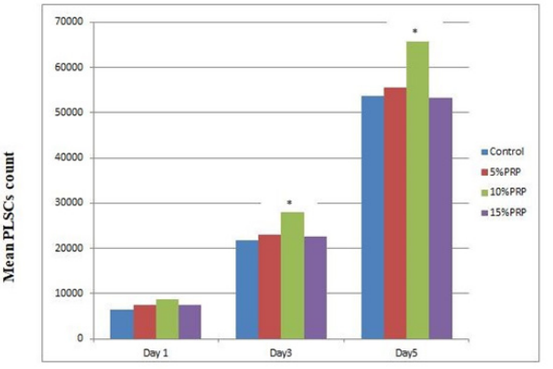

Fig. 2: Bar chart presenting the mean proliferation count of PLSCs at day 1, 3, & 5 for each experimental group.

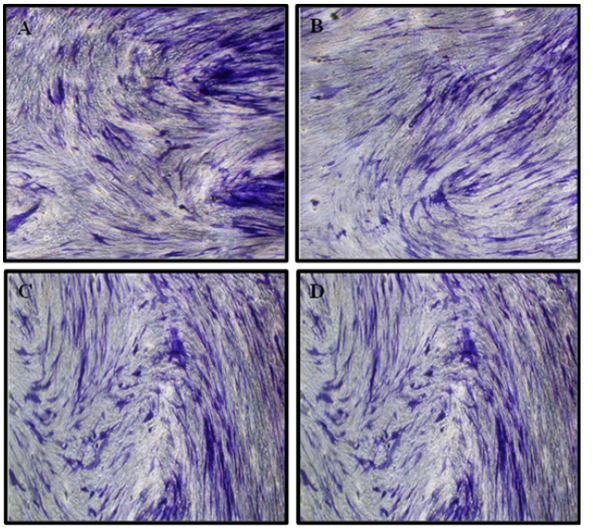

Fig. 3: Micrograph (4X) presenting positive reaction of PLSCS for ALP, blue staining, revealing early mineralization of PLSCs after 7 days of culture in osteogenic media for different experimental groups (A) Control group (10% FBS), (B) 5% PRP, (C) 10% PRP, (D) 15%PRP.

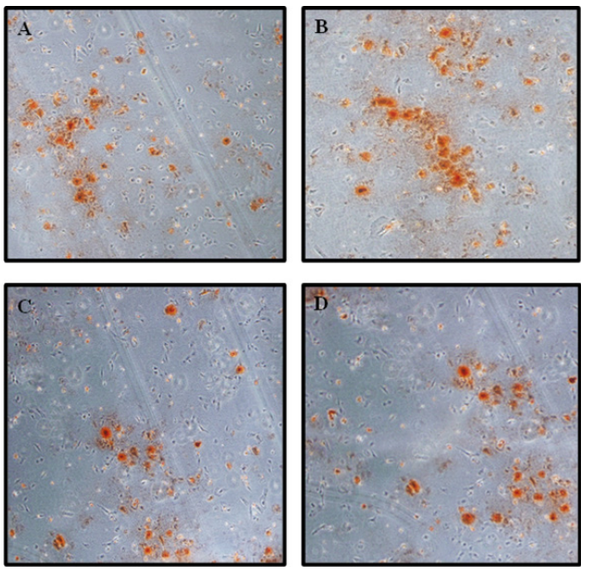

Fig. 4: Micrograph (4X) presenting positive reaction to alizarin red stain (orange-red nodules), revealing calcium accumulation by PLSCs after 2 weeks of culture in osteogenic media for different experimental groups (A) Control group (10% FBS), (B) 5% PRP, (C) 10% PRP, (D) 15% PRP.

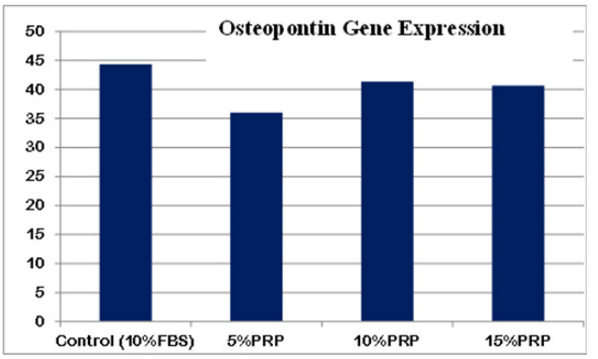

Fig. 5: Bar chart presenting mean values of relative osteopontin gene expression in the control group (10% FBS), 5% PRP, 10% PRP and 15% PRP.

DISCUSSION

Periodontitis is a complex immune-inflammatory disease, described by the destruction of the periodontium. Tooth loss is an inevitable sequela of periodontitis, if left untreated. Conventional periodontal therapy aims at eradicating microbial periodontal infection by removing bacterial biofilm, and thereby reducing inflammation to prevent further disease progression, however it is unable to restore the lost periodontal tissues (**Liang *et al*., 2020**).

The ultimate objective of periodontal therapy is the comprehensive regeneration of entire periodontal tissue comprising of alveolar bone, cementum, along with periodontal ligament, and that is a hard challenge in periodontitis management (**Qiu *et al*., 2020**).

Periodontal regeneration implicates the use of mesenchymal stem cell (MSC) populations for cell-based therapies. Dental tissue derived Stem Cells (DSCs) for instance, periodontal ligament, pulp, apical papilla, in addition to gingival stem cells are self-renewing and multi-potent MSCs. (**Liu *et al*., 2015**).

PLSCs, as the adult stem cell population isolated from the PDL tissue of human teeth, are the most extensively studied and applied for regeneration of the periodontal tissues (**Liang *et al*., 2020**).

In the current study, PLSC's were chosen over another stem cell sources as bone marrow, umbilical cord, or adipose tissue, because dental SCs have many advantages (**Bakopoulou *et al*., 2016**). Contrasted with bone marrow stem cells, they are superior in proliferation and survival rate in culture alongside deferred senescence process. Up till now, in vivo applications resulting in no major adversative consequences, as teratoma development. Most importantly, it can be easily isolated during routine noninvasive clinical dealings (extraction of wisdom teeth or premolars for orthodontic reasons) (**Egusa *et al*., 2012**).

PLSCs have the ability to multidifferentiate in vitro into collagen-forming cells, cementoblast-like cells, osteoblasts, chondrocytes, adipocytes as well **(Tan *et al*., 2015**). Above all, PLSCs possibly will produce cementum/ PDL tissue in vivo, that affords an optimum cell source for regeneration of periodontal tissue (**Iwata *et al*., 2009; Flores *et al*., 2008**).

PDL cells expansion is a mandatory stage to multiply the quantity of prerequisite cells desirable for cell-based therapies, which advocates the utilization of growth factors (**Choudhery, 2021**).

Studies reported the occurrence of anaphylactic reactions by humans transplanted with cells cultivated in the existence of FBS. Additionally, FBS carries a high risk of animal disease transmission. Therefore, there was a call for other sources of growth factors rather than fetal bovine serum.

Platelet rich plasma has been established as a safer substitute to fetal bovine serum, allowing efficient proliferation of human cells under animal serum-free conditions (**Guiotto *et al*., 2020**).

PRP is widely used for various tissue engineering applications, because of its several advantages; first of all, PRP is non- toxic and non-immunoreactive, and can augment bone regeneration. Second, clinical utility of PRP is supported by the relatively easy acquisition process. PRP also advocated proliferation and osteogenic differentiation of dental tissue derived stem cells (**Zhu *et al*., 2012**).

Platelet activation is a critical step that affects the attainability of bioactive molecules. Upon activation, PRP releases a large number of growth factors. Generally, platelets reside in the inactivated, latent state awaiting a trigger signal in order to be activated (**Eker *et al*., 2020**). Platelet activation can be exogenous either by physical methods such as freeze-thaw cycles as implemented in our study or by the addition of collagen, thrombin, and/ or calcium (**Eker *et al*., 2020**). Activation can also be endogenous in which the platelets are physiologically activated inside the body without prior activation of PRP (**Kikuchi *et al*., 2019**).

Our study showed that PRP can effectively induce hPDLC cell proliferation as well as stimulate osteogenic differentiation of hPDLC, these results are consistent with previous studies (**Zhu *et al*., 2012; Reichert *et al*., 2011, Han *et al*., 2007**).

Studies vary considerably regarding which PRP concentrations are optimal in inducing cell proliferation and promoting regeneration (**Gentile and Garcovitch, 2020**). Some recommended a high or very high PRP concentration as the most effective (**Jo *et al*., 2012**). However, others advocated low or moderate platelet concentrations and considered that very high PRP concentrations, not only are counterproductive but also carry a potential risk of cell death (**Giusti *et al*., 2014, Kakudo *et al*., 2008, Zheng *et al*., 2016**). Thus, in the present study, we used different concentrations of PRP (5%, 10% and 15%) aiming to compare their effect on PLSCs proliferation and osteogenic differentiation.

It could reasonably be expected that the higher the PRP concentration, the higher is cell proliferation. However, we found that a PRP concentration of 10% showed the greatest cell proliferation, followed by the 5 % and then the 15%.

Our results are in accordance with previous studies. **Felthaus *et al*. (2017)** which showed an increase in both cell vitality and adipogenic differentiation with a 10% to 20% PRP concentration. While, a PRP concentration more than 20% had inhibitory effects, a PRP concentration of 30% showed a decline in cell vitality and differentiation. Also, **Amable *et al*. (2014)** showed the best adipose stem cells proliferation with 10 % PRP concentration, followed by the 5% concentration and that high concentrations led to a diminished cell vitality (20%, 30%) or even to cell death (40%, 50%). **Wang *et al*. (2019)** found an increase in proliferation with 10% PRP but when 20% PRP was used, cell proliferation decreased.

However, our results are inconsistent with **Chen *et al*. (2012)** who observed maximal proliferation of dental pulp stem cells when using 5%, not 10% PRP concentration. And another study by **Xu *et al*. (2017)** who found that 1% PRP concentration showed the strongest PDLSC's differentiation. They attributed the various effects caused by different PRP concentrations to the growth factors concentrations, and considered that the decreased ability for osteogenic differentiation is as a negative feedback, controlled by the over dosage of growth factors. Therefore, standardization for PRP is very important. The best concentration of PRP has not been decided yet.

In our research, we deduced that platelet rich plasma can be used as a safe alternate for fetal bovine serum in stem cells culture with concentration of 10% and higher concentration does not induce a better result. Nevertheless, further investigates are required to reach optimal PRP concentration.

CONFLICT OF INTEREST

There are no conflicts of interest.

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