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# Possible Proliferative Impact of Human Platelet-Rich Fibrin Obtained From Controlled Type 2 Diabetic Patients on Human Dental Pulp Stem Cells (In Vitro Study)

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# Possible Proliferative Impact of Human Platelet-Rich Fibrin Obtained From Controlled Type 2 Diabetic Patients on Human Dental Pulp Stem Cells (In Vitro Study)

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## *ABSTRACT*

**Purpose**: This study was carried out in order to assess the effect of 10% PRF exudate obtained from controlled type 2 diabetic patients on proliferation capacity of dental pulp stem cells (DPSCs). Then 1% PRF exudate obtained from controlled type 2 diabetic patients was tested for osteogenic capability after 1 week. **Material and methods:** PRF exudates were prepared using the direct method from 8 healthy donors (control group) and 8 controlled type 2 diabetic patients (study group). In 96-well plates, DPSCs harvested from pulp tissue of impacted lower third molar were treated with 10% PRF exudate obtained from healthy donors and controlled type 2 diabetic patients for 1,3 and 5 days. The effects of exudates on proliferation and cell viability were tested using the MTS assay. For osteogenic capability, DPSCs were treated with 1%PRF exudate obtained from healthy donors and controlled type 2 diabetic patients supplemented with osteogenic medium for 1week and the osteodifferentiation assay was held using alizarin red stain with ELIZA reader. All data were subjected into one-way Anova statistical analysis. **Results:** The concentrations of 10% and 1% PRF exudates obtained from controlled type 2 diabetic patients had proliferative and osteoinductive effects on DPSCs comparable to that of the healthy individuals. **Conclusion:** Based on our study, controlled type 2 diabetic patients can be treated similar to their Unaffected peers using 10% PRF exudates as the optimal concentration for DPSCs proliferation and 1% PRF exudates as the optimal concentration for DPSCs osteodifferentiation and formation of mineralized nodules in 7 days.

***KEYWORDS***

*Diabetes mellitus, PRF, DPSCs*

## INTRODUCTION

Diabetes mellitus is a wide-ranging term for diverse metabolic disorders in which chronic hyperglycemia is the core finding. Either defect in insulin secretion or defect in insulin action or both are

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probably the main cause of the disease[1]. Diabetic wounds display a tenacious inflammatory state associated with decreased levels of several growth factors. Some of the significant growth factors are; Insulin-like growth factor-1 (IGF-1), Platelet-derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1)[2, 3].

Excogitation in the concept of tissue engineering has resulted in a dramatic enhancement in the process of tissue healing. Numerous pathological etiologies contributes to dysfunction of oral tissues as well as development of bone defects, hence influencing the life quality of the population suffering from these pathoses. The pivotal objective of periodontal and maxillofacial tissue regeneration is to repair these defects which resulted in the quest for a biofuel. Emphasis has always been on creating a "wonder material" with the greatest regenerative potential[4].

Several platelet-derived products also known as platelet concentrates have been developed and serve as biological mediators in the healing process. PRF is one such product that has proven its worth and has edged past the other. PRF has opened the floodgates in the dentistry, aiming primarily at improved healing and regeneration[4].

Platelet-rich fibrin (PRF), is a leucocyte- and platelet-rich fibrin biomaterial that discharges great amounts of three chief growth factors: TGFβ-1, vascular endothelial growth factor (VEGF), PDGF and a significant coagulation multicellular glycoprotein (thrombospondin-1, TSP-1) throughout 7 days[5].

Stem cells are considered to be the main source of differentiated cells for generation of tissues during development and the restoration of postnatally diseased or damaged tissues. Recently, stem cell research has tremendously expanded due to the realization that stem cell based therapies hold the ability to improve the quality of life of the patients with diseases ranging from Alzheimer's disease to cardiac ischemia to bone or even loss of teeth. An attractive source for tissue engineering applications, is the dental pulp being enriched with mesenchymal stem cells[6].

It is now well established that dental pulp stem cells (DPSCs) hold the ability to give rise to numerous types of cells, such as odontoblasts, neural progenitors, osteoblasts, chondrocytes, and adipocytes. DPSCs being extremely proliferative, aids in ex vivo expansion and improves these cells translational potential. Dental pulp is considered the most easily available reservoir of postnatal stem cells owing to its multipotency, high proliferation rates, and accessibility[6].

## MATERIAL AND METHODS

### Isolation and growth of DPSCs:

DPSCs were isolated from 4 impacted third molars extracted from 4 donors who had visited the oral surgery department, Mansoura University, Egypt after obtaining informed consent approved by the Ethics committee of Mansoura University, Egypt[7]. The extracted teeth were immediately and consecutively irrigated for 15 seconds with normal saline, 70% ethyl alcohol, and phosphate buffered saline (PBS). The teeth were kept in sterile falcon tubes holding pasteurized whole milk **,** then transported for processing to Nile Experimental Center[8]. All the following steps were performed under careful aseptic conditions in the laminar flow hood. Curette was used to scrap all the soft tissues attached to the extracted teeth, then chlorhexide was used for extracted teeth irrigation followed by PBS. Using the handle of Hand Held Pulp Isolator, the tooth is divided into two portions, then transferred to sterile dishes. The pulp was excavated from the surrounding hard tissue then was cut horizontally for outgrowth expansion technique into 10 fragments[8]. In $25cm^2$ flask(Greiner) , the pulp fragments were cultured with 10ml DMEM/F12; lonza supplemented with 1% penicillin and streptomycin(Gibco) as well as 10% FBS (Gibco), then the cultured fragments were kept at 5% $CO_2$ atmosphere in addition to $37^0C$[9]. Every three days,

complete Media was changed, then the cells were transported into 75 $cm^2$ flask when the cell colony formation units have reached 70-80 % confluence. With the protocol of cell culturing, feeding was continued until the cells were ready to be used after reaching the 4th passage[8].

**Preparation of PRF exudate**

For the control group and in accordance with our institutional ethics committee, 8 healthy donors have been chosen from the outpatient clinic at Faculty of Dentistry, Horus University in Egypt. They were nonsmokers and nonalcoholic. 4 of them were females and the others were males aged between 18 to 30 years. For the diabetic group PRF exudates were prepared from 8 controlled type 2 diabetic patients with HbA1c levels below 6.5%[10], with similar criteria to the control group with age average ranging from 40 to 50 years. Informed consents were obtained from both groups[8]. 30mm of blood were quickly collected without being exposed to air from each donor at Nile Experimental Center. In centrifugation machine (Sigma Aldrich), the blood specimens were centrifuged for 12 min at 2700 rpm[7]. PRF clot was held and detached from red blood corpuscles (RBCs) using sterile scissor and then pressed against the walls of a glass tube, it was then converted after 1 min into PRF membrane while collecting the exudates in the bottom of the glass tube. At 1800 rpm for 5 min, the PRF exudates were centrifuged to acquire exudates only without RBCs. The exudates were then filtered by 0.22μm syringe filter unit (Millipore Corporation, Bedford, MA 01730). PRF exudates were kept in eppendorfs at -20°C. The final concentrations of PRF exudates (1% and10%) were calculated on the basis of the volume of PRF exudates that was added to the whole volume of the culture media[8].

**Flowcytometry:**

Two millions of DPSCs were trypsinized and harvested at fourth passage. They were washed and resuspended in PBS supplemented with 3% FB containing saturating concentrations (1: 100) dilution of the following fluorescein isothiocynate-conjugated anti-human monoclonal antibodies: anti CD34- phycoerythrin (PE), anti-CD105 and anti-CD 90[8].

**Proliferation assay**

DPSCs were treated with 10% PRF exudates obtained from normal individuals and controlled type 2 diabetic patients. 96-well plates were used with cell density of $2\times10^3$ for each well**.** Both groups were incubated at 5% $CO_2$ and $37^0$C. Cells were daily examined with the aid of inverted light microscope (Olympus Optical Co., Ltd., Japan). MTS assay was used to assess the proliferation rate after 1, 3 and 5 days. Every 3days, the culture media was changed and the experiment was repeated at least 3 times[11].

**Osteogenic differentiation assay**

DPSCs were treated with 1% PRF exudates obtained from normal individuals and controlled type 2 diabetic patients. 6-well plates were used with cell density of $5\times10^4$ for each well. Both groups were incubated $37^0$C and 5% $CO_2$. Cells were daily examined with the aid of inverted light microscope (Olympus Optical Co., Ltd., Japan). Alizarin red stain was used to assess the formation of mineralized nodules after 7 days. Every 3days, the osteogenic media was changed and The experiment was repeated at least 3 times[11].

**Statistical analysis**

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0**.** (Armonk, NY: IBM Corp).

Descriptive statistics were measured using Mean ±Standard deviation (SD) form. In the statistical comparison between the different groups, the significance of difference was evaluated utilizing one-way ANOVA (analysis of variance) to compare between more than two different groups of numerical (parametric) data. Statistical significances accepted for probability (P) values $<0.05$ for all tests.

## RESULTS

Morphology of cultured DPSCs: At first cells had a rounded floating appearance during first feeding. During second feeding, some cells began to show spindle-like appearance while others revealed a fibroblast-like appearance demonstrating the diversity of pulp cells morphologies. After 2 weeks of isolation, the cells started to interlace with each other exhibiting a spindle shape appearance. (Fig.1)

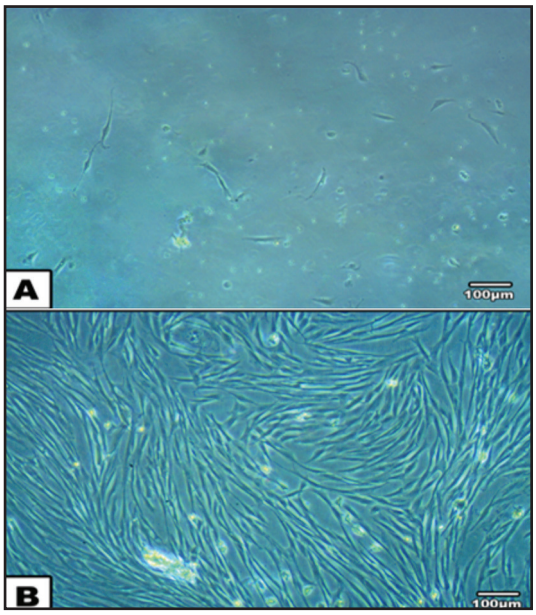


Figure (1): Inverted light microscopic photographs demonstrating colony formation capacity of DPSCs

### Flowcytometry results

Surface antigen analysis for HDPSCs at 4th passage revealed that cells exhibited positive results to CD90 (92.5%) as well as CD105 (95.9%) while cells expressed negative results (10.1%) for the hematopoietic marker (CD34). (Fig.2)

### Proliferation results

In the control group, the highst mean value for viability of cells was on day 5 (0.33±0.09) followed by day 1(0.25±0.06) while the lowest mean value for cell viability was on day 3 (0.22±0.04).(Table 1) Diabetic group showed decreased mean values when compared to theat of the control group. Day 5 recorede the highest value for cell viabilty (0.28±0.03) followed by day 1 (0.21±0.05) then day 3 (0.18±0.04).(Table 1)

### Statistical analysis:

Statistically, one way-ANOVA showed no significant difference between control and diabetic group regarding cell viability. Within the same group there was significant difference between different time periods. Post hoc test for the control group showed a significant difference between day 1 and day 5 and between day 3 and day 5 and a non-significant difference between day 1 and day 3. Post hoc test for the diabetic group showed a significant difference between day 1 and day 5 and between day 3 and day 5 and a non-significant difference between day 1 and day 3. **(Table 1)**

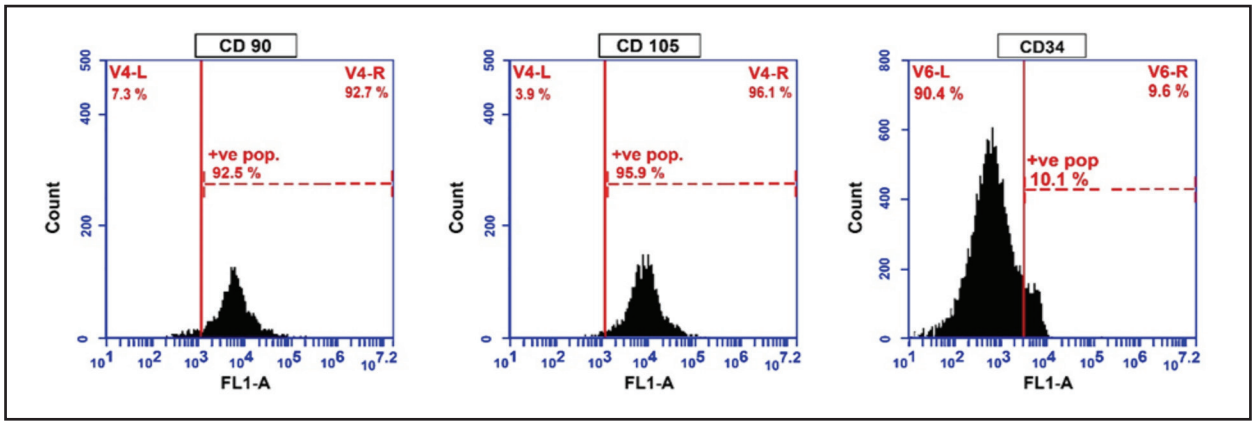


Figure (2): Photographs showing flowcytometry results

**Table (1):** *Comparison between the three studied time periods regarding cell viability*

| | Day 1 | Day 3 | Day 5 | p |
|---|---|---|---|---|
| **Control (n = 8)** | | | | |
| Mean ± SD. | 0.25±0.06 | 0.22±0.04 | 0.33±0.09 | <0.001* |
| **Sig. bet. periods.** | $p_1$=0.092, $p_2$=0.019*, $p_3$=0.003* | | | |
| **Diabetic (n = 8)** | | | | |
| Mean ± SD. | 0.21±0.05 | 0.18±0.04 | 0.28±0.03 | <0.001* |
| **Sig. bet. periods.** | $p_1$=0.095, $p_2$=0.021*, $p_3$<0.001* | | | |

*Significance between periods was done using Post Hoc Test (adjusted Bonferroni)*

*$p_1$, $p_2$, $p_3$ to compare between Day 1 and Day 3, Day 1 and Day 5 and Day 3 and Day 5 respectively while p value is statistically significant at ≤ 0.05*

**Osteogenic differentiation results:**

Nodules of mineralization were detected and stained with Alizarin red. Control group showed large red areas of calcification with scattered minute calcium deposits, while the diabetic group showed smaller calcified areas with traces of calcium deposits when compared to the control group (Fig.3). The intensity of calcium compound staining was higher in the control group than diabetic group. The mean optical density for control group and diabetic group were (0.19 ±0.02) & (0.17±0.02) respectively. Statistically there was a non-significant difference between both control and diabetic groups regarding the intensity of the formed calcium compounds.

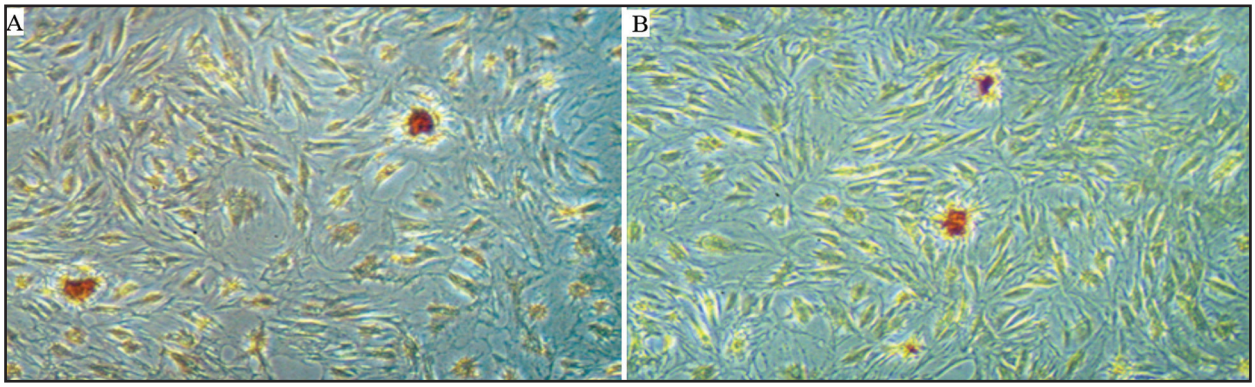


Figure (3): Inverted light microscopic photographs showing mineralized nodule formation of DPSCs stained with Alizatin Red staining and treated with 1% PRF exudates (A) control group (B) diabetic group

## DISCUSSION

Human tissues vary tremendously in their regenerative potentials. Epithelial defects can be repaired and regenerated on their own after injury, cartilage and bone small defects can be restored by connective tissue under certain conditions[12].

In response to tissue injury, abundant growth factors and cytokines stored in platelet granules are released from activated platelets[13]. Such growth factors play a key role in the integration of tissue physiology, wound healing, tissue repair, maturation and in growth. Growth factors, in some tissues affected by diabetes, are induced by a relative deficit or excess[14].

The structural changes that are characteristic to diabetic microangiopathy, and may be referred to as abnormal growth and impaired regeneration, strongly indicate a role for a group of abnormally

expressed growth factors, most likely acting together in the production of these complications[14].

In addition, in vitro cell and tissue mechanistic studies revealed that returning the abnormal level and function of the growth factor in diabetes into normal will result in a decrease in tissue pathology and dysfunction[15].

Dental pulp is considered one of the main sites for mesenchymal stem cells collection as the procedure is non-invasive with very minimal tissue sacrifice. It has been established that, transplantation of newly developed bone tissue derived from DPSCs has led to vascularized adult bone development along with good integration between the graft and the surrounding tissues in vivo [16].

It has been established that FBS is a common cell and tissue culture media growth supplement. However, serious ethical concerns regarding the welfare of the donor fetuses have been raised [17]. The present study used PRF exudate derived from normal individuals and controlled type 2 diabetic patients as a FBS alternative. Based on a previous study, PRF exudates when used as FBS alternative, also promote in vitro mineralized nodule formation and the proliferation rate of rat osteoblast[18].

It has been demonstrated that PRF exudate of 10 % concentration was the optimal concentration for in vitro proliferation of DPSCs. Whereas PRF exudate of 1 % concentration was shown to be the optimal concentration for the DPSCs in vitro osteodifferentiation[8].

In the current study, the proliferation rate of the diabetic group using 10% PRF exudates on day1 was comparable to that of the control group which showed a slightly higher rate this is in agreement with a previous study which stated that glycemic control as judged by HA1c levels along with modern diabetes care, principally intensified insulin regimens, might result in improved metabolic control in diabetic patients similar to that of their unaffected peers[19].

In agreement with another previous study which stated that after three days of culture, an inhibition in the proliferation rates of DPSCs treated with 10% PRF was observed, our study showed decreased proliferative capacity on day 3 compared to day1 in both diabetic and control groups even though slightly higher rate was recorded in control group[17].

On day 5 the proliferation rates increased again for both groups with the control group showing a slightly higher rate of proliferation than that of the diabetic. This is in accordance with the previous studies that showed significant increase in proliferation rates at the same time period[8, 20].

On the contrary to our results, a study held in 2020, suggested that 5% injectable platelet-rich fibrin (iPRF) stimulated better proliferation rates of gingival stem cells than 10% concentration. The same study agreed that iPRF had inhibitory effect on the proliferation rates in the same time periods (1, 3, and 5 days) which may imply that growth factors might need certain time to induce local stimulation[20].

In the previous studies, other platelet concentrates were used as FBS alternatives with different concentrations. The optimum PRP concentration ranged from 10% to 50%[21, 22] in addition to less than 1%[23]. For cellular proliferation and mineralization rates, In 2004, it was reported that 0.5~1% PRP is the optimal concentration[23].

However, a study held in 2005, reported that the optimum concentration for osteoblast proliferation was 50% PRP[21]. In contrast, 10% PRP was adequate to stimulate a noticeable proliferation of MSC obtained from adipose tissue[24]. Additionally, 5% PL was reported to be the optimal concentration for proliferation and osteogenesis of DPSCs and MSC[24].

Regarding the osteogenic capacity, 1% PRF exudates stimulated the osteogenic differentiation of DPSCs for both control and diabetic groups on day 7 in agreement with the previous study which

stated that it is endorsed to use this concentration, particularly in wound healing of bone and tissue engineering[17].

The study held in 2020, also considered 10% iPRF as un favorable concentration for osteogenic differentiation of gingival stem cells and stated that standard exhibited better osteogenic differentiation[20].

The previous studies also demonstrated that the optimal concentration for formation of calcium deposits for DPSCs and periodontal ligament stem cells was 2% PRP. Whereas ,1% PRP contributed to the highest rates of calcium deposits formation in human exfoliated deciduous teeth[25, 26].

So it's safe to say that appropriately managed diabetic patients with strict glycemic control as demonstrated by HA1c levels being below 6.5%[27] along with modern diabetes care, particularly intensified insulin regimens, might achieve an improved metabolic control comparable to that of normal individuals. Therefore, they could be given the same chance to be treated with the advanced dental treatment options as their unaffected peers[19].

## CONCLUSION

Based on our study, controlled type 2 diabetic patients can be treated similar to their un affected peers using 10% PRF exudates as the optimal concentration for DPSCs proliferation and 1% PRF exudates as the optimal concentration for osteodifferentiation of DPSCs and formation of mineralized nodules in 7 days only.

## RECOMMENDATIONS

We recommend utilizing PRF exudates as an alternative to FBS in supplementation of the culture media of DPSCs to avoid the risk of zoonoses in controlled type 2 diabetic patients similar to normal individuals. Preparing PRF exudates with the direct method was simple, easy and cheap.

10% Platelet rich fibrin (PRF) exudates was the optimum concentration for DPSCs proliferation while, 1% PRF exudates was the optimum concentration for DPSCs osteodifferentiation in a very short period of time in controlled type2 diabetic patients similar to their un affected peers so we recommend it in treating bone defects by cell therapy for that particular group of patients.

We also recommend conducting further studies on the effect of PRF exudates obtained from uncontrolled type 2 diabetic patients on the proliferation and osteodifferentiation capacity of DPSCs in comparison to that obtained from the controlled population.

## CONFLICT OF INTEREST

All authors deny any conflict of interest

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