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Research Article

Personalized Culture of Human Dental Pulp Stem Cells

Miroslav Tolar[1]* and Marie M Tolarova[2]

[1]Pacific Regenerative Dentistry Laboratory, Departments of Orthodontics and Biomedical Sciences, University of the Pacific, Arthur A Dugoni School of Dentistry, San Francisco, California, CA, USA

[2]Pacific Craniofacial Team and Cleft Prevention Program, Departments of Biomedical Sciences and Orthodontics, Arthur A. Dugoni School of Dentistry, University of the Pacific, San Francisco, California, USA

***Corresponding Author:** Miroslav Tolar, Pacific Regenerative Dentistry Laboratory, Departments of Orthodontics and Biomedical Sciences, University of the Pacific, Arthur A Dugoni School of Dentistry, San Francisco, California, CA, USA.

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Abstract

Introduction: We studied interactions of human dental pulp-derived stem cells (hDPSC), human platelet-rich plasma (PRP), human platelet-rich fibrin (PRF) and fibrin in vitro. These factors are known to participate in repair and healing of a wound and PRP and PRF have been used clinically. Optimized combinations of PRP, PRF and a low oxygen atmosphere in vitro enhance multiplication of hDPSC and are highly important for their successful clinical applications.

Methods: Our in vitro model involved hDPSC isolated from extracted premolars and grown in MSC cultivation medium. Human PRP, PRF, fibrin and serum were prepared from venous blood. The cultures were maintained in normal 21% oxygen or in a low (5%) oxygen atmosphere (Biospherix). Properties of hDPSC and their responses in culture were examined by flow cytometry, growth dynamics, and histochemical staining of cells. The study has a valid IRB approval Nr. 09-99.1.

Results: We show that PRP, PRF, fibrin itself and low oxygen atmosphere markedly increase multiplication rate of human hDPSC in culture and support their osteogenic differentiation potential.

Conclusion: The personalized (optimized in respect to a patient) cultivation conditions can be used for accelerated preparation of patient's hDPSC suitable for implantation to facilitate growth of a new bone.

Keywords: *Dental Pulp Stem Cells; Platelet Rich Plasma; Platelet Rich Fibrin; Regenerative Dentistry*

Abbreviations

MSC: Mesenchymal Stem Cells; hDPSC: Human Dental Pulp-Derived Stem Cells; PRP: Platelet-Rich Plasma; PRF: Platelet-Rich Fibrin; PPP: Platelet-Poor Plasma; PDF: Platelet-Derived Factors

Introduction

Wound healing is a complex process comprising hemostasis (involving activation of platelets and formation of fibrin), inflammatory processes, and restoration of tissue integrity by proliferating and differentiating stem cells.

With the aim to improve tissue healing, platelet growth and adhesion factors combined with fibrin were clinically applied in a form of platelet-rich plasma (PRP) [1] and platelet-rich fibrin (PRF) [2,3]. Since then, PRP and PRF have been widely used in periodontal and maxillofacial surgeries to improve wound healing and new bone formation. For larger bone defects, MSC are collected, expanded in culture and then applied to a host site.

We focused on this critical *in vitro* period of MSC proliferation. The goal of this study was to optimize the culture conditions for expansion of hDPSC utilizing important intrinsic factors of a wound microenvironment, i.e. platelet-derived factors, fibrin and low oxygen tension. Low oxygen tension is one of the factors constituting microenvironment of MSC [4] and can increase their proliferation in culture [5].

Materials and Methods

Human hDPSC were isolated from healthy premolars (IRB approval Nr. 09.91.1). They were positive for CD29, CD90 and CD105 (> 95%) and negative for CD34 and CD45 (< 1%) by flow-cytometric analysis and passages 3 – 6 were used.

They were grown as monolayer on the following substrates: polystyrene, fibrin and fibrin with platelet-derived factors (PDF). The growth medium consisted of alphaMEM supplemented with human adult serum (10%), 2 mM L-glutamine, 100µg/ml Streptomycin, 100 units/ml Penicillin (Thermo Fisher Scientific, USA). The osteogenic differentiation medium consisted of alpha MEM supplemented with human adult serum (10%), 2 mM L-glutamine, 10 nM dexamethasone, 100 µM L-ascorbic acid, 1.8 mM KH_2PO_4 and antibiotics. The cells were grown at 37 °C, 98% humidity, 5% carbon dioxide and 21% or 5% oxygen (using Biospherix chamber, NY, USA).

Human adult serum without platelets (S) was prepared from coagulated blood using BD vacutainers and was inactivated at 56°C for 30 minutes. Using EDTA-uncoagulated blood, platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were separated by 150 g centrifugation followed by 1660g centrifugation. Platelets in a small volume of plasma were activated by mixing in 4:1 (v/v) ratio with calcium chloride water solution (3 mg/mL $CaCl_2.2H_2O$) causing a release of platelet-derived factors (PDF). Platelet-rich serum (PRS) contained 10% of PDF fraction. PP-fibrin layer was prepared by coagulation of platelet-poor plasma mixed in 4:1 (v/v) ratio with 3 mg/mL $CaCl_2.2H_2O$ water solution. PR-fibrin layer was prepared by the same procedure using the platelet-rich plasma.

Growth curves were obtained by counting cells in circles (1 mm in diameter) inscribed exteriorly on the bottom of cultivation dishes. Cultures of osteogenically differentiated hPDSC were stained for intracellular alkaline phosphatase by a tetrazolium method (Sigma, St. Louis, MO, USA) and for extracellular calcium deposits by alizarin red S (Sigma, USA).

Descriptive statistics was performed using Microsoft Excel statistical tool pack. Two-way t-test was used for calculation of statistical significance of differences between means ($p < 0.05$ was considered statistically significant).

Results

Fibrin and platelet-derived factors strongly enhance proliferation of hDPSC in culture

The same batch of platelet-poor plasma for preparation of human fibrin and of platelet-rich plasma for preparation of platelet-derived factors was used for parallel experiments. Controls were cultivated on polystyrene substrate in medium with serum containing no fibrin or platelet factors. We found that fibrin and platelet-derived factors statistically significantly accelerated cell division rate of hDPSC (Table 1).

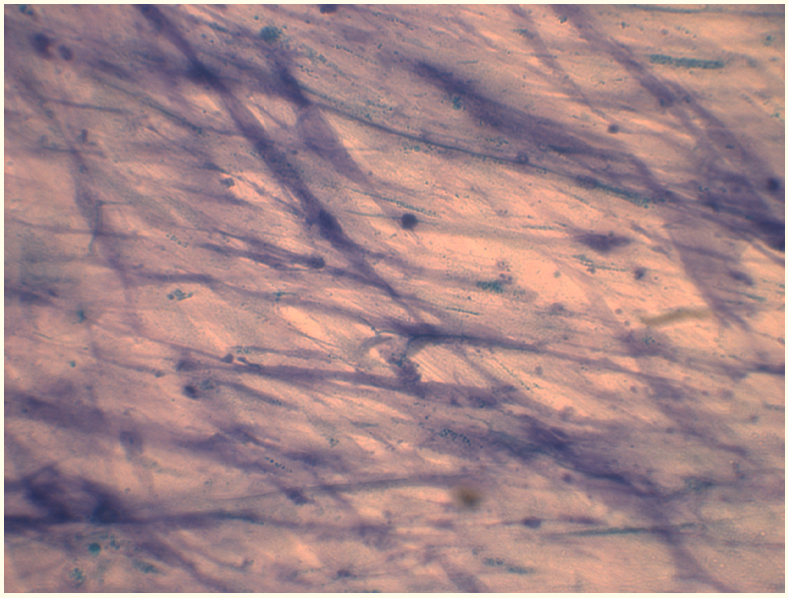
Culture conditions	hDPSC 4 days in culture (n = 6)
On polystyrene + S	8.67 ± 4.59
On PP-fibrin + S	29.67 ± 13.05 ($p < 0.02$)
On PR-fibrin + S	53.34 ± 31.16 ($p < 0.05$)
On polystyrene + PRS	9.67 ± 3.33
On PP-fibrin + PRS	24.67 ± 17.35 ($p < 0.02$)

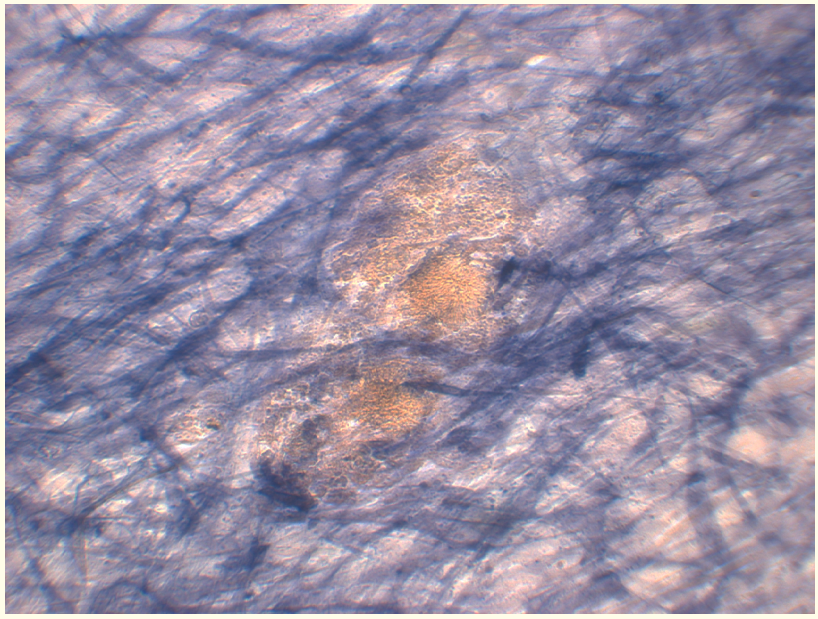
Table 1: *Growth of hDPSC.*

Legend: *Proliferation of hDPSC was measured for four days starting from the same number of seeded cells. Here we show the results for day 4. Each point represents a mean of 6 counts. Abbreviations: S = Serum, PRS = Serum with platelet-derived factors, PP-fibrin = Fibrin prepared from platelet-poor plasma, PR fibrin = Fibrin prepared from platelet-rich plasma.*

Fibrin enhances differentiation of hDPSC in culture

To show that increased proliferation mediated by the optimized culture conditions did not interfere with functionality of hDPSC, we induced their osteogenic differentiation. Our data show that the hDPSC activated by fibrin were fully functional and exhibited osteogenic markers (intracellular alkaline phosphatase and extracellular calcium deposits) within three weeks of cultivation (Figure 1).





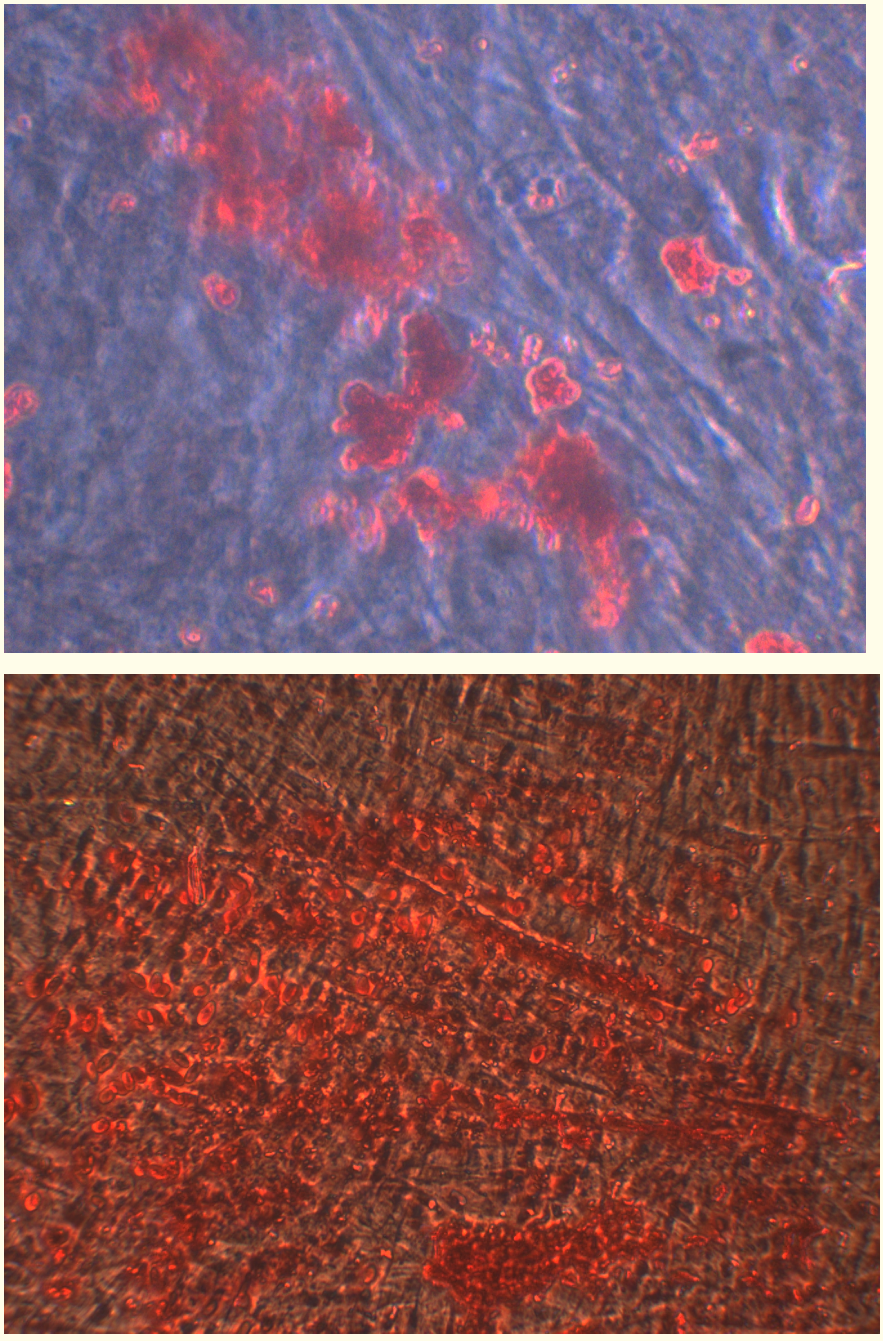


Figure 1: *Osteogenic differentiation of hDPSC.*

Legend: *Mineralized tissue (osteogenic) phenotype of hDPSC: A. Light expression of cellular alkaline phosphatase when hDPSC were grown on polystyrene. B. Strong expression of cellular alkaline phosphatase and increased cell density when hDPSC were grown on PP-fibrin. C. Few calcium deposits stained with alizarin red S among hDPSC cultivated on polystyrene. D. Extensive calcium (Magnification 400x) deposits stained with alizarin red S and increased cell density of hDPSC cultivated on PP-fibrin.*

Low (5%) oxygen atmosphere enhances proliferation of hDPSC in culture

In another experiment, we compared proliferation of hDPSC on polystyrene substrate in normal (21%) and low (5%) atmospheric oxygen. In comparison with starting day, number of hDPSC on day 9 of culture increased by 188.83 ± 19.90% in 21% oxygen atmosphere but increased by 326.13 ± 67.26% in 5% oxygen atmosphere ($p < 0.03$).

Discussion

We showed that fibrin substrate significantly enhanced proliferation of hDPSC *in vitro*. The highest rate of multiplication and differentiation of hDPSC was seen on a platelet-rich fibrin in a growth medium containing human serum. The lowest proliferation and differentiation rates were found when hPDSC were cultivated on polystyrene in medium containing serum.

We also showed that a low (5%) oxygen partial pressure allowed a higher proliferation rate of hDPSC in comparison with normal (21%) oxygen partial pressure in the atmosphere.

It is generally accepted that human adult MSC represent a valuable resource for building the tissues in the oral and maxillofacial regions and elsewhere [6,7]. Time to obtain a sufficient number of cells needed for implantation is 3 - 4 weeks [8,9]. We have shown that this time can be cut approximately by half, if fibrin substrate and/or low oxygen are applied as culture conditions.

Standard methods of MSC cultivation involve use of polystyrene substrate, homologous human or animal sera, atmospheric oxygen concentration. All these factors can lead to endoplasmic reticulum stress and unfolded protein response in cultured cells, thus decreasing their vitality or leading to their death.

Using our optimized culture protocol, hDPSC are less stressed, proliferate faster and time spent in culture is shortened. Less stress and shorter time in culture to obtain a sufficient number of hDPSC increase general vitality of hDPSC population and increase their potential to successfully adapt to host environment after implantation.

The studied intrinsic factors (hDPSC, serum, fibrin, PRP, PRF, PDF) can be obtained from the same patient, to whom hDPSC are planned to be applied. Personalized medicine and dentistry lead to better clinical outcomes and patient satisfaction. Our optimized culture protocol is also personalized.

Clinical use of stem cells is always connected with safety concerns. A recent study [10] concluded that no neoplastic complication was recorded in 339 patients treated with autologous cultivated MSC and followed up for three years. It seems reasonable to suppose that personalized culture protocol will increase safety of hDPSC clinical applications.

The aim of this short communication was to point to the important personalization area that can benefit a patient undergoing periodontal or maxillofacial surgical procedure involving bone tissue engineering.

Conclusion

We evaluated effects of three components of cellular microenvironment – low oxygen, platelet-derived factors and fibrin substrate - on proliferation and osteogenic differentiation of hDPSC.

Proliferation rate of hDPSC was significantly increased when maintained in 5% oxygen on polystyrene substrate with medium containing serum.

Proliferation rates were significantly increased when hDPSC were grown on fibrin substrate with or without platelet-derived factors in medium containing serum.

Our findings have practical implications for preparation of tissue-engineered grafts using hDPSC. If autologous cells, fibrin, platelet-derived factors, and serum are used, a faster multiplication *in vitro* can be achieved and a number of cells required for implantation can be obtained in a much shorter time and with a higher vitality than using contemporary techniques, thus minimizing patient's waiting time as well.

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