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ORIGINAL PAPER

Injectable platelet-rich fibrin influences the behavior of gingival mesenchymal stem cells

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Abstract

In this study, we examined the effects of injectable platelet-rich fibrin (iPRF) on proliferation and osteodifferentiation in mesenchymal stem cells (MSCs) isolated from human gingiva. Gingival MSCs (gMSCs) were grown in experimental culture media with different concentrations of iPRF [5%, 10%, and replacement of fetal calf serum (FCS) in the standard media with 10% iPRF–10% iPRF-FCS]. Immunophenotyping of gMSCs was performed after seven days by flow cytometry, and their proliferation was examined after three and seven days using the Cell Counting Kit-8 method. After 14 days in culture, spontaneous osteogenic differentiation of gMSCs was evaluated *via* real-time polymerase chain reaction. All gMSCs were positive for cluster of differentiation (CD) 105, CD73, CD90, and CD44, and negative for CD34/45, CD14, CD79a, and human leukocyte antigen, DR isotype (HLA-DR). Reduced expression of some surface antigens was observed in the gMSCs grown in 10% iPRF-FCS medium compared to the other groups. After three days, gMSCs grown in 10% iPRF had proliferated significantly less than the other groups. After seven days, proliferation was significantly higher in the 5% iPRF cells compared to the control, while proliferation in the 10% iPRF and 10% iPRF-FCS groups was significantly lower. No spontaneous osteogenic differentiation was observed in the presence of iPRF, as observed by low runt-related transcription factor 2 (*RUNX2*) expression. Some expression of secreted protein acidic and cysteine rich (*SPARC*) and collagen 1 alpha (*COL1A*) was observed for all the gMSCs regardless of the culture medium composition. gMSCs grown in 10% iPRF had significantly lower *SPARC* expression. In conclusion, 5% iPRF stimulated gMSC proliferation, and an excessively high concentration of iPRF can impair osteogenic induction.

***Keywords*:** mesenchymal stem cells, platelet-rich fibrin, growth factors, proliferation, differentiation, gingiva.

Introduction

Periodontitis is a prevalent disease [1], which induces the destruction of tooth-supporting tissues and affects oral function and quality of life. Although highly expected [2, 3], regeneration of lost periodontal tissues remains unpredictable. Current biomaterials used in periodontal treatment [3, 4] generally induce repair [3], and could be associated with risks of disease transmission [5].

New periodontal therapies using autologous mesenchymal stem cells (MSCs) [6] of different oral origin [7–13] may stimulate periodontal regeneration, but they are associated with certain drawbacks that limit the clinical use of *ex vivo*-manipulated MSCs [14]. Growth factors could eventually be used to stimulate the intrinsic regenerative capacity of the periodontal tissues [15].

Platelet concentrates are blood-derived products containing a 3- to 5-fold higher concentration of platelets compared to whole blood and contain many growth factors essential for bone growth and regeneration [16]. Platelet concentrates are cost effective, available in large quantities, reduce inflammation, provide healing, are non-immunogenic [16] and could replace fetal calf serum (FCS) in culture media [17]. Different production protocols have resulted in different leukocyte content and fibrin matrix architecture and density of the products [18–20].

Platelet-rich plasma (PRP) enhanced osteoprogenitor cells [21] and induced positive *in vitro* and oral surgery outcomes [22–24], but a rapid release of growth factors has also been reported [25]. Moreover, some hazards are associated with bovine thrombin and other additives used

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to handle PRP [26]. The development of platelet-rich fibrin (PRF) as a second generation of platelet concentrate, which is rich in various growth factors and capable of releasing them continuously and slowly, offers a solution for appropriate growth factor delivery [27]. Moreover, PRF is produced without the use of anticoagulants or coagulation factors [28] and, due to reduced centrifugation G-force, it induces an increase in leukocyte numbers [29] and growth factor concentrations [25]. These products both share the same principle of preparation through activation of the intrinsic coagulation cascade [30]. PRF products have complete immune biocompatibility and induce rapid angiogenesis of tissues, leading to faster wound healing [31]. Platelets harbor potent growth factors important for tissue regeneration and MSC maintenance [32, 33], such as platelet-derived growth factors (PDGFs), vascular endothelial growth factor (VEGF), transforming growth factor-beta (TGF-β), and insulin-like growth factor (IGF) [34, 35], which can be released only after platelet aggregation [36]. The leukocytes in PRF-based biomaterials improve regeneration [37], fight infection, significantly influence the biological behavior of these products [38] and contribute to the cross-talk between local cells [39, 40].

Initially, PRF was designated as leukocyte- and PRF (L-PRF) [28]. The preparation protocol was later modified to produce injectable PRF (iPRF) [34]. iPRF [34] is a recently developed leukocyte-enriched platelet concentrate, which could better assist tissue regeneration and wound healing phenomena [41]. Although initially in a liquid phase, iPRF forms a dynamic fibrin gel embedding platelets, leukocytes, type 1 collagen (COL1), osteocalcin (OC), and growth factors [38] and providing slow release of growth factors [42].

iPRF may stimulate intrinsic tissue regeneration capacity by stimulating the proliferation of human MSCs *in vitro* [18, 43–46] and by inducing osteogenic differentiation of MSCs [18, 44, 46, 47]. Additionally, it has been reported that iPRF has greater antimicrobial activity against several periodontal pathogens [48]. When implanted along with a bone graft, iPRF increases the incorporation of the graft and the signaling local molecules [49]. Despite these promising results, a more comprehensive physicochemical and biological characterization of iPRF is needed before its clinical use in dentistry. Currently, literature describing the use of iPRF in dentistry is scarce [41, 42, 50–52]. The current data seems to suggest that iPRF would be beneficial when used in periodontal-implant procedures [53], but other studies are needed to fully characterize its behavior and reveal any added advantages or drawbacks [42].

Oral MSCs are important players in the process of periodontal regeneration, and stimulation of these cells by surgical approaches is highly beneficial and expected. Using growth factors to promote periodontal regeneration is an important strategy for the reestablishment of structure and function in periodontitis patients. iPRF is a new type of platelet concentrate that may provide improved periodontal regeneration due to its supraphysiological doses of growth factors and cells. The aim of the present study was to observe the effects of iPRF on MSCs isolated from human gingiva, specifically in terms of proliferation and osteodifferentiation capability in comparison with standard growing conditions. Our null hypothesis is that there will be no differences in gingival MSCs (gMSCs) grown in different concentrations of iPRF-supplemented culture media *versus* standard culture medium.

Materials and Methods

Study design

The protocol used in this study was approved by the Ethics Board of the Iuliu Haţieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania (Nos. 359/13.10.2014 and 330/26.07.2018). Before signing the informed consent, the patients from which tissue samples and blood were collected were given explanations regarding the study protocol and procedural details. This study protocol adhered to the principles outlined in the Declaration of Helsinki on experimentation involving human subjects. The experiments using stem cells were conducted in accordance with European Union and national laws.

MSCs previously isolated from gingival tissues and fully characterized [12] were used in the present experiment. Experimental culture media were prepared by supplementing the standard culture medium with different concentrations of iPRF. gMSCs were grown in experimental culture media for seven and 14 days, and then their surface antigen expression, proliferation capacity, and eventually spontaneous osteogenic differentiation capacity were assessed. Immunophenotyping of surface antigens was performed by flow cytometry, and the proliferation capacity of gMSCs was tested using the Cell Counting Kit-8 (CCK-8) method. The spontaneous osteogenic differentiation of gMSCs cultivated in different experimental culture media for 14 days was evaluated through real-time polymerase chain reaction (RT–PCR) analysis of gene expression. gMSCs cultivated in standard culture medium were used for the control group. All experiments were performed in triplicate.

Preparation of iPRF and experimental culture media

All blood samples were obtained from a 27-year-old female volunteer donor who was a member of the research team (SI), a non-smoker, had no systemic disease, had no history of using antiplatelet or anticoagulant medication, and had given informed consent for the procedure.

The procedure was performed every three days, corresponding to culture medium replacement, for a total of five times. Each time, four tubes (iPRF Collection Tubes, Process for PRF, Nice, France) containing 10 mL of whole blood without anticoagulant were centrifuged according to a pre-established protocol [42, 54] at 700 rpm for three minutes (60×g), at room temperature, in a Duo Centrifuge (Process for PRF, Nice, France) (Figure 1A). After centrifugation, the whole blood was separated into three main parts: a yellow upper part, a buffy coat in the middle, and a red blood cell-containing lower part (Figure 1B). An automatic pipette was used to collect the iPRF (approximately 1 mL from the upper layer). Particular attention was given to collect only the superficial yellow coat (above the leukocyte-rich buffy coat) because

even minimal changes in the fractionation protocol could alter the physical and biological properties of the final product [49]. The process was performed as quickly as possible.

Figure 1 – ***Preparation of iPRF: (A) Duo Centrifuge; (B) iPRF tube after centrifugation: iPRF is the upper yellow layer. iPRF: Injectable platelet-rich fibrin.***

The three experimental culture media were prepared for the experiment as follows: two media were prepared by supplementing the basal culture medium [Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12), Sigma-Aldrich, St. Louis, MO, USA; plus 10% FCS, EuroClone, Milano, Italy; plus 1% antibiotic/ antimycotic, Gibco Life Technologies, Paisley, UK] with iPRF in two concentrations – 5% (5% iPRF) and 10% (10% iPRF). The third propagation medium was obtained by replacing FCS in basal medium with 10% iPRF (10% iPRF-FCS).

Gingival MSCs

For this experiment, gMSCs obtained from a previously isolated and fully characterized cell line were used [12].

The gMSCs were obtained from a 26-year-old woman from normal gingival wastes removed during a tooth extraction. After isolation, cells were kept in complete DMEM/F12 culture medium containing 20% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Gibco Life Technologies, Paisley, UK).

After several passages and characterization (immunophenotyping of cell surface antigens by flow-cytometry, transcription factors by immunocytochemical staining and the stemness gene expression by RT–PCR), the cell line was cryopreserved. The characterization of putative MSCs isolated from gingival tissues followed the achievement of the standard minimal criteria suggested by the *International Society for Cellular Therapy* [55], as well as some supplementary investigations. Cells were characterized by immunophenotyping of cell surface antigens [positivity for cluster of differentiation (CD) 90, CD105, CD73 and negative expression of CD45, CD34, CD14, CD79, and human leukocyte antigen, DR isotype (HLA-DR)] and transcription factors, stemness gene expression [Nanog homeobox (*NANOG*) and sex determining region Y (SRY)-box 2 (*SOX2*)], as well as by trilineage mesenchymal differentiation potential (osteogenic, chondrogenic, adipogenic) [12]. After thawing, passage 4 cells were expanded until passage 6 using a protocol detailed elsewhere [12, 56]. Briefly, 1.5×10^6 gMSCs were cultured in normal propagation DMEM/F12 medium (Gibco Life Technologies, Paisley, UK), supplemented with 10% FCS and 1% antibiotic/antimycotic (Gibco Life Technologies, Paisley, UK), and plated in a 25 cm^2 tissue flask in a humidified atmosphere with 5% CO_2, at 37°C. After reaching 70–80% confluence (five days), the monolayer cell culture was trypsinized using 0.25% Trypsin–Ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes, at 37°C, and was passaged at a 1:4 ratio until passage 6. The medium was replaced every three days.

Immunophenotyping of gMSCs

After passage 6, the basal culture medium was exchanged with experimental culture media. After seven days of growing in experimental media, gMSCs were characterized by immunophenotype analysis as described previously [12]. The following monoclonal antibodies (BD Biosciences, San Jose, CA, USA) were used according to the manufacturer's recommendations: CD105 Allophycocyanin-A (APC-A), CD73 Fluorescein isothiocyanate-A (FITC-A), CD90 Forward scatter-A (FSC-A), CD44 APC-A, CD34/45 Phycoerythrin-A (PE-A), CD14 FITC-A, CD79a PE-A, and HLA-DR PE-A. Negative and positive controls were used. The cells were trypsinized (0.25% Trypsin–EDTA solution, Sigma-Aldrich, St. Louis, MO, USA), washed twice with phosphate-buffered saline (PBS), and then fixed in 4% paraformaldehyde (Fluka, Buchs, Switzerland) for 15 minutes. 1×10^5 cells per sample were stained at room temperature with isotype control monoclonal antibodies (mAbs) and then incubated with 3% FBS albumin. The cells were washed twice with PBS containing 2% FBS and centrifuged for six minutes, at 1800 rpm. The cells were resuspended in 500 μL of cell wash solution (BD Biosciences, San Jose, CA, USA). A BD FACSCanto™ II 6-color flow cytometer (BD Biosciences, San Jose, CA, USA) and BD FACSDiva™ software (version 6.1.3) were used for cytometric analysis [10].

CCK-8 proliferation assay

A CCK-8 (Sigma Aldrich, St. Louis, MO, USA) was used for this assay, after three and seven days of growing gMSCs in experimental media. To evaluate the proliferation of gMSCs, 100 μL of each cell suspension (5000 cells/well) was cultivated in 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA). After three and seven days, a total volume of 10 μL of the CCK-8 solution was added to each well. The plate was then incubated for four hours. The optical densities of each well were read at 450 nm with a BioTek Synergy 2 microplate reader (Winooski, VT, USA).

RT–PCR analysis

Evaluation of osteogenic gene expression was assessed by RT–PCR after 14 days of gMSC culture in experimental media. The cells were harvested in Trizol and processed for total ribonucleic acid (RNA) extraction using the classical method with phenol–chloroform. The obtained RNA was evaluated quantitatively using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and qualitatively with an Agilent

2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only RNAs which had a RNA integrity number (RIN) greater than 8 were used in subsequent analyses. From each sample, 500 ng total RNA was used for complementary deoxyribonucleic acid (cDNA) synthesis with the Transcriptor First Strand cDNA Synthesis Kit according to the manufacturer's protocol (Hoffmann–La Roche, Basel, Switzerland). cDNA was then 1:10 diluted and amplified with the LightCycler® TaqMan® Master Kit using a LightCycler 480 (Hoffmann–La Roche, Basel, Switzerland). C*t* values were calculated using the Absolute Quantification/Second Derivative Maximum method, and the expression levels for each gene were normalized to *18S* housekeeping gene levels. Fold changes were calculated using the *Δ*C*t* method relative to the control samples. Genes with C*t* values above 35 were considered to be not expressed. Expression levels of collagen 1 alpha (*COL1A*), runt-related transcription factor 2 (*RUNX2*), secreted protein acidic and cysteine rich (*SPARC*; osteonectin), Nanog homeobox (*NANOG*), bone morphogenetic protein 4 (*BMP4*), and *18S* were analyzed using the primer sequences listed in Table 1.

Table 1 – *Primer sequence used for gene expression analysis of gMSCs*

Gene	Forward sequence (5'→3')	Reverse sequence (5'→3')
COL1A	CAGGCAAACCTGGTGAACA	CTCGCCAGGGAAACCTCT
RUNX2	GCCTAGGCACATCGGTGA	CCGTCCATCCACTCTACCAC
SPARC	TTCCCTGTACACTGGCAGTTC	AATGCTCCATGGGGATGA
NANOG	ATGCCTCACACGGAGACTGT	CAGGGCTGTCCTGAATAAGC
BMP4	GAGGAGTTTCCATCACGAAGA	GCTCTGCCGAGGAGATCA
18S	GCAATTATTCCCCATGAACG	GGGACTTAATCAACGCACGC

gMSCs: Gingival mesenchymal stem cells; *COL1A*: Collagen 1 alpha; *RUNX2*: Runt-related transcription factor 2; *SPARC*: Secreted protein acidic and cysteine rich (osteonectin); *NANOG*: Nanog homeobox; *BMP4*: Bone morphogenetic protein 4; A: Adenine; C: Cytosine; G: Guanine; T: Thymine.

The primers' design was performed at the laboratory where the RT–PCR analysis was made, using Universal ProbeLibrary Assay Design Center, Roche Life Science, software.

Statistical analysis

Data from the CCK-8 analysis were summarized as mean ± standard deviation (SD). The comparisons between the control group and each experimental group were evaluated with a Mann–Whitney test at a significance level of 5%. The data were plotted as individual values (circles) and the median (the line) using the template provided by Weissgerber *et al.* [57].

RT–PCR assays were analyzed with GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). The differences were considered statistically significant at $p<0.05$.

Results

Characterization of surface markers of gMSCs grown in iPRF-conditioned experimental media

In this experiment, passage 6 gMSCs were grown for seven days in experimental media for evaluating surface antigen make-up (Figure 2). Under optic microscopy, gMSCs grown in all experimental conditions were firmly attached to the plastic culture plates and showed a fibroblast-like morphology with an increased proliferation rate. From passage 4 to passage 6, the cells maintained their proliferation rates without changing their morphology.

The *in vitro* expression of gMSC surface markers was evaluated by flow cytometry. gMSCs were assessed for several MSC surface antigens proposed as possible standards, as well as one supplementary antigen. The results revealed relatively similar expression patterns of surface markers across all four groups. Irrespective of the media composition, all gMSCs were positive for CD105, CD73, CD90, and CD44, and negative for CD34/45, CD14, CD79a, and HLA-DR, indicating a MSC phenotype (Table 2).

However, some differences in antigen expression were observed. A reduced expression of some positive surface antigens (CD105, CD73, and CD90) was recorded for gMSCs grown in 10% iPRF-FCS medium compared to the other groups. Additionally, an increased expression of CD79a, which is usually not expressed on gMSCs, was also observed in this group.

Table 2 – *Flow cytometry analysis of passage 6 gMSCs grown in different experimental media*

Experimental medium	Surface antigens [%] of gMSCs							
	CD105 APC-A¥	CD73 FITC-A‡	CD90 FSC-A†	CD44 APC-A	CD34/45 PE-A§	CD14 FITC-A	CD79a PE-A	HLA-DR PE-A
5% iPRF	93	95.1	95.3	95.5	0.1	0	3.1	0
10% iPRF	94.1	91.1	97.1	94.8	0.1	0	4.1	0
10% iPRF-FCS	88.2	79.2	86.6	94.9	0.1	0	8.6	0
Control	91.3	90.3	97.4	96.8	0.1	0	0.5	0.2

gMSCs: Gingival mesenchymal stem cells; iPRF: Injectable platelet-rich fibrin; FCS: Fetal calf serum; CD: Cluster of differentiation; APC-A¥: Allophycocyanin-A; FITC-A‡: Fluorescein isothiocyanate-A; FSC-A†: Forward scatter-A; PE-A§: Phycoerythrin-A; HLA-DR: Human leukocyte antigen, DR isotype.

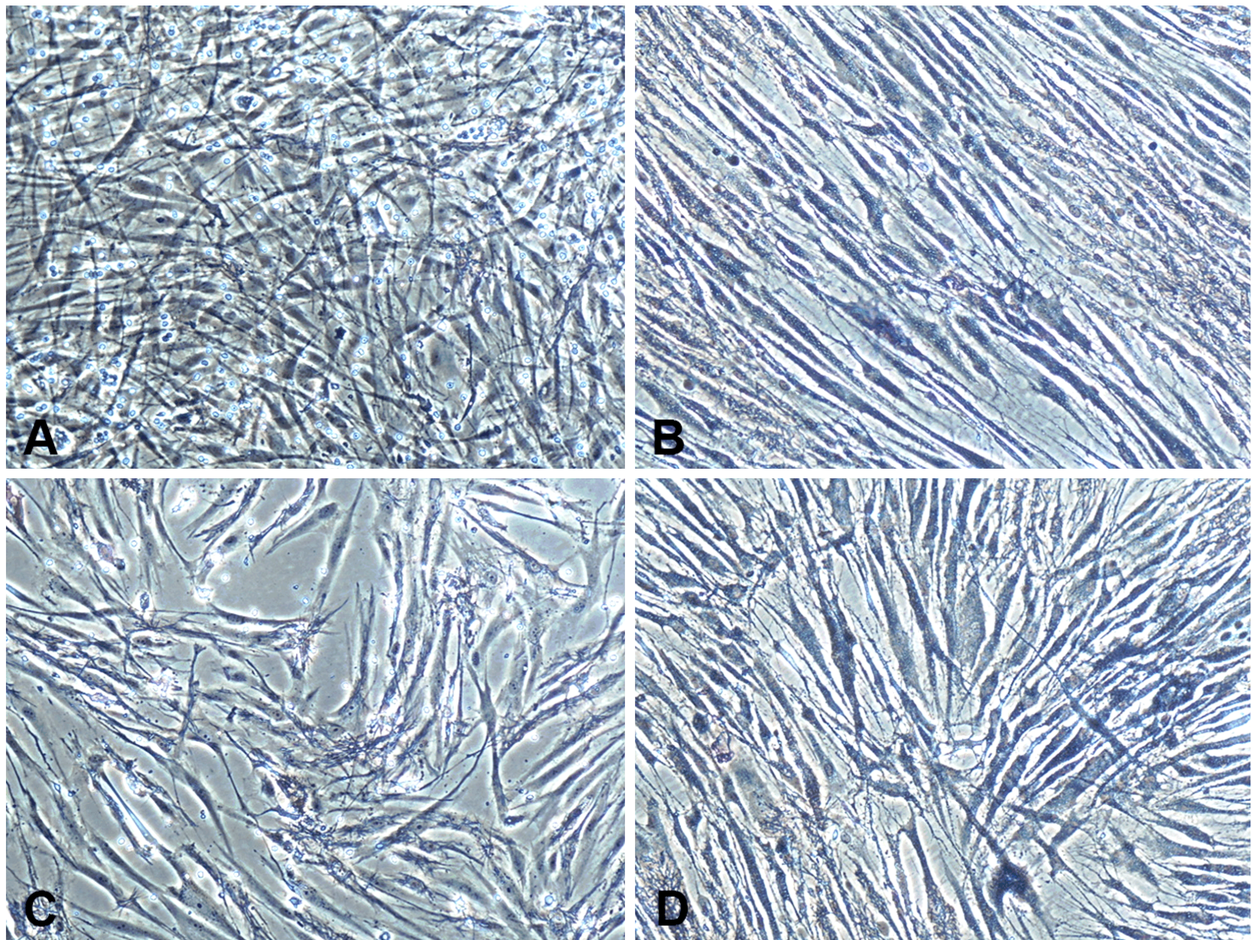

Figure 2 – *Microscopic aspects of gMSCs after seven days of culture in experimental media (10×): (A) 5% iPRF; (B) 10% iPRF; (C) 10% iPRF-FSC; (D) Control. gMSCs: Gingival mesenchymal stem cells; iPRF: Injectable platelet-rich fibrin; FCS: Fetal calf serum.*

CCK-8 proliferation assay

To assess the effects of iPRF on proliferation in gMSCs, proliferation after three and seven days of culture in iPRF-supplemented media was investigated. Generally, iPRF stimulation resulted in significantly lower proliferation as revealed by optical density values after three days of culture: 0.773±0.045 (control conditions) *vs.* 0.732±0.021 (5% iPRF) *vs.* 0.64±0.04 (10% iPRF) *vs.* 0.568±0.209 (10% iPRF-FCS). After three days, the control group had the highest rate of proliferation (Figure 3), with no significant differences between the control and 5% iPRF group (Mann–Whitney test: *Z*-statistic = 1.09) or the 10% iPRF-FCS group (Mann–Whitney test: *Z*-statistic = 1.53), but with a significantly lower proliferation in the 10% iPRF group (Mann–Whitney test: *Z*-statistic = 1.96) (Figure 4A).

After seven days, the optical density values indicating cell proliferation were as follows: 1.338±0.123 (control group) *vs.* 1.493±0.04 (5% iPRF) *vs.* 0.97±0.068 (10% iPRF) *vs.* 0.741±0.078 (10% iPRF-FCS). The cell proliferation was significantly higher in the 5% iPRF group compared to the control group (Mann–Whitney test: *Z*-statistic = -1.96), while the proliferation in the 10% iPRF (Mann–Whitney test: *Z*-statistic = 1.96) and 10% iPRF-FCS (Mann–Whitney test: *Z*-statistic = 1.96) groups was significantly lower (Figure 4B).

Characterization of osteogenic markers

To evaluate the effect of iPRF on spontaneous osteogenic differentiation of gMSCs, *COL1A*, *RUNX2*, *SPARC*, *NANOG*, and *BMP4* expression was measured by RT–PCR after 14 days of gMSC culture in experimental media. An overall decrease in the expression of all investigated osteogenic genes was observed for gMSCs grown in iPRF-containing media in comparison with the controls. A significantly lower expression of *RUNX2* was observed in gMSCs grown in 10% iPRF and 10% iPRF-FCS in comparison with gMSCs grown in standard culture medium. Also, a significantly lower expression of *SPARC* was observed in gMSCs grown in 10% iPRF in comparison with the controls (Figure 5).

Discussions

PRF is an autologous fibrin-based living biomaterial rich in growth factors that can modulate regenerative processes [58], possessing improved biological properties compared to other platelet concentrates. In this study, MSCs of gingival origin at passage 6 were grown in experimental iPRF-based culture media in order to observe the influence of the platelet concentrate on cell proliferation, stemness, and osteogenic differentiation. To the best of our knowledge, this is the first study investigating the influence of iPRF on gMSCs. iPRF was chosen because this product demonstrated the ability to release higher

concentrations of growth factors and to induce improved cell behavior *versus* the standard PRP variants [42]. Moreover, the process used to produce iPRF is completely natural, with no use of anticoagulant during blood harvest nor bovine thrombin and calcium chloride for platelet activation and fibrin polymerization [42].

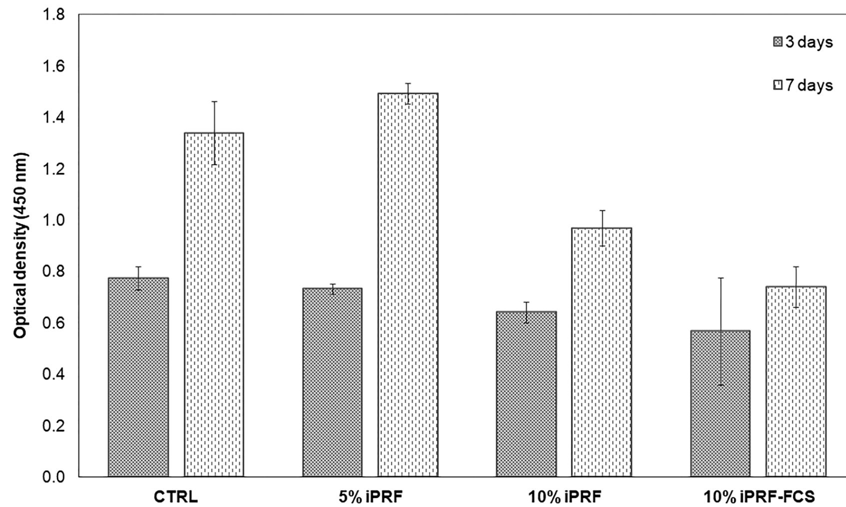

Figure 3 – ***Proliferation after three and seven days of culture in iPRF-containing media. The height of the column indicates the value of arithmetic mean for optical density, and the whiskers correspond to the standard deviation. CTRL: Control; iPRF: Injectable platelet-rich fibrin; FCS: Fetal calf serum.***

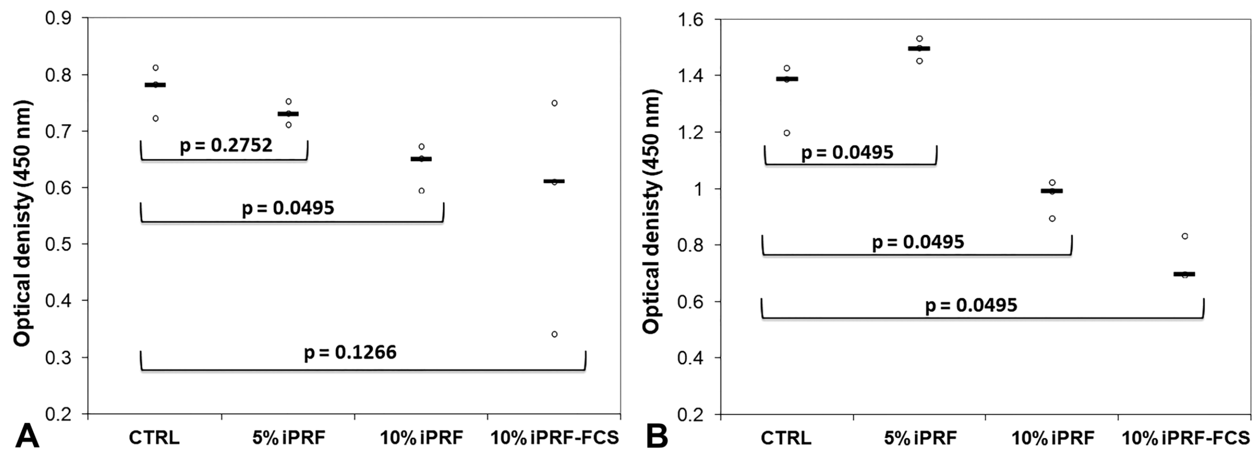

Figure 4 – ***Proliferation of gMSCs grown in the investigated culture media: (A) After three days; (B) After seven days. The circles correspond to the individual values while the line corresponds to the value of the median. gMSCs: Gingival mesenchymal stem cells; CTRL: Control; iPRF: Injectable platelet-rich fibrin; FCS: Fetal calf serum.***

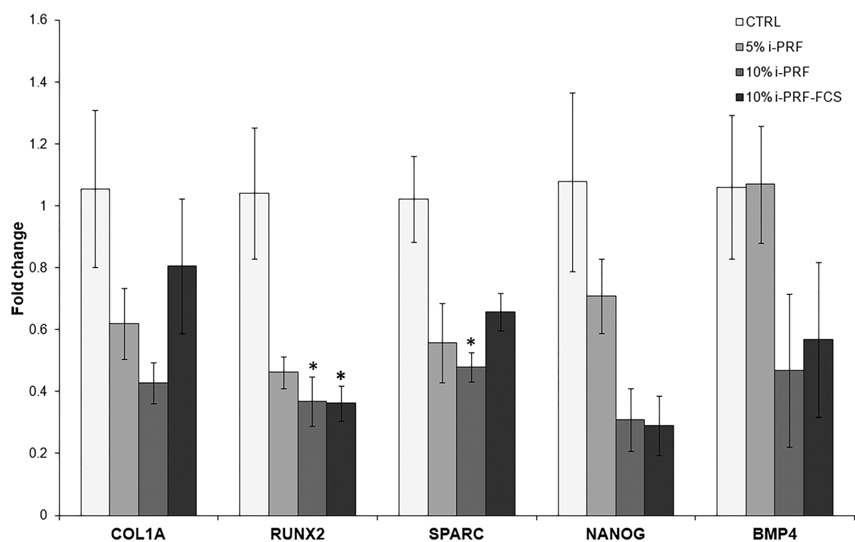

Figure 5 – ***RT-PCR results. Relative gene expression in gMSCs grown in iPRF-supplemented culture media. gMSCs: Gingival mesenchymal stem cells; CTRL: Control; iPRF: Injectable platelet-rich fibrin; FCS: Fetal calf serum; COL1A: Collagen 1 alpha; RUNX2: Runt-related transcription factor 2; SPARC: Secreted protein acidic and cysteine rich (osteonectin); NANOG: Nanog homeobox; BMP4: Bone morphogenetic protein 4.***

After three days of culture, a decrease in proliferation was observed in gMSCs grown in experimental media, although significance was only reached in the 10% iPRF group. Five percent iPRF enhanced gMSC proliferation after seven days of cultivation while retaining cell stemness. After seven days of culture, 10% iPRF or the replacement of FCS with 10% iPRF significantly decreased the proliferation of the gMSCs. The higher concentration of iPRF

seems to have an inhibitory effect on gMSC proliferation. This is in agreement with other studies, which reported that higher concentrations (10%, 20%) of PRP induced significantly less proliferation compared to lower concentrations (2%, 5%) [59, 60] possibly due to the inhibitory effect of prostaglandin E2 released by the platelet concentrate [61]. Two percent PRP seems to be the ideal concentration for the growth of exfoliated deciduous tooth MSCs [60]. The most surprising results in the current study were those of the 3-day cell proliferation assay. We would have expected a proliferative benefit induced by at least the lower concentration of iPRF, since it has been demonstrated that PRF products release high quantities of growth factors and matrix proteins for at least seven days [62]. Moreover, previous studies have reported that iPRF significantly stimulates *in vitro* proliferation of MSCs in a dose-dependent manner [18, 46] and stimulates proliferation of gingival fibroblasts [41]. It is likely that the induction of proliferation by iPRF needs time, thus explaining our results and further demonstrating the slow-release of growth factors by iPRF. One limitation of this study is that we did not use iPRF concentrations lower than 5%, and other studies have reported that 5% PRP inhibited cell proliferation [63]. However, the most beneficial iPRF concentration for inducing proliferation is still controversial [44, 59, 60, 63].

Some studies have suggested that FBS can be replaced with PRP to induce stem cell proliferation [14], with 15% PRP having the highest proliferation rate [14, 64]. The results of our study do not sustain this idea.

After seven days of culture in iPRF-containing media, a decrease in the expression of several cell surface markers was generally observed, with a more important decrease recorded for CD105, CD73, and CD90 in the 10% iPRF-FCS group. Increased CD79a positivity was seen in the 10% iPRF-FCS group. CD79 is a disulfide-linked heterodimer found on the surface of B-lineage leukocytes and its expression is likely caused by leukocytes in the iPRF conditioned media [18].

RT–PCR was used to measure important osteogenesis-related genes, including *COL1A*, *RUNX2*, and *SPARC*. We did not observe any spontaneous osteogenic differentiation in the presence of iPRF, as revealed by only low expression of *RUNX2* in all the experimental groups and a significantly lower expression of *RUNX2* in the gMSCs grown in 10% iPRF and 10% iPRF-FCS compared to the other groups. Expression of *SPARC* and *COL1A* was recorded for all gMSCs regardless of the culture medium composition. Additionally, a significantly lower expression of *SPARC* was observed in the gMSCs grown in 10% iPRF. These specific genes are expressed at different stages of osteogenic differentiation. During bone development, *RUNX2* plays an essential role in the direct activation of other osteogenic genes, including *COL1* [65]. COL1, which is the most predominant extracellular protein in bone, initially provides a structural framework for inorganic deposits [66]. *RUNX2* is a key factor that leads to MSC osteoblastic differentiation [60] and functions at the center of many other osteogenesis-related pathways, including the BMP signaling pathway [67].

Our study revealed some seemingly conflicting results, such as high co-expression of *NANOG* and *BMP4* genes implicated in pluripotency and self-renewal on one hand, and on the other some osteogenic gene expression in the MSC control cell cultures and 5% iPRF cultures. It is well known that gMSCs have a unique developmental origin (perifollicular mesenchyme and neural crest) [68], which is responsible for their high self-renewal capacity and proliferation rates compared to bone-marrow derived MSCs. Pluripotency markers, including octamer-binding transcription factor-3/4 (*OCT-3/4*), *NANOG*, and *SOX2*, are expressed by gMSCs in a manner very similar to dental pulp pluripotent-like stem cells, and their long-term telomerase expression is similar to that of embryonic stem cells [69]. *BMP4* was initially described as a major effector of the self-renewal mechanisms in mouse embryonic stem cells, accomplished by inhibition of both the extracellular receptor kinase and p38 mitogen-activated protein kinase pathway [70]. The osteoblastic differentiation process is connected to the p38-mediated phosphorylation and extracellular receptor kinase pathway, which promotes the progression of osteogenesis by enhancing the activity or expression of osteoblast-specific transcription factor genes, such as *RUNX2* [71]. These observations may explain the lower expression of osteogenic genes (*COL1A*, *RUNX2* and *SPARC*) in our samples cultivated with 10% iPRF.

The results of studies examining the effects of iPRF on osteogenic differentiation of MSCs are conflicting. Our results are consistent with studies showing that platelet concentrates appear to be inefficient in promoting osteogenic differentiation [5, 45, 72, 73]. Other studies have conversely reported that iPRF promotes significant proliferation and differentiation of bone MSCs [18, 54] in a dose-dependent manner.

Our study rejects the null hypothesis, since significant differences in terms of proliferation and gene expression in the different gMSC groups were observed. The results of the present study should be regarded with caution. It seems that proliferation and osteogenic differentiation are two processes which are activated at different concentrations of platelet concentrates. Ten percent PRF increases the proliferation of MSCs, but 1% PRF is recommended for bone wound healing and tissue engineering [44]. Moreover, there is heterogeneity in stem cell properties and in the composition of platelet concentrates due to intra-patient variation [5]. In addition, the true periodontal microenvironment was not mirrored in the present study and thus we could not measure the biological benefits of iPRF on gMSCs *in vivo*.

Conclusions

Five percent iPRF stimulated gMSC proliferation after seven days of culture but not after three days, which could indicate that a certain time is needed for growth factors to induce local stimulation. This highlights the importance of using slow-releasing growth factor products, such as iPRF, in order to assure biological stimulation for clinical purposes. Additionally, an excessively high concentration of iPRF could impair osteogenesis. Further studies are needed before iPRF can be implemented in clinical dental practice.

Conflict of interests

The authors declare that they have no conflict of interests.

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Authors' contribution

Sofia Iozon, Gabriela Valentina Caracostea and Emőke Páll equally contributed to the present study and can be regarded, therefore, as being main authors.

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