Choukroun’s platelet-rich fibrin (PRF) stimulates in vitro proliferation and differentiation of human oral bone mesenchymal stem cell in a dose-dependent way

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Abstract

Background: Choukroun’s platelet-rich fibrin (PRF) is an autologous leukocyte- and platelet-rich fibrin biomaterial. The purpose of this study was to analyse the in vitro effects of PRF on human bone mesenchymal stem cells (BMSC), harvested in the oral cavity after preimplant endosteal stimulation.

Materials and methods: BMSCs from primary cultures were cultivated with or without a PRF membrane originating from the same donor as for the cells, in proliferation or osteoblastic differentiation conditions. After 7 days, the PRF membranes were removed. A series of cultures were performed using 2 PRF membranes, in order to measure the dose-dependent effect. Cell counts, cytotoxicity tests, alkaline phosphatase (ALP) activity quantification, Von Kossa staining and mineralisation nodules counts were performed at 3, 7, 14, 21 and 28 days. A last independent series was carried on up to 14 days, for a morphological scanning electron microscope (SEM) observation.

Results: PRF generated a significant stimulation of the BMSC proliferation and differentiation throughout the experimental period. This effect was dose-dependent during the first weeks in normal conditions, and during the whole experimentation in differentiation conditions. The cultures without PRF in differentiation conditions did not rise above the degree of differentiation of the cultures in normal conditions with 1 or 2 PRF up to the 14th and 28th day, respectively. The SEM culture analysis at day 14 allowed to show the mineralisation nodules which were more numerous and more structured in the groups with PRF compared to the control groups.

Discussion and conclusions: This double contradictory proliferation/differentiation result may be due to the numerous components of PRF, particularly the presence of leukocytes: any culture with PRF is in fact a coculture with leukocytes. It could be the source of differential geographic regulation processes within the culture. The combination of oral BMSC and PRF might offer many potential clinical and biotechnological applications, and deserves new studies.

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1. Introduction

The use of platelet concentrates in oral and maxillofacial surgery, particularly in implant dentistry, is a current and interesting trend. A large number of production protocols have been developed and marketed, and many other custom-made protocols have been tested in basic research studies.\textsuperscript{1,2} The name “Platelet-Rich Plasma” (PRP) is often used to refer to these products,\textsuperscript{3} by analogy with the platelet concentrates used in haematology; however this global designation is controversial as these products show very different compositions, particularly concerning the leukocyte content (some techniques discard the leukocytes) and the fibrin matrix architecture and density.\textsuperscript{3,4} Recently, a global classification was published in order to clarify this denomination issue.\textsuperscript{3}

These autologous concentrates have been tested on many different cell types, and in numerous clinical situations.\textsuperscript{1} Nevertheless, the results are difficult to sort and interpret due to the differences between the tested PRPs. Some of these protocols have been used in periodontal surgery, with quite mixed results.\textsuperscript{5} The effects of these preparations on osteoblast and fibroblast cell cultures are contradictory, some inducing a stimulation of proliferation\textsuperscript{6} (and sometimes of differentiation), while others show reverse effects.\textsuperscript{7}

These PRPs were also tested on the bone mesenchymal stem cells (BMSC), when attempting for bone tissue engineering.\textsuperscript{8,9} PRPs give excellent results when used as a cell culture medium, in order to eliminate the fetal calf serum (FCS) from the preparations intended to be reimplanted on patients.\textsuperscript{10} The purpose of numerous studies would be to create an in vitro grafting material by combining BMSC (most often originating from the iliac crest, harvested in a less invasive manner by aspiration), and PRP as a culture medium and stimulating component,\textsuperscript{11–17} sometimes combined with a bone substitute.\textsuperscript{18–21} Nevertheless, the first results demonstrate that the tested PRPs generate a strong proliferation, but inhibit the differentiation of the BMSC.\textsuperscript{22–24} Moreover, no other easier accessible source for BMSC has yet been tested for these applications, and the use of iliac crest BMSCs remains inappropriate in everyday practice.

Choukroun’s platelet-rich fibrin (PRF) is defined as an autologous leukocyte- and platelet-rich fibrin (L-PRF) biomaterial.\textsuperscript{1,4,25,26} This easy and open-access procedure was developed in France by Choukroun et al.\textsuperscript{27} Blood is collected in 9 mL tubes and gently centrifuged during 12 min in order to divide the blood sample in three layers: a base of red blood cells at the bottom, acellular plasma on the top, and a clot of fibrin in the middle. One of the main difference between the PRF concept and most PRPs systems is that PRF production process is completely natural, with no use of anticoagulant during blood harvest nor bovine thrombine and calcium chloride for platelet activation and fibrin polymerisation. PRF is often simply considered as a natural optimised blood clot.\textsuperscript{28}

PRF clots can easily be transformed in dense fibrin membranes (Fig. 1) or cylinders by the use of the adequate tools (such as the PRF Box, Process, Nice, France). The PRF membranes release high quantities of growth factors (such as transforming growth factor TGF\textsubscript{β}-1, platelet-derived growth factor PDGF-AB or vascular endothelial growth factor VEGF) and matrix proteins (such as thrombospondin-1, fibronectin, vitronectin) during at least 7 days.\textsuperscript{29} The effects of PRF membranes on the proliferation of many different cell types have already been assessed in vitro, and the influence of the leukocytes on both cell reactions and growth factor release were already hypothesised.\textsuperscript{30} Several applications of PRF have already been described in oral surgery,\textsuperscript{31–36} ear–nose–throat\textsuperscript{37} and plastic surgery.\textsuperscript{38,39} PRF seems particularly efficient as osteoconductive filling material during sinus-lift procedure.\textsuperscript{32,40} In all clinical applications, PRF has to be considered and used as a fibrin-based living biomaterial, and not only as a simple source of growth factors.\textsuperscript{41} This novel approach on platelet concentrates opens a new field of biotechnological applications.

The purpose of this first study was to analyse the in vitro effects of Choukroun’s PRF on human BMSC, harvested in the oral cavity after preimplant endosteal stimulation.

![Fig. 1 – Choukroun’s platelet-rich fibrin membrane (original length: 4 cm).](image)

2. Materials and methods

2.1. Bone stimulation and bone cell harvesting

In order to perform human cell cultures in the presence of PRF, we had to harvest tissue specimens from patients volunteering to undergo later further blood collection for PRF production. Indeed, for immune compatibility purposes, PRF membranes must come from the same donor as the cultivated cells. For this reason, the human harvest was performed on a volunteer experimenter, a healthy 54-year-old man. The patient was planned to undergo implant placement in the maxilla in order to replace a first molar. The clinical and radiographic analysis showed a low quality bone, and decision was made to perform an endosteal stimulation prior to implant placement. Fifteen days before implant placement, a 3 mm diameter drilling (9 mm long) was performed in the future implant site, in order to activate the bone site. On the day of implant placement, the priorly performed drilling hole was carefully curetted. The harvested cells are various bone healing cells gathered on the site of the bone defect.
2.2 Preparation of human BMSC primary cultures

The explants were transported and stored in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Invitrogen, USA) at +4 °C, then placed in culture according to the explant technique. All the cells originating from the primary in vitro cultures have a fibroblastic structure in the beginning, and are morphologically similar. Primary cultures were morphologically characterised and validated as human BMSC after the induced osteoblastic differentiation of samples. After the third confluence passage, the collected cell lineages (called Chouk/Strom) were trypsinised, then frozen at −80 °C.

2.3 Cell cultures and proliferation assessment

30 culture plates (diameter 60 mm, Nunc, Germany) were cultivated (20,000 cells per plate): 5 cultures were carried in standard conditions (control group), 5 with a PRF membrane in standard conditions (test group (PRF)), 5 with 2 PRF membranes in standard conditions (test group (2 PRF)), 5 in differentiation conditions (control group D), 5 with a PRF membrane in differentiation conditions (test group D (PRF)), and 5 with 2 PRF membranes in differentiation conditions (test group D (2 PRF)). The PRF membranes originated from the same donor as for the explant. The PRF was produced according to the protocol described above, using a collection kit and table centrifuge specifically designed for this application (Process protocol, Nice, France). The PRF clot was harvested with pliers and gently pressed into a membrane, using soft compression during 10 s between 2 sterile gauzes in order to keep the membrane wet (Fig. 1). PRF membranes were added to the cultures at the first day of experimentation. Then, one culture plate of each group was removed for counting at each experimental time, i.e. on days 3 (D3), 7 (D7), 14 (D14), 21 (D21) and 28 (D28). The cell count was performed after trypsinisation, using a Malassez cell technique. All the cells originating from the primary cultures have a fibroblastic structure in the beginning, and are morphologically similar. Primary cultures were morphologically characterised and validated as human BMSC after the induced osteoblastic differentiation of samples. After the third confluence passage, the collected cell lineages (called Chouk/Strom) were trypsinised, then frozen at −80 °C.

2.4 MTT assay

At each experimental time, the cell suspension was diluted to the concentration of 5000 cells/mL. Then, 200 μL of cell suspension was seeded into a 96-well tissue culture plate (Nunc, Germany), 20 wells for the control group and another 20 wells for the test group. Cells were placed in the incubator for 24 h to obtain a monolayer cell growth. After overnight attachment, each well was washed twice with sterile phosphate buffer saline solution (PBS) and the MTT assays were performed (MTT, Sigma Chemical Co., USA). The experiments were repeated 3 times to ensure reproducibility. The spectrophotometric absorbance (optical density) was read at 540 nm using a microplate reader (BIO-TEK Instruments Inc., USA). Detailed procedures for these measurements have been previously described by Mosmann.42

2.5 Osteoblastic differentiation assessment: quantification of the mineralisation nodules and measurement of the alkaline phosphatase (ALP) activity

At each experimental time, ALP activity assessment was performed, after the cell counting, by using a commercially available kit (ALP reagent, Sigma, USA). The production of p-nitrophenol was measured at 410 nm. ALP activity was expressed as nU/cell and mU/plate.

Three series were fixed with a neutral phosphate buffered 2% glutaraldehyde solution during 1 h at 4 °C. A Von Kossa staining was then performed to detect mineralisation. Mineralisation nodules appeared as dark brown/black spots. The cultures displayed a large surface, and therefore, a complete count of all the nodules of the culture plate seemed difficult. Therefore, the nodules of significant size and shape (homogeneous and well defined dark spots) were counted on a square area of 3 cm² in the middle of the culture plate.

2.6 Scanning electron microscope (SEM) study

Scanning electron microscopy of BMSC cultures undergoing differentiation was performed using a JEOL JSM-5310 LV SEM (Jeol, Tokyo, Japan). This examination was associated with an Energy dispersive X-ray analysis (EDX, Oxford probe) in order to identify the chemical composition of potential nodules. The cultures were first fixed with a solution of 2.5% glutaraldehyde for 1 h, then dehydrated in ethanol and gold sputter-coated (with a 20 nm gold layer, Auto Sputter Coater, Agar Scientific Ltd., Stansted, UK). SEM images were captured at accelerating voltages between 15 and 25 kV.

2.7 Statistical analysis

For the MTT assay, the mean optical density of the control group was set to represent a 100% viability. Results of the test groups were expressed as percentages of the control. Statistical analysis was performed by one-way analysis of variance, and in case of significant difference, the Tukey’s test was used. Statistical significance was assigned when p < 0.05.

Cell numbers, ALP activity per cell and the number of mineralisation nodules in each group were expressed in means and standard deviation, and their evolution in time was analysed by a variance test (ANOVA) compared to the control cultures. The 2 test groups (with 1 or 2 PRF) were also compared one to each other according to the same modalities.
The results were considered statistically significant for $p < 0.01$.

3. Results

3.1. Cell proliferation and cytotoxicity testing

In the presence of 1 or 2 PRF membranes, the number of BMSC in culture was significantly higher ($p < 0.01$) compared to the control cultures at the 5 experimental times, whatever the culture conditions (Fig. 2).

In standard culture conditions, the stimulation of proliferation ranged between 160% and 210% with 1 PRF, between 190% and 380% with 2 PRF (Fig. 3). The level of stimulation was stable with 1 membrane, but we noticed a stimulation peak during the first week (330% at D3, 380% at D7) with 2 membranes. The cultures with 2 membranes underwent significantly stronger stimulations than those with only 1 membrane ($p < 0.01$) up to at least 14 days. This dose-dependent effect disappeared beyond that time (Fig. 2).

In differentiation culture conditions, the stimulation of proliferation ranged between 160% and 210% with 1 PRF, between 240% and 310% with 2 PRF (Fig. 3). The stimulation level was stable with 1 or 2 membranes (no marked stimulation peak). Nevertheless the cultures with 2 membranes underwent significantly stronger stimulations than those with only 1 membrane ($p < 0.01$) during the whole experimental time (Fig. 2). Finally, we noticed that starting from day 14, the group in differentiation conditions with 2 PRF was the one which showed the strongest proliferation stimulation among all the groups ($p < 0.01$).

Choukroun’s PRF was thus able to significantly stimulate BMSC proliferation in vitro, both in standard or differentiation conditions, in a dose-dependent manner.

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**Fig. 2 – Proliferation assessment of human bone mesenchymal stem cells (BMSC) primary cultures in 6 different experimental conditions and at 5 experimental times. Results are expressed as mean ± SD (standard deviation).**

**Fig. 3 – Evolution of the proliferation increase ratio due to PRF during the experiment and MTT assays results. Results of the test groups are expressed as mean percentages of the control. In standard conditions (test groups), the proliferation ratio ranged between 1.6 and 2.1 with 1 PRF, and 1.9 and 3.8 with 2 PRF. In differentiation conditions (test groups D), this ratio ranged between 1.6 and 2.1 with 1 PRF, and between 2.4 and 3.1 with 2 PRF. In the MTT viability test of BMSC in contact with 1 or 2 PRF membranes, values were non-significantly different from the control ($p > 0.05$); no cytotoxicity effect was detected.**
Moreover, the series of cytotoxicity tests confirmed the absence of cytotoxicity of PRF for BMSC in standard or differentiation conditions, with 1 or 2 membranes (Fig. 3).

3.2. Osteoblastic differentiation

The osteoblastic differentiation analysis of the BMSC was performed by the assessment of the ALP activities per cell (Fig. 4) and the number of mineralisation nodules in culture (Fig. 5). The nodules were counted in plates stained with Von Kossa (Fig. 6). In the presence of 1 or 2 PRF membranes, the ALP activities and the number of nodules were significantly higher \((p < 0.01)\) compared to the values of the respective control cultures at the 5 experimental times, whatever the culture conditions (Figs. 4 and 5).

In standard culture conditions, stimulation of the differentiation was significantly higher \((p < 0.01)\) with 2 membranes than with only one, up to the 21st day. In differentiation culture conditions, stimulation of the differentiation was significantly higher \((p < 0.01)\) with 2 membranes than with only one during all the experimentation (Figs. 4 and 5). There was thus a significant dose-dependent effect of PRF on the differentiation of the BMSC in culture.

We noticed that the ALP activities of control group D (differentiation conditions, without PRF) do not reach the level of the test group (PRF) and the test group (2 PRF), both in standard culture conditions, up to the 14th and 21st day, respectively (Fig. 4). Moreover, the ALP activities and number of nodules obtained in differentiation conditions, using 1 or 2 PRF membranes, were much higher than those of all the other groups, starting from D21: at this stage, even the test group (2 PRF), although highly differentiated, could not compete with the differentiation signs of test groups D (Figs. 4 and 5).

![Fig. 4 - Alkaline phosphatase activity quantification (ALP, nU/cell) in human BMSC primary cultures in 6 different experimental conditions and at 5 experimental times. Results are expressed as mean ± SD.](image)

![Fig. 5 - Numeration of critical sized mineralisation nodules in human BMSC cultures in 6 different experimental conditions, after Von Kossa staining. Results are expressed as mean ± SD.](image)
The morphological analysis of the cultures observed using SEM at 14 days allowed to visualise the architecture of the mineralisation nodules (Fig. 7), with a calcium phosphate content confirmed by the EDX analysis. The control group barely showed any nodule of significant size, which confirmed the relatively non-differentiation of the cultivated BMSC. In the presence of PRF, numerous nodules strewed the cultured cell layer. This phenomenon was amplified in the cultures using differentiation conditions.

Choukroun’s PRF was thus able to stimulate significantly BMSC differentiation in vitro, both in standard or differentiation conditions, in a dose-dependent way.

4. Discussion

All previous studies on BMSC cultures in the presence of PRP have demonstrated that the platelet concentrate generates a
strong proliferation, but inhibits differentiation.\textsuperscript{22–24} These BMSC cultures always originated from the iliac crest. In our study, the addition of Choukroun’s PRF in the primary cultures of human oral BMSC in standard conditions (test group (PRF)) seemed to stimulate simultaneously, in a dose-dependent way, the proliferation and some kind of differentiation characterised by a strong activity of alkaline phosphatase, and the formation of mineralisation nodules. BMSCs placed in differentiation medium without PRF (control group D) did not reach the same level of differentiation before D14 (test group (PRF)) or even D21 (test group (2 PRF)). Finally, the BMSC which received PRF and the differentiation medium (test groups D) showed differentiation characteristics which were highly superior to all the other groups. However, it was somewhat difficult to imagine that the same cells could proliferate and differentiate. The most logical explanation would be that the presence of PRF induced the separation of the culture into two cell profiles: one part of the cells reacted to the growth factors by proliferating, while another group reacted to other signals by a strong differentiation. This explanation is logical when one knows the non-homogenous nature of PRF.\textsuperscript{28}

The non-homogeneity of the BMSC primary cultures could be hypothesised and may explain some of our results. However, this may not be the most relevant hypothesis. Indeed, the double contradictory proliferation/differentiation profiles assessed in this study have already been described with osteoblasts in primary cultures, and the notion of coculture with leukocytes has been highlighted in a previous study.\textsuperscript{30}

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The dose-dependent effect of PRPs on cell cultures has already been tested in numerous experimental situations with osteoblasts and fibroblasts.\textsuperscript{6} Two previous studies have already demonstrated a dose-dependent effect for a PRP on BMSC cultures.\textsuperscript{22,24} The increase of the platelet concentration generated an increase of the proliferation, and a decrease of the differentiation. With Choukroun’s PRF, we observed a very different dose-dependent effect: when we doubled the number of membranes in culture (therefore, we doubled the volume of fibrin, of platelets, of leukocytes, and numerous other molecules), we stimulated significantly the proliferation and the differentiation during at least the first 14 days in standard culture conditions, and during all the experimentation in differentiation culture conditions.

Some high concentrations of platelets could also have an inhibiting, indeed toxic effect, on the cultured cells, as demonstrated with the osteoblasts and PRPs.\textsuperscript{6} But with PRF, no such thing has been observed. The MTT tests demonstrated the absence of cytotoxicity of PRF at the two tested doses. Moreover, the dose-dependent stimulation of the PRF on the BMSC cultures was highly significant. A previous study had already demonstrated the absence of cytotoxicity during the very first hours of culture (12 and 24 h of culture) with human preadipocytes, prekeratinocytes, fibroblasts and osteoblasts.\textsuperscript{43} This result was even confirmed recently in slightly different experimental conditions.\textsuperscript{30}

All the results obtained with PRF are quite different from the data previously reported in the literature about PRPs. These differences between PRF and PRF have already been pointed out and discussed with osteoblast cell cultures.\textsuperscript{30,44} One significant reason may be that in this study PRF was obtained from the same donor as for the BMSCs, in order to avoid any bias related to immune incompatibility, what is particularly important with a L-PRF, a concentrate rich in leukocytes:\textsuperscript{30} in most studies about PRPs, cells and platelet

Fig. 7 – Scanning electron microscope examination of human BMSC after 14 days of culture in different experimental conditions. In the control group, mineralisation nodules were very difficult to isolate (A). In the test group with PRF, many mineralisation nodules were identified on the surface of the cultured cells layer (B). After magnification, we observed significantly big nodules (B1, B1’ and B2).
concentrates are not coming from the same donor. But the main reason is probably more related to the intrinsic nature, content and architecture of the PRF biomaterial. As previously explained, PRF is a natural optimised blood clot. It contains aggregated platelets, associated with leukocytes, and trapped within a dense fibrin network. Several studies have already demonstrated the osseointductive effect of a gelled fibrin matrix in certain experimental conditions with the BMS. For bone tissue engineering using BMSC, the use of platelet-rich fibrin glues gives much better results than a standard PRF (platelet concentrate only). But, in addition to the role of fibrin, it would be interesting to investigate the role of the leukocytes which are released in the culture by the membrane. To some aspects, any cell culture with a PRF membrane is first of all a coculture with autologous leukocytes. Unfortunately, the literature concerning platelet concentrates much too often tends to totally forget the role of the leukocytes within these preparations, although some recent studies have discussed this notion of immune concentrate, antimicrobial properties of platelet concentrates, and even the influence of the platelet concentrate on the leukocytes of the concerned surgical site. Moreover, leukocytes in platelet concentrates seem to produce high quantities of some growth factors. Finally, we must recall that the true effect of platelet releasates on healing is to be analysed cautiously. Indeed, the platelet concentrates include many different factors with a wide range of effects. For example, the platelet granules contain angiostimulating factors (VEGF, basic fibroblast growth factor (bFGF)) and angiostatic factors (endostatin, thrombospondin-1) in equivalent proportions, which implies quite contrasted results. In the same way, the effects of the two key platelet cytokines, TGFβ1 and PDGF-AB, are very variable according to the initial state of the cells: the TGFβ1 acting rather on the differentiation process, and the PDGF-AB on the proliferation process. Therefore, a platelet concentrate can be analysed accurately only as a whole, like a complex combination of platelets, fibrin and leukocytes, in various quantities and configurations. Anyhow, it seems that Choukroun’s PRF presents properties which differ from those of the PRPs tested up to this day on BMSC.

The presence of BMSC in the oral cavity is already well-known, but the harvesting modalities of these cells remain undetermined. In this study, the stimulation of the alveolar bone using a simple drilling allowed to harvest enough cells to prepare primary cultures of BMSC. The cultures in the control group only showed very few signs of differentiation (which differed from control group D placed in differentiation medium or from the test groups): therefore, we were in the presence of BMSC lineages which were ready for use and easy to obtain. A contamination of the drilling site with osteoblasts and other bone healing cells was probable. However, if different bone cell profiles can be suspected in the initial cell harvest, the competition between the cells during the proliferation cycles of the primary culture production can only lead to the final selection of the most proliferative cell profile, considered to be BMSC. This statement was carefully checked during the primary culture preparation process, both with morphological characterisation and osteoblastic differentiation induction of cell samples. It could be interesting to characterise in a detailed way the phenotype of these BMSCs, since numerous markers of the degree of differentiation are progressively elucidated today.

The Choukroun’s PRF presents a general aspect and a composition which are quite different from the other platelet concentrates. For tissue engineering applications, PRF could be used in two ways: the membrane as a biomaterial, and the acellular serum as a culture medium. Several authors have already advocated the idea of using platelet concentrates originating from patients as a culture medium for human cells, in order to replace the fetal calf serum, and thus to limit the possible reimplantation problems of the cultivated tissue. This option is to be tested with the PRF.

In the same way, the fibrin seems to be a relevant matrix to support the osteoblastic growth and differentiation, and it is frequently used during bone tissue engineering experiments. This option has already been tested by combining PRF, BMSC and fibrin glues, with promising results in animals and humans as well. The PRF, due to its composition, its consistency, and its easiness of production, may be adequate for such indications. The dose-dependent stimulation of the proliferation and differentiation of the BMSC also suggests the possibility to fabricate a conglomerate PRF/BMSC with several clots of PRF, and a relatively low volume of oral BMSCs. The harvesting of BMSCs in an oral site, easy of access and little invasive, may be an interesting option for biotechnological applications. It might also be attractive to harvest such cells before performing a large bone graft, and to mix them with PRF in order to obtain an optimised grafting material. However, one limitation of the present study was that cells were collected from one individual only; the validation of this BMSC harvesting protocol would thus require to be repeated in a series of patients.

5. Conclusion

This study shows the remarkable combined proliferation and differentiation reaction of oral BMSC when associated with Choukroun’s PRF. The potential clinical and biotechnological applications of a PRF and oral BMSC conglomerate are numerous, and deserve new studies in order to understand more clearly the underlying biological mechanisms, particularly concerning the role of leukocytes, and to master the preparation of this conglomerate.

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