

Effect of PRP and PPP on proliferation and migration of human chondrocytes and synoviocytes *in vitro*

Research Article

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Abstract: Cartilage tissue engineering can provide substantial relief to people suffering from degenerative cartilage disease, such as osteoarthritis. The autologous platelet-rich plasma (PRP) application appears to improve cartilage healing due to its ability to positively influence cellular mechanisms, mainly in cells from synovium and cartilage. Primary cultures of human synovial fluid stem cells (synoviocytes, SCs) and chondrocytes (CCs) were exposed to various concentrations of non-activated PRP and platelet-poor plasma (PPP) prepared by apheresis. Cell proliferation and migration were evaluated in real-time with the non-invasive xCELLigence System. It was found that PRP had a similar effect on the growth of cells as fetal bovine serum (FBS). Surprisingly, our proliferation assay results indicated that 50% PPP had the largest effect on both cell types, with a statistically significant increase in cell number ($P < 0.001$) compared to the (0% FBS) in vitro control. The migratory ability of SCs was significantly enhanced with 10% PRP and 0.8% hyaluronic acid (HA). HA also augmented migration of CCs. In summary, these results demonstrate that directed cell proliferation and migration are inducible in human articular CCs and SCs, and that both platelet-derived fractions may exert a positive effect and modulate several cell responses that are potentially involved in tissue integration during cartilage repair.

Keywords: Platelet-rich plasma • Platelet-poor plasma • Synoviocytes • Chondrocytes • Proliferation • Migration • Impedance based real-time assay

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1. Introduction

Osteoarthritis (OA) is one of the most prevalent musculoskeletal disorders, generally characterized by a catabolic and inflammatory joint environment. OA is associated with the loss of articular cartilage, intra-articular bone hypertrophy and many different immunological and morphological effects. Cartilage tissue is composed of chondrocytes and is embedded in the dense extracellular matrix (ECM). It has poor autonomous regeneration capacity, mainly due to its avascular nature. Another factor contributing to poor regenerative capacity of articular cartilage is the restricted number of ECM producing cells. The

percentage of highly specialized chondrocytes (CCs) in cartilage tissue is only 1–3% [1]. CCs are embedded in extracellular matrix, rendering them unable to migrate to a site of injury; they are able to synthesize fibrous repair tissue, but not sufficiently to fill even small defects (<3 mm in diameter) with a cartilage-like matrix [2]. Cartilage defects in OA are associated with major loss of performance. Various treatments, including cell therapy, nonsteroidal anti-inflammatory medications, corticosteroid injections, glucosamine and chondroitin supplements, as well as topical analgesics are used to treat inflammation and cartilage degeneration [3,4]. Intra-articular injection of HA is widely used as a safe and effective therapy for knee OA treatment,

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but it is considered only as an alternative therapy to corticosteroid injections with disease-modifying attributes [5]. Unfortunately, effective approaches for cartilage repair, especially in a strong inflammatory micro-milieu, are still limited.

The use of platelet-rich plasma (PRP) can augment the healing process, primarily in avascular tissues, and thus can be particularly effective in treating OA [6]. PRP is a relatively inexpensive, simple, and minimally invasive method of obtaining a natural concentration of autologous beneficial growth factors and other bioactive molecules. Components of PRP have an anabolic effect on different cell types, like chondrocytes, synoviocytes, and local stem cells with positive affect on cell proliferation, matrix production, mitogenic action and chemotaxis. Moreover, anti-inflammatory and antinociceptive effects via downregulation of known catabolic signaling pathways have been observed [7,8]. In OA synoviocytes, PRP results in an increased synthesis of hyaluronan, which then leads to both decreased inflammation and increased anabolic activities in neighboring chondrocytes [9]. The most prominent growth factors in PRP are platelet-derived growth factor (PDGF), transforming growth factor (TGF- β 1 and TGF- β 2), epidermal growth factor (EGF), insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), all of which positively influence cell proliferation and differentiation [10]. Platelets also contain adenosine diphosphate, thromboxane A2, and thrombin, which lead to further platelet activation, increased platelet aggregation, and ultimately hemostasis. However, PRP preparations contain a mixture of anabolic and catabolic mediators [11]. Cytokines and matrix metalloproteinases (MMPs) obtained in PRP can harm articular cartilage. PRP was shown to contain a significantly higher number of anabolic and catabolic cytokines relative to platelet-poor plasma (PPP) [12]. Browning *et al.* [12] demonstrated that fibroblast-like synoviocytes treated with PRP produced an increase in matrix metalloproteinases (MMPs) secretion, which may accelerate cartilage catabolism. It was concluded from this study that PPP did not cause similar responses, and that synoviocytes produced lower concentrations of the above mentioned mediators. Currently, only a few studies have attempted to evaluate the effect of PPP, which is a by-product of the platelet-rich plasma preparation process [12,13]. Because PPP is rich in plasma proteins and contains native levels of fibrinogen, it can be utilized as a sealant, with adhesive and hemostatic powers. In addition, like PRP, PPP is an autologous product that is relatively inexpensive, readily available, and simple to prepare. Donor variability, different preparation methods for the

cells and PRP as well as culture conditions have been shown to affect the levels of growth factor release, or adequate cell response. Consequently, the platelet concentration is critical, and therefore concentrations that are too low or too high are not beneficial [14,15], and can even be disadvantageous [16].

In the current study, we have set up *in vitro* assays for exploring the activity of synoviocytes and chondrocytes treated with different concentrations of PRP in order to study its effect on cellular events relevant in arthritis, such as cell proliferation and migration ability. These experiments were repeated with PPP to assess the effect on cells in relation to PRP in the same model. We have conducted experiments with the new xCELLigence system, which offers dynamic live cell monitoring and high data acquisition rates.

2. Experimental Procedures

2.1 Isolation and cultivation of cells

Synovial stem cells (synoviocytes, SCs) were isolated by centrifugation at 150 x g for 7 minutes at 4°C from synovial fluid (SF). SF was obtained aseptically by syringe aspiration from patients in the initial stages of osteoarthritis (with informed consent) before the intra-articular drug application. Human SCs derived from synovial fluid were cultured for maintenance in cell culture medium based on MEM Alpha Medium (Invitrogen, GIBCO®, USA), 10% fetal bovine serum (FBS, Invitrogen, GIBCO®, USA) and 1% antibiotic/antimycotic solution (10,000 units mL⁻¹ penicillin, 10,000 µg mL⁻¹ streptomycin, and 25 µg mL⁻¹ amphotericin B, Invitrogen, GIBCO®, USA). To obtain a homogenous population of SCs [17], the subpopulation of CD105+ cells was separated by magnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany) using an anti human-CD105 antibody (Miltenyi Biotec) after two passages. SCs were incubated with colloidal MicroBeads, (Miltenyi Biotec, Germany) coated with CD105 monoclonal antibodies for 15 min at 10°C. CD105+ cells were then enriched using the MINI MACS System (Miltenyi Biotec), according to the manufacturer's specifications. Primary chondrocytes (CCs) were isolated from human articular cartilage specimens obtained from total knee replacements. Cartilage tissue was placed into the transport medium containing sterile Dulbecco's modified Eagle's medium (DMEM; Invitrogen, GIBCO®, USA) supplemented with 1% antibiotic/antimycotic solution (10,000 units mL⁻¹ penicillin, 10,000 µg mL⁻¹ streptomycin, and 25 µg mL⁻¹ amphotericin B; GIBCO BRL). Cartilage pieces (1x1x1 mm) were digested with 0.1% collagenase type

II (Invitrogen, GIBCO®, USA) in Ham's F-12 (Biochrom AG) for 16–20 h at 37°C. The obtained cell suspension was passed through a 40 µm nylon cell strainer (BD Falcon, Biosciences, Bedford, MA) and centrifuged at 150 x g for 7 min. Cells were resuspended in cell culture medium containing Ham's F-12 (Biochrom AG), 10% fetal bovine serum (FBS, Invitrogen, GIBCO®, USA), 1% antibiotic/antimycotic solution (10,000 units mL⁻¹, 10,000 µg mL⁻¹ streptomycin, 25 µg mL⁻¹ amphotericin B, Invitrogen, GIBCO®, USA) and 1% Insulin-Transferrin-Selenium-A supplement (Invitrogen, GIBCO®, USA). All cells were cultured as a monolayer for expansion in a 37°C humidified incubator with an atmosphere of 95% air and 5% CO₂. The medium was changed 2 times weekly. Confluent cultures were dissociated with trypsin-EDTA (Invitrogen, GIBCO®, USA). All experiments with CCs were performed with cells from the first or second passage, and the subpopulation of CD105+ SCs after the second passage were used for further analysis. All procedures in this study were in accordance with the ethical standards of our hospital's (L. Pasteur University Hospital, Košice, Slovakia) committee on human experimentation.

2.2 Cell characterization

To evaluate multilineage differentiation of human synoviocytes *in vitro*, a differentiation kit (Human mesenchymal stem cell functional identification kit, R&D Systems, Inc.) was used. Cells cultured in complete culture medium were used as a negative control, which included a subpopulation of CD105+ cells after 2 passages that were seeded at a density of 2.0 x 10⁴ cells/cm² for differentiation in glass chamber slides (Lab-Tec®, Nalgene Nunc International, Naperville, IL). To evaluate multilineage differentiation, cells were allowed to become near-confluent and then cultured for 2 to 3 weeks in an appropriate induction medium. Expression of adipogenic, chondrogenic and osteogenic differentiation was determined using standard techniques recommended by the manufacturer (data not shown). Differentiation into osteoblasts was visualized by von Kossa staining. Alcian blue staining was used to assess chondrogenic differentiation. Adipocytes after adipogenic differentiation were stained with Oil Red O.

To analyze cell-surface expression of typical markers, cultured cells were labeled with monoclonal antibodies targeting human antigens. After detaching cells from the flasks, 100,000 cells were incubated with mouse anti-human CD90-PE (Miltenyi Biotec Inc., USA), mouse anti-human CD44-PE (Miltenyi Biotec Inc., USA), mouse anti-human CD105-PE (DakoCytomation, Inc., USA) and mouse anti-human CD45-FITC (Miltenyi Biotec Inc., USA) for 30 min in the dark. Fluorescein isothiocyanate

(FITC) and phycoerythrin (PE)-conjugated mouse IgG antibodies were used as isotype-matched controls. Flow cytometric analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson) and CellQuest (Becton Dickinson) software.

2.3 Preparation of PRP

PRP was obtained from the venous blood of a 40-year-old healthy male volunteer from the National Blood Center, Košice, Slovakia. The blood collection was approved by the Ethic Committee and the volunteer signed the written contents. At the Blood Center, PRP was collected by plateletpheresis with automated instruments (SYSMEX KX21N). Leukodepleted plasma was used in the experiment as platelet-poor plasma. The platelets present in PRP were counted by an automated hematology analyzer (Sysmex). The platelet concentration in the PRP was 969 x 10⁹ platelets L⁻¹. Products from whole blood were used immediately for the proliferation and migration assay.

2.4 Real-time cell proliferation and migration assay

To continuously monitor the effect of whole blood products on cell proliferation and migratory behavior, we used the xCELLigence System (according to supplier instructions (Roche Applied Science) [18]), a novel real-time cell monitoring system that measures electrical impedance and displays the results as cell index (CI) values. xCELLigence technology measures impedance changes in a meshwork of interdigitated gold microelectrodes located at the well bottom (E-plate), or at the bottom side of a microporous membrane (CIM-plate 16).

After reaching 80% confluence, human chondrocytes and synoviocytes were detached from the tissue-culture flasks by a brief treatment with trypsin/EDTA and then used for further analysis. Subsequently, cells were seeded at five different densities: 20,000; 15,000; 10,000; 8,000 and 5,000 cells/well in E-Plate 16 microtitre plate devices (E-Plate™, as suggested by supplier's manual) in order to determine the optimum cell concentration for the proliferation assay. Initially, 100 µL of cell free culture medium (with 10% FBS) was added into each well at room temperature and the background impedance was measured. 100 µL of each cell suspension was then added to the 100 µL medium wells on E-plate 16. After leaving the plates at room temperature for 30 minutes to allow cell attachment, they were transferred to the RTCA DP device in the incubator and the impedance value of each well was automatically monitored by the xCELLigence system and expressed as cell index (CI). Cell attachment and proliferation were continuously monitored every 15 min for a period of up

to 48 h. It was demonstrated that optimal density of cells for proliferation study was 10,000 cells/well in the case of SCs, and 15,000 cells/well in the case of CCs (data not shown).

Detection of proliferation kinetics of human SCs and CCs in the presence of different concentration of PRP (50% and 10%) and PPP (50% and 10%) was performed by the xCELLigence system. The optimal number of cells/well in 100 μ L of culture medium (with 0.1% FBS) in E-Plate 16 was exposed to 100 μ L of medium containing the indicated concentrations of PRP and PPP. Controls received either medium only (containing 0% FBS), or medium with 10% FBS. CI was monitored every 15 min during the first hour and every hour for the rest of the period. All experiments were run for 48 h.

Cell migration experiments were performed using modified 16-well plates (CIM-plate16). An upper and a lower chamber of the CIM-Plate 16 is separated by a microporous membrane containing randomly distributed 8 mm-pores. This protocol is optimized for human SCs (30,000 cells/well) and CCs (20,000 cells/well) to assess chemotactic migration of these cells to medium containing PRP (50%, 10%), PPP (50%, 10%), and 0.8% hyaluronic acid (Sinovial®, Laboratoires Genévrier, S.A.). Lower chambers containing medium with 10% FBS and 0% FBS served as positive and negative control, respectively. Initially, 160 μ L of serum-free medium (0% FBS) with chemoattractant was added to the lower chambers and the CIM-Plate 16 was assembled by placing the top chamber onto the bottom chamber and snapping the two together. Then, 30 μ L of culture medium (with 0.1% FBS) was placed in the top chamber to hydrate and pre-incubate the membrane for one hour in the CO₂ incubator at 37°C before obtaining a background measurement. Once the CIM-Plate 16 was

equilibrated, it was placed in the RTCA DP station and the background CI values were measured. To initiate an experiment, cells were detached using trypsin/EDTA, resuspended at the indicated cell densities in culture medium (with 0.1% FBS) and seeded in the upper chamber by applying the desired cell number in 120 μ L of medium. CIM-Plates 16 were incubated for 30 min at room temperature in the laminar flow hood to allow the cells to settle onto the membrane according to the manufacturer's guidelines. The CIM-Plate 16 was placed in the RTCA DP station and migration was monitored every 15 min for up to 21 hours. All data have been recorded by the supplied RTCA software.

2.5 Statistical data evaluation

All calculations were obtained using the RTCA-integrated software of the xCELLigence system. The cell index was calculated from one experiment, performed seven times for all time points. Statistical analysis was performed with Wilcoxon-rank sum test and differences with $P < 0.05$ were considered significant.

3. Results

3.1 Phenotype characterization of cells

Human synoviocytes isolated from synovial fluid were cultured for one to two passages and grown to confluence, at which point they appeared to be exclusively fibroblast-like based on their morphology (Figure 1A). Freshly isolated human chondrocytes were seeded as a suspension and grown in basic culture medium supplemented with 10% FBS. Chondrocytes at passage P0 reached confluence within 2 to 3 weeks and appeared polygonal. (Figure 1B).

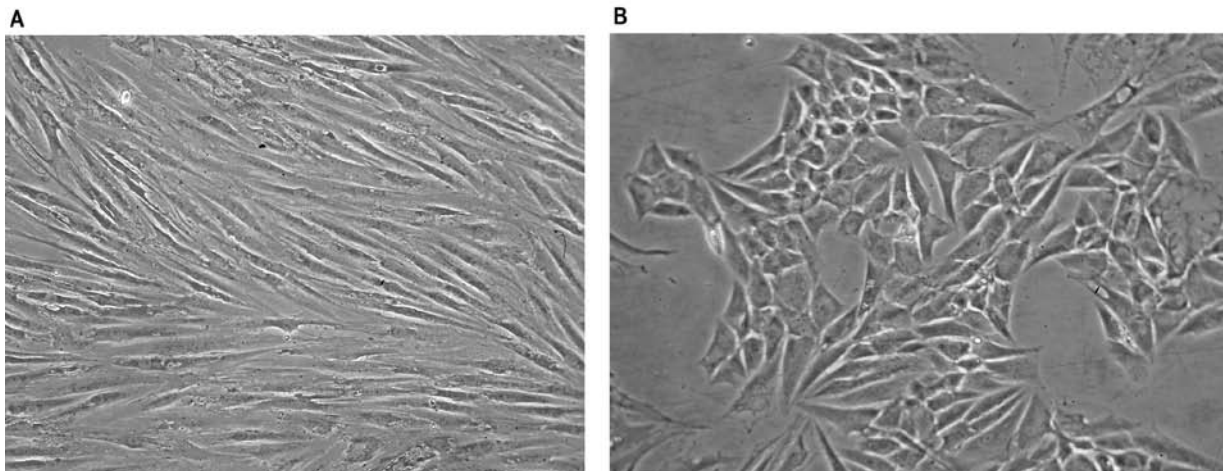


Figure 1. Representative phase-contrast photomicrographs (magnification: $\times 100$) of cultured human A) SCs, 20 days of cultivation at P0; the morphology of cultured synovial fluid cells (synoviocytes) was adherent fibroblast-like cells and B) CCs, 10 days of cultivation at P0; chondrocytes were polygonal.

In vitro differentiation studies were performed to verify the ability of SCs to differentiate into additional mesodermal specific cell lines (osteoblasts, chondrocytes, and adipocytes). Our data demonstrated that these cells are pluripotent for mesoderm cell types and displayed typical characteristics of MSCs (data not shown). Figure 2 indicates the data representative of immunophenotype in SCs and CCs. Flow cytometric analysis showed that phenotype of culture-expanded SCs and CCs after passage P1 was positive for CD44, CD90 and CD105 antigens. The cells did not express haematopoietic marker CD45. The expression profile of these two cell types was similar.

3.2 Effect of PRP and PPP on cell proliferation and migration in real-time

This study investigated the proliferation and migration ability of human chondrocytes and synoviocytes in the presence of different concentrations of PRP and PPP. The effects of products from whole blood on cell function were evaluated with the xCELLigence System.

First, we determined the optimal concentrations for cell proliferation and viability measurements. Different cell numbers (20,000; 15,000; 10,000; 8,000 and 5,000 cells/well) were seeded in the E-plate 16, and the impedance was determined (data not shown). The starting cell concentration of 10,000 cells/well for SCs and 15,000 cells/well for CCs was defined as the optimum concentration for the proliferation experiment. The optimal cell concentrations for the migration assay measured in CIM-plate16 were 30,000 SCs cells/well and 20,000 CCs cells/well.

The ability of cells to proliferate was stimulated with two different concentrations of PRP and PPP (10% and 50%) or with 10% FBS (positive control). The effects of PRP and PPP concentrations on SCs and CCs during 48 h are shown in Figure 3A and 3B, respectively.

Both, SCs and CCs displayed rapid attachment over the first 2 h followed by a relatively short lag phase, and finally entered an exponential growth phase. Cell proliferation was stimulated with the highest concentrations of PRP and PPP.

In the case of SCs, at the lower percentage PPP and PRP concentrations, there were insufficient nutrients to support adequate cell adhesion and growth (as demonstrated by much lower cell index plots) (Figure 3A). Consistently, 10% PRP and 10% PPP had rarely no additional effects on SCs proliferation compared with 0% FBS (as control). Here we found that 50% PRP and 10% FBS (as control) had nearly the same effect on the growth of SCs with statistically significant increase in cell index compared to 0% FBS ($P < 0.001$). Interestingly,

the 50% PPP concentration had the largest effect on SCs proliferation during 48 h with statistically significant increase in cell number compared to control (0% FBS) cells ($P < 0.001$).

As shown in Figure 3B, all concentrations of PPP and PRP had a stimulatory effect on proliferation of CCs. Concentrations of 50% ($P < 0.001$) and 10% ($P < 0.001$)

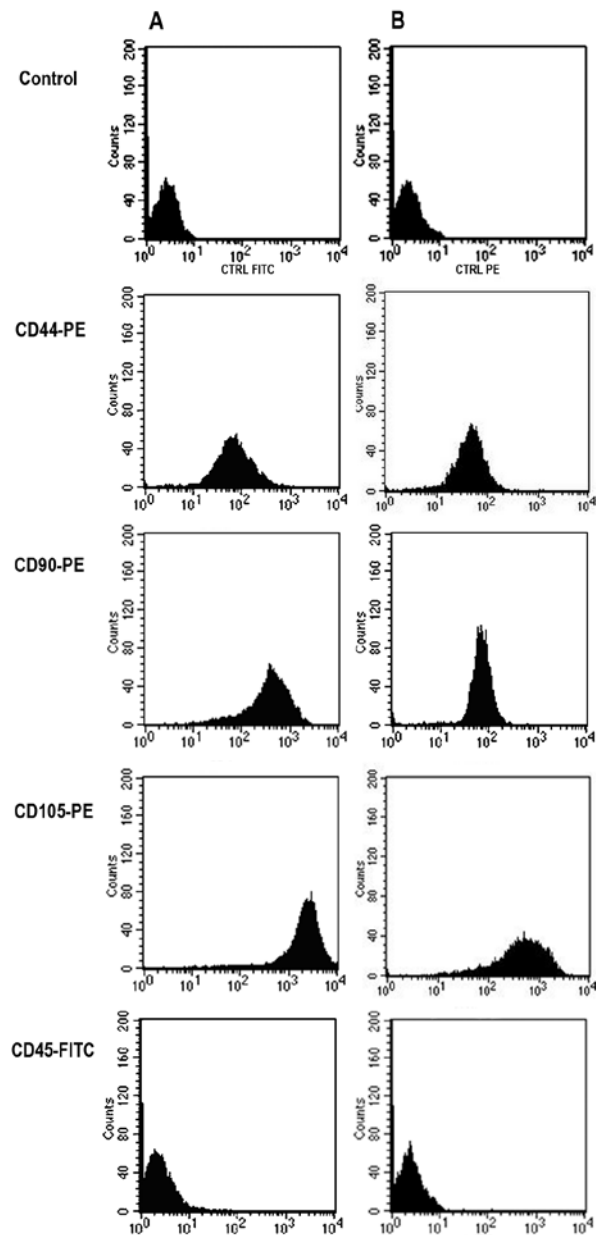


Figure 2. Flow cytometric analysis for expanded cells A) SCs and B) CCs. Cells were labeled with specific monoclonal antibodies for indicated molecules (positive for CD44, CD90, CD 105 and negative for CD 45). These experiments were repeated at least three times, and similar results were observed.

PPP and PRP, respectively significantly enhanced proliferation of CCs compared to control (0% FCS) at 48 h. The highest values of cell index were observed at 50% PPP concentration. Our results of proliferation assay indicated that 50% PPP positively affect the proliferation of both cell types used in this study.

To analyze whether PRP also constitutes a chemotactic stimulus of GFs, we evaluated the ability of CCs and SCs to migrate toward a PRP and PPP gradient compared with hyaluronic acid by a cell migration assay using the CIM-Plate 16. CCs at densities of 20,000 and SCs at densities of 30,000 cells/well in CIM-Plates 16 were seeded and observed for a period of 21 h. Growth curves obtained by repeated xCELLigence measurements (four times for each concentration of additives) are shown in Figure 4.

As shown in Figure 4A, 10% PRP had a significantly higher stimulation on the migratory capacity of SCs as 0% FBS ($P<0.05$). A comparable stimulation was achieved by addition of 10% FBS ($P<0.05$). Furthermore, we found that 0.8% hyaluronic acid also significantly enhanced cell migration compared to the negative control ($P<0.05$). No stimulation of SCs migration was observed by addition of 10% and 50% PPP and 50% PRP, respectively. Decrease in the cell index was statistically significant in comparison to the negative control with 0% FBS.

Results of the migration assay of CCs showed that 0.8% hyaluronic acid significantly augmented ($P<0.05$) cell migration, whereas different concentrations of PPP and PRP reduced cell migration in comparison to basal medium without FBS after 21 h (Figure 4B).

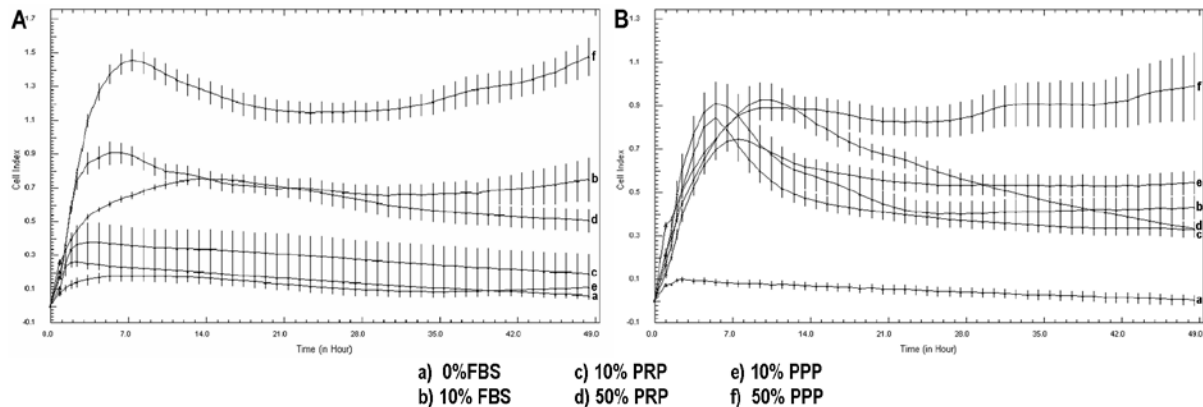


Figure 3. Real-time dynamic monitoring of cell proliferation using impedance technology. Human A) SCs and B) CCs were seeded in E-Plates 16 at a density of 15,000 cells/well and 10,000 cells/well, respectively. The adhesion, spreading and proliferation of the cells in culture medium supplemented with PRP (50%, 10%), PPP (50%, 10%), 10% FBS and without FBS were dynamically monitored every 30 minutes using the RTCA® system.)

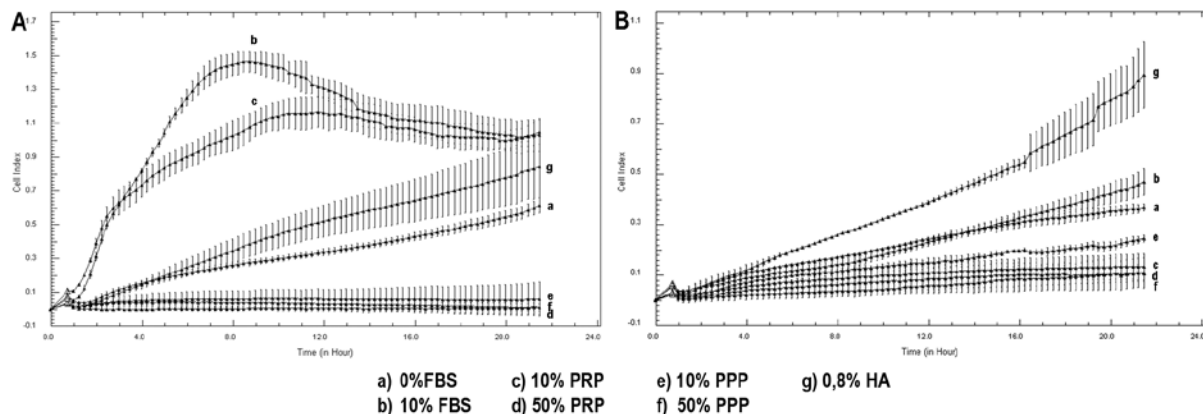


Figure 4. Real-time dynamic monitoring of cell migration using impedance technology. Human A) SCs and B) CCs were seeded in CIM-Plates 16 at a density of 30,000 cells/well and 20,000 cells/well, respectively. Migratory capacity of cells in response to different concentrations of PRP (50%, 10%), PPP (50%, 10%) and 0.8% hyaluronic acid were monitored and CI values were measured over 21 hours using the RTCA® system.

4. Discussion

In recent years, the use of PRP has gained considerable popularity for the purpose of delivering growth factors to the tissue healing site in order to promote regeneration in a variety of applications. The application of PRP for cartilage repair or intra-articular therapy is relatively new, so reliable clinical evidences are still missing, and only few papers are dealing with successful treatment applications in the orthopedic field [19–21].

We hypothesized that the delivery of a natural mixture of biologically active molecules incorporated in PRP within the joint compartment may target synovial cells as well as chondrocytes, inducing positive changes in the whole joint micro-environment. In this study, we investigated the effects of platelet-rich and platelet-poor plasma on proliferation and migration of human SCs and CCs, the two cell types critical for cartilage regeneration.

Platelet-derived fractions such as PRP and PPP are used as autologous preparations to initiate, enhance, or accelerate tissue regeneration. To compare *in vivo* and *in vitro* effects of two inexactly defined preparations is almost impossible. PRP is a source of different factors supporting activity of cells of mesenchymal origin. The whole blood from subchondral bone is widely used in orthopaedic surgery to support cartilage healing [22]. PRP is a step forward in standardization of non drug based cartilage regeneration. However long-term controlled studies are needed to confirm the reliability of PRP therapy for human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) and other blood infections prior to wide-use clinical application. We recently used PRP as a safe and effective biological approach in the early phases of cartilage degeneration, without side effects [22]. Long-term anti-inflammatory and antinociceptive treatment of joint inflammation caused by cartilage degeneration can also cause inhibition of function of mesenchymal stem cells, which represent an important cell population of synovium and are able to enhance cartilage regeneration. The autologous regeneration approach should be superior to the systemic or intra-articular use of synthetic drugs, and therefore preferentially considered.

Platelet-rich preparations are easily obtained from patient's blood after a centrifugation process. However, it is therefore critical to recognize that platelet-rich plasma prepared with different methods could have differences in platelet number, platelet activation, and growth factor profiles. The lack of suitable standardization protocol has caused the appearance of many different platelet-rich products with controversial therapeutic effects. Products of whole blood used in this

study were prepared by an automated cell separator, and non-activated PRP was used for analysis. It is well documented, that platelets release their growth factors almost immediately after activation. Approximately 70% of growth factors are released in the first ten minutes and almost 100% within the first hour [23]. The platelet counts in the whole blood and PRP was $250 \times 10^9 \text{ L}^{-1}$, and $969 \times 10^9 \text{ L}^{-1}$ respectively. The platelet concentration (enrichment of 3.80 x) used in our study was similar to that of other protocols and consistent with those reported in the literature [24]. PRP and PPP preparations were directly applied to the cells to mimic the clinical scenario. A number of studies have addressed the effect of PRP concentration on the proliferation of osteoblasts, periodontal cells, or mesenchymal stem cells [25–29]. Some authors have reported that PRP at higher concentrations failed to promote proliferation, and in some cases even suppressed it [14,30]. On the other hand, there are only a few studies on the effect of different concentrations of PRP on the proliferation of human chondrocytes [9,31,32] and synoviocytes [33]. The results of our proliferation assay indicated that PRP is capable of inducing proliferation of both cell types. The proliferation assay showed that PRP enhanced cell proliferation, in keeping with other reports [14,34,35], with a concentration of 50% being the most effective. We demonstrated that treatment with 50% PRP can induce the rate of proliferation of SCs and CCs *in vitro* to a level which is comparable to that observed with the conventional 10% FBS. Unexpectedly good results in the proliferation assay, however, were also obtained for both cell types treated with PPP. Our results showed that PPP significantly enhanced proliferation, with the formulation of 50% PPP being equally effective as the most effective PRP concentration of 50%. We suggest that this could be explained by the fact that PPP preparations contain significant amounts of certain growth factors at levels comparable to those found in PRP, namely IGF-1 and BMP-2 [36]. This finding was consistent with a previous report describing PPP promoting proliferation. [13]. Creeper *et al.* [13] demonstrated a positive effect of PRP on osteoblast and periodontal ligament cell function. Furthermore, PPP appears to also have the ability to significantly promote wound healing associated cell function. The clinical benefits of PPP in wound healing have been previously reported by Withman *et al.* [37]. Browning *et al.* [12] have shown that in contrast with PPP, PRP can accelerate catabolic process in joint-microenvironments due to the presence of catabolic cytokines in PRP and the upregulation of MMPs in fibroblast-like synoviocytes exposed to PRP. In our study there was no characterization of PPP to

identify which factor or factors are responsible for PPP-induced cell proliferation, and only one donor was used to generate the blood products for this study. Further *in vitro* experiments are necessary to confirm the beneficial effect of PPP on human SCs and CCs.

Another focus of interest in this study was to examine the potential influence of PRP, PPP and HA on the migratory capacity of SCs and CCs. Numerous *in vitro* studies have demonstrated a mitogenic effect of PRP on various cell types, such as human trabecular-bone-derived cells [38], human osteoblasts [39], human oral fibroblasts and osteoblasts [14] and human foreskin fibroblasts [40]. There are also several reports of mitogenic stimulation of human MSC by platelet releasates [41]. In our study, the large chemotactic response was observed with FBS, which is known to contain multiple chemotactic factors. We found that cell migration ability was significantly augmented by hyaluronic acid in CCs, confirming the expression of CD44 by CCs and the important role of HA in maintaining normal joint function by providing support and lubrication, as well as regulating biochemical processes [42]. Low cell index values were observed for CCs exposed to different concentrations of PRP and PPP, demonstrating that these whole blood products had no significant effect on this type of cell migration. Conversely, our migration data showed that PRP possess biological activity on SCs, even at the lowest utilized concentration of 10%, and that PRP had a greater mitogenic effect compared to the various concentrations of PPP. This finding was in contrast to the findings of Creeper *et al.* [13], who showed that 50% PPP also had a significant migratory stimulus compared to 0% FCS, and that there were no significant differences between the various concentrations of PRP. Differences in the mitogenic effect of PRP on articular SCs and CCs indicated that nonchondrocytic cell lineages are more responsive to a boarder spectrum of factors.

This work was undertaken as a basic *in vitro* study investigating the potential therapeutic role of PRP

and PPP in the joint environment, including biological effects on cartilage and synovial stem cells. However, the present study also contains some limitations. The presence and concentration of growth factors and other bioactive molecules included in PRP and PPP responsible for cellular-level mechanisms could not be analyzed and standardized. In addition, proliferation and migration assays of SCs and CCs represent an allogeneic use of PRP, whereas PRP is widely used in clinical practice as autologous product. It is important to note that not all PRP is the same, and that platelet preparation and activation represents a key factor in cartilage regeneration. In our study only one preparation procedure of PRP was tested. PRP used in our experiments was not activated because it has been found to be effective *in vivo* in a recent human study [21,43], as well as *in vitro* [29]. In future investigations, the activation status of PRP used in clinics should be reported and compared *in vitro* and *in vivo*. Further study of PRP with or without PPP should also be undertaken in the future to determine whether there is a synergistic effect; our findings will be supplemented with the results of comparative study between the effect of degranulated and whole platelets on cartilage and synovial stem cells.

In conclusion, results reported here indicate that both PRP and PPP have a strong effect on the proliferation of human SCs and CCs, and demonstrated the ability of these cells to migrate in response to serum as well as PRP, supporting the potential of PRP and PPP to affect basic cell functions in cartilage repair.

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References

- [1] Fritz J., Gaissmaier C., Schewe B., Weise K., Cartilage repair in the knee joint [Biologische Knorpelrekonstruktion im Kniegelenk], Unfallchirurg, 2006, 109, 563-576 (in German)
- [2] Kim H.K., Moran M.E., Salter R.B., The potential for regeneration of articular cartilage in defects created by chondral shaving and subchondral abrasion. An experimental investigation in rabbits, J. Bone. Joint. Surg. Am., 1991, 73, 1301-1315
- [3] Ho S.T., Yang Z., Hui H.P., Oh K.W., Choo B.H., Lee E.H., A serum free approach towards the conservation of chondrogenic phenotype during *in vitro* cell expansion, Growth Factors, 2009, 27, 321-333
- [4] Vinatier C., Mrugala D., Jorgensen C., Guicheux J., Noel D., Cartilage engineering: a crucial combination of cells, biomaterials and biofactors, Trends Biotechnol., 2009, 27, 307-314

- [5] Namiki O., Toyoshima H., Morisaki N., Therapeutic effect of intra-articular injection of high molecular weight hyaluronic acid on osteoarthritis of the knee, *Int. J. Clin. Pharmacol. Ther. Toxicol.*, 1982, 20, 501–507
- [6] Hildner F., Albrecht C., Gabriel C., Redl H., van Griensven M., State of the art and future perspectives of articular cartilage regeneration: a focus on adipose-derived stem cells and platelet-derived products, *J. Tissue Eng. Regen. Med.*, 2011, 5, 36–51
- [7] Murphy M.B., Blashki D., Buchanan R.M., Yazdi I.K., Ferrari M., Simmons P.J., et al., Adult and umbilical cord blood-derived platelet-rich plasma for mesenchymal stem cell proliferation, chemotaxis, and cryo-preservation, *Biomaterials*, 2012, 33, 5308–5316
- [8] van Buul G.M., Koevoet W.L., Kops N., Box P.K., Verhaar J.A., Weinans H., et al., Platelet-rich plasma releasate inhibits inflammatory processes in osteoarthritic chondrocytes, *Am. J. Sports. Med.*, 2011, 39, 2362–2370
- [9] Bendinelli P., Matteucci E., Dogliotti G., Corsi M.M., Ban G., Maroni P., et al., Molecular basis of anti-inflammatory action of platelet-rich plasma on human chondrocytes: mechanisms of NF-kappaB inhibition via HGF, *J. Cell Physiol.*, 2010, 225, 757–766
- [10] Anitua E., Andia I., Ardanza B., Nurden P., Nurden A.T., Autologous platelets as a source of proteins for healing and tissue regeneration, *Thromb. Haemost.*, 2004, 91, 4–15
- [11] Sundman E.A., Cole B.J., Fortier L.A., Growth factor and catabolic cytokine concentrations are influenced by the cellular composition of platelet-rich plasma, *Am. J. Sports. Med.*, 2011, 39, 2135–2140
- [12] Browning S.R., Weiser A.M., Woolf N., Golish S.R., SanGiovanni T.P., Scuderi G.J., et al., Platelet-rich plasma increases matrix metalloproteinases in cultures of human synovial fibroblasts, *J. Bone Joint Surg. Am.*, 2012, 94(23), e1721–1727
- [13] Creeper F., Lichanska A.M., Marshall R.I., Seymour G.J., Ivanovski S., The effect of platelet-rich plasma on osteoblast and periodontal ligament cell migration, proliferation and differentiation, *J. Periodontal Res.*, 2009, 44, 258–265
- [14] Graziani F., Ivanovski S., Cei S., Ducci F., Tonetti M., Gabriele M., The in vitro effect of different PRP concentrations on osteoblasts and fibroblasts, *Clin. Oral. Implants Res.*, 2006, 17, 212–219
- [15] Uggeri J., Belletti S., Guizzardi S., Poli T., Cantarelli S., Scandroglio R., et al., Dose-dependent effects of platelet gel releasate on activities of human osteoblasts, *J. Periodontol.*, 2007, 78, 1985–1991
- [16] Choi B.H., Zhu S.J., Kim B.Y., Huh J.Y., Lee S.H., Jung J.H., Effect of platelet-rich plasma (PRP) concentration on the viability and proliferation of alveolar bone cells: an in vitro study, *Int. J. Oral Maxillofac. Surg.*, 2005, 34, 420–424
- [17] Harvanová D., Tóthová T., Šarišský M., Amrichová J., Rosocha J., Isolation and Characterization of Synovial Mesenchymal Stem Cells, *Folia Biologica*, 57, 119–124
- [18] Roche Diagnostics GmbH., Introduction of the RTCA SP Instrument. RTCA SP Instrument Operator's Manual, A. Acea Biosciences Inc., 2008, 14–16
- [19] Sampson S., Reed M., Silvers H., Meng M., Mandelbaum B., Injection of platelet-rich plasma in patients with primary and secondary knee osteoarthritis: a pilot study, *Am. J. Phys. Med. Rehabil.*, 2010, 89, 961–969
- [20] Kon E., Buda R., Filardo G., Di Martino A., Timoncini A., Cenacchi A., et al., Platelet-rich plasma: intraarticular knee injections produced favorable results on degenerative cartilage lesions, *Knee Surg. Sports Traumatol. Arthrosc.*, 2010, 18, 472–479
- [21] Spaková T., Rosocha J., Lacko M., Harvanová D., Gharaibeh A., Treatment of knee joint osteoarthritis with autologous platelet-rich plasma in comparison with hyaluronic acid, *Am. J. Phys. Med. Rehabil.*, 2012, 91, 411–417
- [22] Falah M., Nierenberg G., Soudry M., Hayden M., Volpin G., Treatment of articular cartilage lesions of the knee, *Int. Orthop.*, 2010, 34, 621–630
- [23] Marx R.E., Platelet-rich plasma (PRP): what is PRP and what is not PRP?, *Implant. Dent.*, 2001, 10, 225–228
- [24] Foster T.E., Puskas B.L., Mandelbaum B.R., Gerhardt M.B., Rodeo S.A., Platelet-rich plasma: From basic science to clinical applications, *Am. J. Sports Med.*, 2009, 37, 2259–2272
- [25] Gruber R., Karreth F., Kandler B., Fuerst G., Rot A., Fischer M.B., et al., Platelet-released supernatants increase migration and proliferation, and decrease osteogenic differentiation of bone marrow-derived mesenchymal progenitor cells under in vitro conditions, *Platelets*, 2004, 15, 29–35
- [26] Han J., Meng H.X., Tang J.M., Li S.L., Tang Y., Chen Z.B., The effect of different platelet-rich plasma concentrations on proliferation and differentiation of human periodontal ligament cells in vitro, *Cell Prolif.*, 2007, 40, 241–252
- [27] Markopoulou C.E., Markopoulos P., Dereka X.E., Pepelassi E., Vrotsos I.A., Effect of homologous

- PRP on proliferation of human periodontally affected osteoblasts: in vitro preliminary study. Report of a case, *J. Musculoskelet. Neuronal Interact.*, 2009, 9, 167–172
- [28] Slapnicka J., Fassmann A., Strasak L., Augustin P., Vanek J., Effects of activated and nonactivated platelet-rich plasma on proliferation of human osteoblasts in vitro, *J. Oral Maxillofac. Surg.*, 2008, 66, 297–301
- [29] Mishra A., Tummala P., King A., Lee B., Kraus M., Tse V., et al., Buffered platelet-rich plasma enhances mesenchymal stem cell proliferation and chondrogenic differentiation, *Tissue Eng. Part C Methods*, 2009, 15, 431–435
- [30] Cho H.S., Song I.H., Park S.Y., Sung M.C., Ahn M.W., Song K.E., Individual variation in growth factor concentrations in platelet-rich plasma and its influence on human mesenchymal stem cells, *Korean J. Lab. Med.*, 2011, 31, 212–218
- [31] Spreafico A., Chellini F., Frediani B., Bernardini G., Niccolini S., Serchi T., et al., Biochemical investigation of the effects of human platelet releasates on human articular chondrocytes, *J. Cell Biochem.*, 2009, 5, 1153–1165
- [32] Pettersson S., Wettero J., Tengvall P., Kratz G., Human articular chondrocytes on macroporous gelatin microcarriers form structurally stable constructs with blood-derived biological glues in vitro, *J. Tissue Eng. Regen. Med.*, 2009, 6, 450–460
- [33] Anitua E., Sanchez M., Nurden A.T., Zalduendo M.M., de la Fuente M., Azofra J., et al., Platelet-released growth factors enhance the secretion of hyaluronic acid and induce hepatocyte growth factor production by synovial fibroblasts from arthritic patients, *Rheumatology (Oxford)*, 2007, 12, 1769–1772
- [34] Weibrich G., Hansen T., Kleis W., Buch R., Hitzler W.E., Effect of platelet concentration in platelet-rich plasma on peri-implant bone regeneration, *Bone* 2004, 34, 665–671
- [35] Lucarelli E., Beccheroni A., Donati D., Sangiorgi L., Cenacchi A., Del Vento A.M., et al., Platelet-derived growth factors enhance proliferation of human stromal stem cells, *Biomaterials*, 2003, 24, 3095–3100
- [36] Schmidmaier G., Herrmann S., Green J., Weber T., Scharfenberger A., Haas N.P., et al., Quantitative assessment of growth factors in reaming aspirate, iliac crest, and platelet preparation, *Bone*, 2006, 39, 1156–1163
- [37] Whitman D.H., Berry R.L., Green D.M., Platelet gel: an autologous alternative to fibrin glue with applications in oral and maxillofacial surgery, *J. Oral Maxillofac. Surg.*, 1997, 55, 1294–1299
- [38] Gruber R., Varga F., Fischer M.B., Watzek G., Platelets stimulate proliferation of bone cells: involvement of platelet-derived growth factor, microparticles and membranes, *Clin. Oral Implants Res.*, 2002, 13, 529–535
- [39] Celotti, F., Colciago A., Negri-Cesi P., Pravettoni A., Zaninetti R., Sacchi M.C., Effect of platelet-rich plasma on migration and proliferation of SaOS-2 osteoblasts: role of platelet-derived growth factor and transforming growth factor-beta, *Wound Repair Regen.*, 2006, 14, 195–202
- [40] Lanas A., Haggerty P., Hirschowitz B.I., Ingestion of aspirin prevents platelet-induced human fibroblast growth. Implications for peptic ulcer healing, *Scand. J. Gastroenterol.*, 1994, 29, 17–22
- [41] Vogel J.P., Szalay K., Geiger F., Kramer M., Richter W., Kasten P., Platelet-rich plasma improves expansion of human mesenchymal stem cells and retains differentiation capacity and in vivo bone formation in calcium phosphate ceramics, *Platelets*, 2006, 17, 462–469
- [42] Laurent T.C., Fraser J.R., Hyaluronan, *FASEB J.*, 1992, 6, 2397–2404
- [43] Mishra A., Pavelko T., Treatment of chronic elbow tendinosis with buffered platelet-rich plasma, *Am. J. Sports Med.*, 2006, 34, 1774–1778