Platelet gel supernatant as a potential tool to repopulate acellular heart valves

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Abstract

Objective: The aim of this study was to repopulate decellularized heart valve matrices with ovine mesenchymal stem cells (oMSCs) by the use of platelet gel (PG) supernatant, a storage vehicle for growth factors.

Methods: oMSCs were exposed to different concentrations of PG-released supernatant and cell proliferation was evaluated using the MTS assay. oMSC motility and invasiveness were assayed using a Boyden chamber. A quantitative sandwich enzyme immunoassay was used to examine amounts of bFGF and TGF- β 1 in the PG supernatant. Repopulation of acellular heart valve matrices was stimulated by seeding matrices with oMSCs supplemented with the PG supernatant.

Results: The most significant increase in proliferation induced by PG supernatant appeared at 1×10^5 plts/ml concentration. Higher concentrations evoked reduction of the stimulatory process. oMSC motility was most significantly stimulated at 1×10^6 plts/ml. Stimulating invasiveness of oMSCs needed the much higher concentration of 2×10^6 plts/ml. Immunoassays revealed that sheep PG supernatant contains 184.8 pg/ml bFGF and 60.5 ng/ml TGF- β 1. Moreover, repopulation of acellular heart valve matrices was significantly enhanced by PG supernatant addition and resulted in upregulation of the myofibroblast marker α -smooth muscle actin.

Conclusions: Growth factors released from platelets had the potential to induce cell repopulation in a heart valve tissue engineering procedure, through stimulation of mesenchymal stem-cell migration and invasion.

Introduction

Cell-based therapies belong to the more recent approaches in regenerative medicine that aim to replace or repair organs and tissues. For this purpose, mesenchymal stem cells (MSCs) can be used, as they are multi-potent progenitor cells. The most commonly used source for MSCs is the bone marrow (BM), although MSCs can also be obtained in adipose tissue (1), foetal tissues (2), amniotic fluid (3), umbilical cord blood (4), Wharton's jelly of the umbilical cord (5), limbal stroma of the eye (6) and dental tissues (7). MSCs have a self-renewal capacity, long-term viability and the potential to differentiate into diverse cell types, such as adipogenic, osteogenic and chondrogenic lineages (8,9).

Currently, MSCs are of particular interest for their potential use in heart valve tissue engineering. Although the cells reside in low frequencies in various tissues, they can readily be expanded to high quantities that are required for clinical applications. Several polypeptide growth factors have already been implicated as active mitogens in MSC proliferation (10,11).

Maintaining physiological proportional relationship between growth factors is thought to be important in tissue regeneration. One potential method for providing the entire complex of growth factors involved in tissue repair and regeneration might be through the use of platelet-gel (PG). PG is a blood component obtained from platelet (PLT) concentrates, which can be activated with calcium and thrombin. Platelets contain concentrated levels of naturally occurring growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF- β), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and insulin growth factor (IGF) (12–15).

In the last few years, there has been a considerable increase in the use of PG in reconstructive, cosmetic, orthopaedic, cardiovascular, oral maxillo-facial and dermatological surgery, in the attempt to improve tissue healing (16,17). Use of PG for tissue engineering applications is appealing as it is safe (often autologously derived), easily obtainable and a cost-effective source of

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bioactive molecules. Most importantly, PG not only provides increased concentration of several growth factors, but also maintains physiological properties of individual growth factors to each other.

The aim of this study was to isolate primary ovine MSC (oMSCs) cultures from bone marrow aspirates and to investigate the ability of different growth factor-rich PG concentrations to induce oMSC proliferation, migration and invasion. Moreover, our final goal was to repopulate acellular heart valve matrices with oMSCs by the use of PG.

Materials and methods

Cell culture

Bone marrow aspirates were obtained from the sternum of three 1-year-old female Suffolk sheep under sterile conditions after approval by the Regional Ethical Committee. All animals were sedated. After aspiration, nucleate cells from the bone marrow biopsy were isolated with Percoll (Sigma-Aldrich, Bornem, Belgium) by gradient centrifugation at density of 1.073 g/ml, and were resuspended in complete culture medium composed of DMEM-Glutamax (Dulbecco's modified Eagle Medium) (Invitrogen, Merelbeke, Belgium), 20% foetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Resuspended cells (1×10^5 cells/ml) were plated in 25 cm² culture flasks and incubated at 37 °C and atmosphere with 5% CO₂.

After 24-h incubation, non-adherent cells were discarded, and adherent cells were washed twice in phosphate-buffered saline (PBS) and fresh complete medium was added. Cells were incubated for a further 7–10 days before harvesting. Medium was changed every other day. To expand cell numbers, dense cell plaques were trypsinized with 0.25% trypsin and 1 mM EDTA for 5 min at 37 °C, and cells were plated in 75 cm² flasks up to confluence. Cells used for the experiments described were between second and third passages.

Preparation of supernatant released by platelet gel

Whole blood was drawn from Suffolk sheep and mixed at 7:1 ratio [7 parts whole blood to 1 part citrate anticoagulant (ACD-A, Baxter, Lessines, Belgium)] with citrate anticoagulant. Anticoagulated blood was then processed using Angel Whole Blood Separation System (Laguna Health, Alkmaar, The Netherlands). Default program for platelet-rich plasma (PRP) collection was used.

Platelet gel (PG) was produced by adding 330 µl calcium gluconate and leaving the mixture to clot for 5 min at room temperature. Coagulum was then centri-

fuged for 10 min at 250 g to obtain a supernatant rich in growth factors released by the activated platelets. Supernatant was subjected to a series of centrifugations (10 min each, at 250 g) to separate red blood cells, debris and stroma, and then used fresh for the proliferation, migration and invasion assays.

Initial concentration of platelets in the PRP differed between the preparations (40×10^3 – 3.5×10^6 plts/ml). To obtain final platelet concentrations (ranging from 5×10^4 – 2×10^6 plts/ml), PRP samples were diluted with PBS for platelet counting. Afterwards, PG supernatant obtained from appropriate amounts of platelets was added to the cell culture medium.

Quantitative sandwich enzyme immunoassay

A quantitative sandwich enzyme immunoassay (Quantikine; R&D Systems Europe Ltd., Abingdon, UK) was used for examining amounts of bFGF and TGF-β1 in PG supernatant obtained from 2×10^6 plts/ml. For TGF-β1 quantification, samples were activated by adding 10 µl 1 N HCl to 40 µl of plasma. After 10 min incubation at room temperature, samples were neutralized by adding 8 µl 1.2 N NaOH/0.5M N,2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES). Immunoassays were performed in triplicate following the manufacturer's instructions.

Cell proliferation testing

oMSCs were seeded (5000 cells/well) into 96-well plates. Cells were made to adhere in complete medium (DMEM-Glutamax with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin) for 24 h. Subsequently, various concentrations (0 – 2×10^6 plts/ml) of supernatant released from platelet gels were added to the cells. oMSCs treated with complete medium were used as positive controls, whereas negative controls consisted of oMSCs grown in FBS-free DMEM-Glutamax. Cells thus treated were incubated at 37 °C in 5% CO₂ atmosphere. Cell proliferation was evaluated after 72 h using the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) assay (Promega CellTiter 96® AQUEOUS Non-Radioactive Cell Proliferation Assay, Madison, WI, USA). Viable cell numbers were determined indirectly by conversion of MTS into a soluble dye. The MTS assay is based on conversion of MTS into aqueous, soluble formazan by mitochondrial dehydrogenase enzymes, found in metabolically active cells. Quantity of formazan product, as measured by the amount of 490 nm absorbance, is directly proportional to numbers of live cells in the culture. In the test, 20 µl MTS solution was added to

culture wells. After 4 h at 37 °C in a humidified, 5% CO₂ atmosphere, absorbance at 490 nm was recorded using an enzyme-linked immunosorbent assay (ELISA) plate reader (EL 800, Universal Microplate Reader; Bio-Tek Instruments, Drongenbos, Belgium). Every experiment was conducted in triplicate. Data are expressed as mean $\pm$ standard deviation.

Cell migration test

Cell migration assay (QCMTM 24-well colorimetric cell migration assay; Millipore, Overijse, Belgium) enables *in vitro* evaluation of capacity of cells to move towards a soluble chemo-attractant. The assay is performed in a migration chamber, based on the Boyden chamber principle, using an 8 μ m pore size polycarbonate membrane, as this is appropriate for most cell types. Supernatants were the chemo-attractant stimulus used and were added to the lower chamber at various concentrations (0– 2×10^6 plts/ml). FBS-free and 10% FBS medium were used as negative and positive controls respectively. oMSCs were detached, washed in DMEM-Glutamax-0.1% bovine serum albumin (BSA; Invitrogen), resuspended in the same medium at a concentration of 0.5×10^6 cells/ml and added to the upper compartment of the chamber. After 72 h incubation at 37 °C, 5% CO₂ atmosphere, cells that had migrated through the polycarbonate membrane were incubated with cell stain solution, then subsequently extracted and detected on a standard ELISA reader at 560 nm. All experiments were performed in triplicate. Data are expressed as mean $\pm$ standard deviation.

Cell invasion test

Cell invasion assay (QCMTM 24-well colorimetric collagen-based cell invasion assay; Millipore) was performed in an invasion chamber, based on the Boyden chamber principle, using 8 μ m pore size polycarbonate membrane, coated with a thin layer of polymerized collagen. *In vitro*, the assay measures invasive capacity of cells as they must pass through a collagen layer, a compound that mimics composition of the extracellular matrix. The collagen barrier occludes the membrane pores, blocking non-invasive cells from migrating through. The method used is the same as for the cell migration test. Experiments were performed in triplicate. Data are expressed as mean $\pm$ standard deviation.

Valve procurement and decellularization

Porcine aortic valves used in this study were obtained from a slaughterhouse. Immediately after slaughter,

hearts were retrieved and placed on wet ice for transportation. Aortic valves were dissected free as four centimetre-long conduits with a 2-mm rim of myocardium. Valves were decellularized whole using patented detergent-enzymatic treatment, as described by Wilson *et al.* and Zeltinger *et al.* (18,19). Essentially, tissues were rinsed in hypotonic saline containing phenyl-methylsulphonylfluoride (PMSF: 1 μ M; Sigma, Bornem, Belgium) and antibiotics (streptomycin: 100 μ l/l; penicillin: 100 μ l/l mixture; Sigma). Cells were then ruptured by subjecting the tissues to alternating treatments in hypotonic Tris-buffer (pH 8.0) and hypertonic Triton X solution (pH 8.0) (Bio-Rad, Eke, Belgium). All solutions were supplemented with PMSF (1 μ M), penicillin/streptomycin solution (100 μ l/l, respectively) and 50 μ M butylated hydroxyanisole (Sigma). After thorough rinsing, tissues were subjected to a digestive procedure using enzymatic solution containing Dnase1, RnaseA, trypsin, and phospholipases A2, C and D (Sigma). To ensure complete digestion, we employed two enzymatic treatments of 45 min each. Tissues were then washed for 24 h in a magnesium- and calcium-free chelating solution. All steps were performed at 4 °C.

Static culture

Based on results of the *in vitro* assays, effect of the PG-released supernatant on repopulation of acellular porcine aortic valve matrices was assessed by seeding matrices with early passage (2–3) oMSCs. 1×10^6 cells/cm² concentration of was seeded on to matrices ($n = 3$) and maintained in culture for 5 days at 37 °C, using DMEM-Glutamax supplemented with the PG-released supernatant of 2×10^6 plts/ml (100% PG), 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine (Sigma) with daily refreshment of medium. Control matrices ($n = 3$) were also seeded with 1×10^6 cells/cm², maintained in culture for 5 days at 37 °C, using DMEM-Glutamax without PG-released supernatant addition.

Histological staining and immunohistochemistry

After 5 days static culture, PG-oMSC-seeded matrices and control matrices were fixed in 4% phosphate-buffered formaldehyde and were embedded in paraffin wax. Five micrometre sections were cut and stained with haematoxylin/eosin (H&E) and alpha-smooth muscle actin (α -SMA, 1:400; clone HHF35, Dako Ltd, Heverlee, Belgium). Immunohistochemistry involved standard staining techniques with rabbit anti-mouse biotinylated secondary antibody (1:200), peroxidase avidin-biotin complex and 3,3' diaminobenzidine (DAB; Sigma) as chromogen.

Statistics

Descriptive statistics are expressed as mean $\pm$ standard deviation. Significance of differences between test and control groups was assessed using paired Student's *t*-test (SPSS 17.0; SPSS Inc, Chicago, IL, USA). A *P*-value <0.05 was considered significant.

Results

Cell proliferation testing

To evaluate capacity of PG-released supernatant to stimulate proliferation of oMSCs, cells were incubated for 72 h with supernatant recovered after centrifugation of PG and diluting it to obtain different concentrations in the range $0\text{--}2 \times 10^6$ plts/ml. Supernatant was diluted with DMEM-Glutamax resulting in concentrations of 5×10^4 plts/ml (2.5%), 1×10^5 plts/ml (5%), 2×10^5 plts/ml (10%), 4×10^5 plts/ml (20%), 1×10^6 plts/ml (50%) and 2×10^6 plts/ml (100%). FBS-free medium (0% PG) was used as negative control (Neg C) and 10% FBS-medium as positive control (Pos C). Proliferative effect of PG concentrations on oMSCs was assessed using the MTS assay, presented in Fig. 1.

Optical density (OD) $\pm$ SD was measured for negative controls (0% PG): 0.577 ± 0.04 ; positive controls (10% FBS): 0.661 ± 0.02 ; 2.5% PG: 0.612 ± 0.04 ; 5% PG: 0.865 ± 0.05 ; 10% PG: 0.810 ± 0.03 ; 20% PG: 0.823 ± 0.02 ; 50% PG: 0.823 ± 0.01 and 100% PG: 0.789 ± 0.02 .

Figure 1. oMSC proliferation at different PG concentrations ($0\text{--}2 \times 10^6$ plts/ml). Most significant increase in proliferation was exhibited at 1×10^5 plts/ml (5% PG) concentration. Neg C: Negative Controls, Pos C: Positive Controls. **P* <0.05 versus Neg and Pos C; ***P* <0.05 versus 20–50% PG.

Except for 2.5% PG, all PG concentrations led to significant increase in proliferation compared to control cultures (*P* <0.05) as shown in Figure 1. oMSCs exhibited the most significant increase in proliferation at concentration of 1×10^5 plts/ml (5% PG) (*P* = 0.002). Cells exposed to higher PG concentrations ($>5\%$) showed reduction in proliferation (*P* <0.05). Use of 100% PG concentration (2×10^6 plts/ml) led to significant dose-dependent decrease in proliferation compared to 20% PG (*P* = 0.007) and 50% PG (*P* = 0.002).

Cell migration testing

PG-released supernatant was used as chemo-attractant in the migration assay. Supernatant was diluted with DMEM-Glutamax, resulting in concentrations of 5×10^4 plts/ml (2.5%), 1×10^5 plts/ml (5%), 2×10^5 plts/ml (10%), 4×10^5 plts/ml (20%), 1×10^6 plts/ml (50%) and 2×10^6 plts/ml (100%). FBS-free medium (0% PG) was used as negative control (Neg C) and 10% FBS-medium as positive control (Pos C).

Optical density (OD) $\pm$ SD was measured for negative controls (0% PG): 0.074 ± 0.05 ; positive controls (10% FBS): 0.412 ± 0.07 ; 2.5% PG: 0.427 ± 0.05 ; 5% PG: 0.499 ± 0.02 ; 10% PG: 0.593 ± 0.09 ; 20% PG: 0.674 ± 0.02 ; 50% PG: 0.885 ± 0.07 and 100% PG: 0.960 ± 0.02 (Fig. 2). Results show that migration of oMSCs through 8- μ m pore size polycarbonate membrane was stimulated starting at PG concentration of 20% (*P* = 0.045), 50% (*P* = 0.001), then at 100%

Figure 2. oMSC migration through a polycarbonate membrane using different PG concentrations as chemo-attractant ($0\text{--}2 \times 10^6$ plts/ml). The most significant increase in oMSC migration was observed at 1×10^6 plts/ml (50% PG) concentration. Neg C: Negative Controls, Pos C: Positive Controls. **P* <0.05 versus Neg and Pos C.

($P = 0.004$) compared to positive controls. Lower PG concentrations did not stimulate cell migration ($P > 0.05$). No significant differences were observed between 2.5%, 5% and 10% PG concentrations ($P > 0.05$).

The most significant increase in oMSC migration was observed with concentration of 1×10^6 plts/ml (50% PG) ($P = 0.001$). Higher platelet concentrations of 2×10^6 plts/ml (100% PG) did not significantly stimulate migration more compared to 1×10^6 plts/ml (50% PG) ($P = 0.190$).

Cell invasion test

PG-released supernatant was used as chemo-attractant in the invasion assay. Supernatant was diluted with DMEM-Glutamax, resulting in concentrations of 5×10^4 plts/ml (2.5%), 1×10^5 plts/ml (5%), 2×10^5 plts/ml (10%), 4×10^5 plts/ml (20%), 1×10^6 plts/ml (50%) and 2×10^6 plts/ml (100%). FBS-free medium (0% PG) was used as negative control (Neg C) and 10% FBS-medium as positive control (Pos C).

Optical density (OD) $\pm$ SD was measured for negative controls (0% PG): 0.066 ± 0.01 ; positive controls (10% FBS): 0.192 ± 0.03 ; 2.5% PG: 0.121 ± 0.04 ; 5% PG: 0.217 ± 0.05 ; 10% PG: 0.246 ± 0.03 ; 20% PG: 0.296 ± 0.03 ; 50% PG: 0.515 ± 0.01 and 100% PG: 0.711 ± 0.01 (Fig. 3). Results indicate that PG supernatant was able to significantly stimulate oMSC invasion through a polymerized collagen layer starting at platelet

Figure 3. oMSC invasion in a polymerized collagen layer at different PG concentrations (0– 2×10^6 plts/ml). Highest stimulation of invasion was obtained using 2×10^6 plts/ml (100% PG) concentration. Neg C: Negative Controls, Pos C: Positive Controls. * $P < 0.05$ versus Pos C; ** $P < 0.05$.

concentration of 20% ($P = 0.045$), 50% ($P = 0.001$) then at 100% ($P = 0.004$) compared to positive controls. Lower PG concentrations did not induce cell invasion ($P > 0.05$). No significant differences were observed between 2.5%, 5% and 10% PG concentrations ($P > 0.05$).

Highest stimulation of invasion was obtained using concentration of 2×10^6 plts/ml (100% PG). 100% PG significantly stimulated invasion more than 20% and 50% PG ($P = 0.001$). At this concentration, there was approximately 3.7-fold increase in invasion compared to that of positive controls (10% FBS).

Quantitative sandwich enzyme immunoassay

Concentrations of growth factors bFGF and TGF- β 1 in the platelet gel supernatant obtained from 2×10^6 plts/ml were examined. Immunoassays revealed that bFGF concentration in the PG supernatant was 184.8 pg/ml. For TGF- β 1, concentration of 60.5 ng/ml was detected.

Static culture

Control matrices ($n = 3$) seeded with oMSCs using DMEM-Glutamax without PG supernatant supplementation resulted in a multilayer of cells on the surface of the valve. No cell in-growth could be detected. Moreover, cells that adhered to the valve's surface did not express α -SMA (Fig. 4a). Matrices ($n = 3$) seeded with oMSCs using DMEM-Glutamax with PG supernatant addition had cell in-growth, mainly in the ventricularis and spongiosa layers of the valve. Cell invasion was clearly much more compared to control matrices. Moreover, cells which had invaded the matrices, were positive for α -SMA (Fig. 4b).

Discussion

Tissue regeneration is a complex and finely tuned process mediated by action of growth factors and cytokines. Platelet gel, a haemo-component obtained from platelets, is currently being used clinically in an attempt to improve tissue repair and regeneration (12,13,20). Platelets contain concentrated levels of naturally occurring growth factors and other components that have the potential to accelerate tissue repair by influencing several cell processes including chemotaxis, cell proliferation, cell differentiation, angiogenesis, matrix deposition and tissue remodelling (21–23). Unfortunately, the most effective platelet concentration to induce cell repopulation had not yet been determined. To address this issue, here in this study, we investigated effects of PG-released supernatant at different concentrations, on

Figure 4. α -SMA-staining of oMSC seeded matrices α -SMA-stained representative images of oMSC-seeded control matrices (a) and matrices seeded with oMSCs + PG supernatant addition (b) after 5 days static culture. PG addition significantly increased cell in-growth positive for α -SMA (b) compared to controls (a). Scale bar = 50 μ m.

ovine mesenchymal stem cells (oMSCs) using different *in vitro* assays (proliferation, migration and invasion). As concentration of growth factors released from platelets is proportional to initial platelet concentration (expressed as plts/ml), we assumed that supernatant obtained from each preparation had the same concentration of platelets per ml.

Mesenchymal stem cells are an attractive cell population for tissue regeneration. Moreover, ovine bone marrow mesenchymal stem cells have already been successfully isolated and characterized. It has been demonstrated that oMSCs share similar properties with human and rodent MSCs, including their capacity for clonogenic growth and multiple stromal lineage differentiation (24,25). In a previous study, we reported the trilineage differentiation potential of oMSCs (26).

Here, the results of MTS-assay showed that maximum proliferation after 72 h was obtained at 1×10^5 plts/ml (5% PG) concentration, producing 1.5-fold increase in proliferation compared to negative controls, whereas higher concentrations did not stimulate proliferation further. In contrast, higher concentrations (10–50% PG) significantly inhibited the stimulatory process compared to 5% PG. Moreover, at the highest concentration 2×10^6 plts/ml (100% PG), there was a tendency towards inversion of the effect on proliferation, with values similar to those of 2×10^5 plts/ml (10% PG). These results indicate that all concentrations of PG were mitogenic to oMSCs. Hence we determined on 1×10^5 plts/ml to be the optimal concentration to induce proliferation, as higher concentrations suppressed cell viability and proliferation, in a dose-dependent manner. The results of the current study are consistent with those of other investigators who have examined the ability of PRP preparations to increase cell proliferation. Indeed, it has been previously demonstrated that growth factors released by platelets accelerate proliferation of chondrocytes, fibroblasts, endothelial cells and mesenchymal stem cells (27–29).

Results of the Boyden chamber migration assay indicated that ideal PG concentration to significantly stimulate ovine MSC migration after 72 h, was 1×10^6 plts/ml (50% PG) (about 2.3-fold higher than positive controls). Higher concentration of 2×10^6 plts/ml (100% PG) did not significantly enhance motility. When compared to positive controls, migration was only significantly enhanced starting at 4×10^5 plts/ml (20% PG) concentration.

Results of the invasion assay did not parallel those of the migration test. Maximum stimulation after 72 h occurred at 2×10^6 plts/ml (100% PG) concentration. Only 4×10^5 plts/ml (20% PG), 1×10^6 plts/ml (50% PG) and 2×10^6 plts/ml (100% PG) significantly induced MSC invasion compared to positive controls. Exposed to 100% PG, cells showed an approximate 3.7-fold increase in invasiveness compared to positive controls.

Marked increase in proliferative, migratory and invasive activity in presence of platelet-released growth factors has provided some *in vitro* evidence that platelets may play an important role in mesenchymal stem-cell regeneration. Biological effects of PG supernatant may be a reflection of interactive and possibly synergistic effects of multiple platelet growth factors. Previous studies have shown that platelets contain many growth factors such as PDGF, TGF, IGF-I, IGF-II and EGF (12–15).

Importantly, effects of PRP cannot be generalized due to large interspecies variations. It has been indicated that human PRP contains much higher concentrations of growth factors per platelet compared to rat and goat PRP (30). Moreover, van den Dolder *et al.* have demonstrated that TGF- β 1 is the most abundant growth factor in all three species. The results of our immunoassay also showed that TGF- β 1 concentration was much higher than bFGF (60.5 ng/ml versus 184.8 pg/ml). In contrast, Fekete *et al.* (31) have reported that human platelet lysate (HPL) contains 500 ng/ml bFGF and >10 pg/ml TGF- β 1.

Yet, it is unknown whether the stimulatory effect of PRP is related to growth factors present or to other micro-particles from cytoplasm or cell membranes, released from activated platelets (32). However, our results strongly suggest that growth factors present in PG-released supernatant are responsible for the observed effects on proliferation, migration and invasion. Recently, it has been demonstrated that PDGF-BB, TGF- β 1 and bFGF present in HPL are essential stimuli for proliferation of MSCs (31). Their inhibition by neutralizing antibodies reduced MSC growth-stimulating effect of HPL by about 75%. However, a cocktail containing recombinant PDGF-BB, TGF- β 1 and bFGF added to basal medium at concentrations equivalent to the amount present in PL, did not stimulate MSCs. Thus, Fekete *et al.* (31) hypothesized that other components of PL must be important for full biological activity to occur.

Ex vivo expansion of MSCs requires FBS-medium supplementation. Due to increasing safety issues concerning FBS, PL has been proposed as a substitute. Recently, it has been demonstrated by Azouna *et al.* (33) that 5% HLP-supplementation has the most significant effect on culture time of human MSCs, by increasing their growth rate. However, 10% HLP slightly altered their adipogenic differentiation potential. In our study, proliferation of ovine MSCs was also most significantly stimulated by 5% PG supernatant supplementation. Higher concentrations significantly reduced proliferation.

On the basis of results of the migration and invasion assays, we seeded acellular porcine matrices with oMSCs supplemented with PG-released supernatant of 2×10^6 plts/ml, as this platelet concentration significantly stimulated cell invasiveness. Our results showed that after 5 days culture, matrices had significant cell in-growth, which stained positive for α -SMA, a myofibroblast marker; however, cell in-growth was mainly present in the ventricularis and spongiosa layer of the valve. In contrast, control matrices seeded with oMSCs without PG supernatant addition had no cell in-growth and cells did not express α -SMA stress fibres. These results indicate that PG-released supernatant has not only the capacity to enhance acellular matrix recellularization, but also stimulates differentiation into the myofibroblastic phenotype, assessed through production of α -SMA. These results are consistent with those of Caceres *et al.* who demonstrated that platelet-rich plasma promotes migration and invasion of gingival fibroblasts and stimulates myofibroblastic differentiation (34). In contrast, Azouna *et al.* (33) showed that the phenotype of human MSCs was well preserved when expanded in HPL-supplemented media, although low HPL concentration

(5–10%) was used there. In our study, oMSCs seeded on porcine matrices were supplemented with 100% PG supernatant (obtained from 2×10^6 plts/ml).

Recently, we optimized a growth factor cocktail EGF+bFGF+TGF- β , which also stimulates oMSC *in vitro* invasion and upregulates expression of α -SMA stress fibres (20). However, medium supplementation with this triple growth factor addition did not stimulate oMSC invasion in acellular porcine matrices. Therefore, use of PG supernatant is a very promising approach to stimulate repopulation of acellular heart valve matrices.

The beneficial effects of PG supernatant can not yet be completely explained. Probably, proportional relationship maintained between growth factors is important as these growth factors can activate proliferation and invasion of ovine MSCs.

In conclusion in this study, we have identified an optimal concentration of PG to promote *in vitro* proliferation, migration and invasion of ovine MSCs. Moreover, concentration of 2×10^6 plts/ml significantly stimulated acellular matrix recellularization and differentiation of oMSCs into the myofibroblastic phenotype.

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