Platelet-rich plasma induces annulus fibrosus cell proliferation and matrix production

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Abstract

Purpose Platelet-rich plasma (PRP) contains growth factors and creates a 3D structure upon clotting; PRP or platelet lysate (PL) might be considered for annulus fibrosus (AF) repair.

Methods Bovine AF cells were cultured with 25 % PRP, 50 % PRP, 25 % PL, 50 % PL, or 10 % FBS. After 2 and 4 days, DNA, glycosaminoglycan (GAG), and mRNA levels were analyzed. Histology was performed after injection of PRP into an AF defect in a whole disc ex vivo.

Results By day 4, significant increases in DNA content were observed in all treatment groups. All groups also showed elevated GAG synthesis, with highest amounts at 50 % PL. Collagen I and II expression was similar between groups; aggrecan, decorin, and versican expression was highest at 25 % PL. Injection of PRP into the AF defect resulted in an increased matrix synthesis.

Conclusions Platelet-rich preparations increased the matrix production and cell number and may therefore be considered to promote AF repair.

Keywords Annulus fibrosus · Intervertebral disc · Platelet-rich plasma · Platelet lysate · Proteoglycans

Introduction

The intervertebral disc (IVD) has a poor self-repair capacity and undergoes progressive degeneration starting in the second decade of life. Disorganization of proteoglycan (PG) composites in the nucleus pulposus (NP) and breakdown of the organized collagen network within the annulus fibrosus (AF) result in an accelerated degenerative process. Disruption of the AF collagen causes weakening of the AF, which may ultimately lead to disc herniation [1–3].

In the early stages of the degenerative process, anabolic treatment may help to restore cell activity and enhance extracellular matrix (ECM) synthesis, leading to strengthening of the AF and preventing herniation. Several studies have shown the ability of growth factors (GFs) to enhance matrix production and accumulation and stimulate IVD cell proliferation in vitro and in vivo [4]. The application of members of bone morphogenetic proteins (BMPs) has been shown to stimulate cell proliferation and enhance matrix synthesis of AF cells in vitro [5–7]. Several studies furthermore demonstrated stimulatory effects on AF cells by application of transforming growth factor β (TGF- β) and basic fibroblast growth factor (bFGF) [8, 9].

Moreover, administration of platelet-rich plasma (PRP) with the purpose of stimulating the metabolism of degenerative IVD cells has shown positive effects in vitro [10–12] and in vivo [11–14]. Using platelets in the form of PRP offers an easily applicable way to obtain high concentrations of GFs (e.g., platelet derived GF (PDGF), insulin-like GF (IGF), epidermal GF (EGF), transforming GF (TGF), bFGF, and vascular endothelial GF (VEGF)) [15] in a small volume of plasma. PRP is an autologous plasma that has a platelet count above baseline (as compared to normal blood serum), but with a native fibrinogen concentration [16].

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In orthopedic surgery, PRP is commonly used for the repair of muscle, ligament, and tendon injuries [15, 17]. It is also applied in dermatology and plastic surgery for the healing of subcutaneous tissues [18]. One of the benefits of PRP is its natural activity creating a gel-like structure, which helps to maintain the PRP within the treated defect and thus promote wound healing directly at the site of injury. For in vitro application, platelet-rich preparations are often prepared in buffered systems like phosphate buffered saline (PBS), instead of autologous plasma, to avoid gel formation in the culture dishes [19, 20]. As platelet lysates (PL) may support cell growth and proliferation in vitro similar to fetal bovine serum (FBS), the use of such autologous preparations which contain several growth factors seems advantageous [19–21]. However, the effects of PL on IVD cells have not been investigated in direct comparison to PRP preparations, which provide a 3D structure in addition to the anabolic factors. The purpose of this study was to investigate the short term response of AF cells to PRP supplementation in terms of cell proliferation and ECM production and to compare the outcome between PRP and PL preparations in vitro. Furthermore, the feasibility and initial efficacy of PRP was investigated using a whole organ culture model of the bovine IVD.

Methods

Preparation of platelet-rich plasma, platelet-poor plasma, and platelet lysate

Leucocyte depleted ($< 1 \times 10^6$ leucocytes/unit) and pathogen inactivated (INTERCEPT Blood System) platelet concentrate (containing $\sim 1 \times 10^6$ platelets/ μL , $5 \times$ physiological blood concentration) was obtained from the blood bank of the cantonal hospital in Chur, Switzerland. The platelet concentrate was centrifuged for 20 min at 2,000 *g* with weak acceleration and brake speed. The resulting supernatant (platelet-poor plasma, PPP) was removed and stored at -20°C . For preparation of PRP, the pellet was resuspended in 1/10th of the PPPs original volume, sonicated for 15 min and stored at -20°C [19]. For preparation of PL, the pellet was re-suspended in PBS according to the original volume and washed twice with PBS. Thereafter, the concentrate was activated by sonication for 15 min and freezing at -20°C [19]. No exogenous platelet activator was used. Concentrations of PDGF-AB, PDGF-BB, VEGF, and TGF- β were determined by enzyme-linked immunosorbent assays (R&D Systems, Abingdon, UK).

Isolation of annulus fibrosus cells

AF tissue was harvested from caudal discs dissected from five bovine calve tails (6–9 months old; 4–5 IVDs per tail)

within 3 h of death from a local slaughterhouse. To exclude contamination by the surrounding tissue and the NP, the most outer and inner layers of the AF were discarded. Chopped AF was washed with PBS and predigested with 2 mg/mL pronase (Roche, Switzerland) in high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco) for 60 min at 37°C , 5 % CO_2 . The tissue was then washed again $3 \times$ with PBS and digested overnight in 310 U/mL collagenase II (Worthington Biochemical Corporation, USA) in DMEM. The isolated cells were filtered through 70 and 40 μm cell strainers (BD Bioscience), washed with DMEM, counted, and seeded as described below.

Cell culture conditions

AF cells were cultured in 24-well plates at 1×10^5 cells per cm^2 with the following media: (1) 25 % PRP, 75 % DMEM; (2) 50 % PRP, 50 % DMEM; (3) 25 % PL, 75 % DMEM; (4) 50 % PL, 50 % DMEM; (5) 50 % PPP, 50 % DMEM; (6) 10 % FBS, 90 % DMEM; (7) DMEM control. All media were supplemented with 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin (P/S; Gibco), 50 $\mu\text{g/mL}$ ascorbate-2-phosphate and 1 % ITS + 1 (Sigma Aldrich). Cell morphology was analyzed with an upright light microscope Axiovert 2 (Carl Zeiss, Germany), using 4.8 Axiovision software (Carl Zeiss Imaging Solutions, Germany).

Biochemical analysis

After 2 and 4 days, whole samples were digested in 0.5 mg/mL proteinase K solution (Roche) overnight at 56°C . DNA was measured spectrofluorometrically using Hoechst 33258 (Polysciences, Inc.) and calf thymus DNA (Invitrogen) as standard. Glycosaminoglycan (GAG) content in proteinase K digests and media was quantified using dimethylmethylene blue assay (1.9-DMMB, Sigma Aldrich) with chondroitin sulfate (chondroitin-4-sulfate sodium salt from bovine trachea, Fluka BioChemika) as standard.

Gene expression analysis

Gene expression analysis was performed for all platelet preparation groups (PRP, PL, and PPP). TRI reagent (Molecular Research Center) was used for RNA isolation according to the manufacturer's protocol. Reverse transcription was performed using SuperScript VILOTM cDNA Synthesis Kit (Invitrogen) and 500 ng of total RNA. The StepOnePlusTM System (Applied Biosystems) was used for real-time polymerase chain reaction (PCR). Gene expression of collagen type I (COL1A2), collagen type II (COL2A1), aggrecan core protein (ACAN), elastin (ELN), versican (VCAN), biglycan (BGN), and decorin (DCN) were analyzed using primers and TaqManTM probes

Table 1 Oligonucleotide primers and probes and Gene Expression Assays (from Applied Biosystems) used for real-time PCR; fw, forward; rev, reverse; serial number is indicated for Gene Expression Assays

Gene	Abbr.	Primer fw 5'–3'	Primer rev 5'–3'	Probe (5'FAM/3'TAMRA)
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	GGC TGC TTT TAA TTC TGG CAA A	AAT CAT ACT GGA ACA TGT AGA CCA TGT A	TGG ACA TCG TCG CCA TCA ATG ACC
Collagen type I	COL1A2	TGC AGT AAC TTC GTG CCT AGC A	CGC GTG GTC CTC TAT CTC CA	CAT GCC AAT CCT TAC AAG AGG CAA CTG
Collagen type II	COL2A1	AAG AAA CAC ATG TGG TTT GGA GAA A	TGG GAG CCA GGT TGT CAT	CAA CGG TGG CTT CCA CTT CAG CTA TGG
Aggrecan	ACAN	CCA ACG AAA CCT ATG ACG TGT ACT	GCA CTC GTT GGC TGC CTC	ATG TTG CAT AGA AGA CCT CGC CCT CCA T
Biglycan	BGN	Bt03244532_m1		
Decorin	DCN	Bt03230914_m1		
Elastin	ELN	Bt03216594_m1		
Versican	VCN	Bt03217632_m1		

Table 2 Concentrations of growth factors in platelet-rich and platelet-poor preparations as determined by enzyme-linked immunosorbent assay (ELISA)

Growth factor	Platelet-rich preparation (PL) (ng/mL)	Platelet-poor preparation (PPP) (ng/mL)
PDGF-AB	46.4 ± 17.6	14.5 ± 2.7
PDGF-BB	8.4 ± 3.5	5.3 ± 1.1
VEGF	1.4 ± 0.4	0.8 ± 0.6
TGF-β	68.8 ± 16.1	25.3 ± 3.8

(Microsynth, Switzerland) or Gene Expression Assays (Applied Biosystems) (Table 1). The comparative C_T method was used to evaluate differences in mRNA expression between PRP, PPP, and PL groups. The expression was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and to the expression of freshly isolated cells (day 0). The day of cell seeding (day 0) was chosen as reference sample, since completely serum free conditions would induce apoptotic effects in AF cells; thus 100 % DMEM was not considered as an appropriate reference sample [22, 23].

Whole disc organ culture model

Application of platelet preparation was further tested in an organ culture model. PRP was chosen for the organ culture experiment, because injection of PL could not generate reproducible results, due to repeated leakage of PL from the AF defect. Whole discs including the endplates (EPs) were isolated from tails of three bovine calves (6–9 months old). Discs were isolated using a band saw to make parallel cuts through the cranial and caudal EPs. EP surfaces were cleaned with Ringer's balanced salt solution using a Pulsavac Wound Debridement Irrigation System (Zimmer, USA) [24]. Using

a size 15 scalpel blade, a full thickness 8 mm cut was made in the AF of three discs per animal. Then, 100 µL of PRP were injected into two discs per animal, while control discs were left without PRP. Discs were cultured in DMEM containing 1 % ITS + 1, 50 µg/mL ascorbate-2-phosphate and 1 % P/S for 7 days. IVDs were fixed in 4 % buffered formalin, embedded in paraffin, sectioned transversely (5 µm), and stained with Safranin-O. The slides were viewed using 4.8 Axiovision Software in an upright light microscope Axiovert 2 (Carl Zeiss).

Statistical analysis

SPSS 21.0 software was used for statistical analysis. Data sets did not show symmetrical distribution. Biochemical values (DNA, GAG) were compared using nonparametric sample test (one-way ANOVA on Ranks, Kruskal–Wallis test). Gene expression data were normalized to the values of freshly isolated cells (day 0), and groups were compared using nonparametric sample test. For comparison between days 2 and 4 data within treatment groups, Mann–Whitney rank sum test was used.

Results

Preparation of PPP, PRP, and PL

Platelet preparations (PPP, PRP, and PL) from three donors were pooled. Pure platelet concentration was 10×10^6 platelets/µL ± 5 %. The platelet concentration procedure increased the platelet numbers in the PRP preparation on average by 10 times over blood baseline concentration. GF concentrations in platelet-rich and platelet-poor preparations are summarized in Table 2.

Fig. 1 Representative light microscopy images of AF cells in different media at day 2; scale bar 200 μ m. (a), (b) AF cells treated with PRP. The images were taken at the same location at different focuses, indicating that some of the cells were attached to the bottom of the culture dish, while others were embedded in the viscous medium. Within this gel-like structure, AF cells adopted a more

rounded phenotype and were arranged in colonies. (c) AF cells in PL containing medium. Whereas the cells appeared spindle-shaped like in the other monolayer culture conditions, they did form cell conglomerations. (d) AF cells in FBS containing medium. The cell conformation in PPP, FBS, and DMEM media was similar

Cell morphology

Cells cultured in the PRP containing media did not settle at the bottom of the culture dish, but rather adopted a 3D structure within the PRP medium (Fig. 1a, b). The complete clotting of the PRP group appeared after 30–45 min. The 3D environment resulted in more rounded cells which were arranged in colonies or clusters amidst the viscous medium. Cells placed in the PPP, FBS, PL, and DMEM did not experience this 3D-like structure, but rather settled at the bottom of the culture dishes (Fig. 1c, d). This monolayer culture caused a more flattened and spindle-shaped cell morphology.

Biochemical analysis

After 2 days, treatment of AF cells with 25 and 50 % PL resulted in a significant increase in DNA content when compared to DMEM-only treatment (Fig. 2a). After 4 days, the FBS, PL ($P < 0.001$), and PRP groups showed significantly increased DNA contents as compared to the DMEM treatment. With a sevenfold increase, the 50 % PL group showed the largest difference as

compared to the DMEM group. GAG synthesized was higher in the 50 % PRP as compared to the DMEM group after 2 days of culture (Fig. 2b). After 4 days, total GAG content as compared to the DMEM group was significantly increased in the 50 % PL ($P < 0.001$), 25 % PRP, 10 % FBS, 25 % PL, and 50 % PRP treatment groups.

Biochemical parameters were significantly up-regulated at day 4 when compared to day 2 within the treatment groups except in DMEM and PPP groups.

Gene expression analysis

Although collagens I and II and elastin expression showed no difference between the groups, the expression of aggrecan was significantly elevated at day 2 in AF cells treated with 25 % PL when compared with PPP (Fig. 3). Furthermore the expression of versican and decorin showed highest levels after treatment with 25 % PL. Significant up-regulation of versican mRNA was observed after treatment with 25 % PL at day 4, whereas decorin was increased as compared to PPP in the 25 and 50 % PL groups at day 2 and in the 25 % PL group at day 4.

Fig. 2 (a) DNA and (b) GAG content of whole samples after 2 and 4 days of cell culture with platelet-rich plasma (25 and 50 % PRP), platelet-lysate preparation (25 and 50 % PL), platelet-poor plasma (50 % PPP), fetal bovine serum (10 % FBS), and DMEM (100 % DMEM). Asterisk indicates a significant difference ($P < 0.05$) when compared with 100 % DMEM. Mean + SEM, $n = 5$, triplicates per group

Whole disc model

After 7 days of culture, PRP could still be seen attached to the edges of the cut made in the disc. A marked production of GAG was observed in the discs treated with PRP when compared with the disc without PRP (Fig. 4a, b). This was not only limited to the immediate area around the cut, but also within the inner two-thirds of the AF. In the discs treated with PRP, the cells adopted a more spherical morphology (Fig. 4c, d), similar to that described for the in vitro cultures (Fig. 1a, b).

Discussion

By concentrating platelets as in PRP preparations, it is expected that the interplay between diverse GFs shows synergistic effects. In order to evaluate whether the stimulatory effects are influenced by the 3D structure, we added to the AF cells either PRP or PL. This treatment, with all the factors released from the platelets, but without adopting a 3D structure, interestingly increased the cell numbers and GAG concentrations to an extent similar to the treatment with PRP. The gel formation did not significantly reduce the cell growth, although there was a trend towards lower

cell numbers in the 50 % PRP group compared to the PL formulation. The PL treated cells showed furthermore higher mRNA expression for specific ECM molecules, notably the AF proteoglycans decorin and versican [25], as compared to PRP cultures. Facilitated binding to the soluble GFs might be one reason for the more pronounced effects of the PL preparations on the gene expression levels.

Although there has been abundant work testing the efficacy of PRP on soft tissue healing, only few studies have evaluated the impact of platelet-rich preparations on the IVD. PRP effectively stimulated cell proliferation, proteoglycan, and collagen synthesis rates, notably in AF cells cultured in alginate [10]. Obata et al. [12] investigated the reparative effects of PRP-releasate after annular puncture in New Zealand White Rabbits. Although reparative effects were demonstrated, PPP showed similar effects in histological analysis, especially in the posterior inner AF and NP. PPP has also been shown to affect cell proliferation and ECM homeostasis similar to FBS in vitro [12]. In our study, 50 % PPP enhanced cell proliferation and ECM synthesis, although to a markedly lower extent than PRP and PL.

In an ex vivo degenerative whole organ culture model using chymopapain, PRP treatment enhanced chondrogenic differentiation and NP tissue formation similar to the normal control [11]. Using a bovine AF defect model, the present study demonstrates reparative effects of PRP in a similar setting. PRP could be delivered directly into the site of the generated defect and jellified within a short time, thus increasing its local effects.

One aspect to consider when comparing different studies is whether exogenous platelet stimulators were used for PRP activation, which markedly affects the release of GFs. A concern is the risk of developing antibodies against exogenously added thrombin and its alternatives [26]. Type I collagen is known to be as effective as thrombin in stimulating the release of GFs and stabilizing the PRP clot [26]. Endogenous activation of platelets is moreover suggested to result in a slower aggregation and more natural release of GFs [27]. Furthermore, activation through freeze-thawing of platelet suspensions is suggested to induce platelet degranulation by hypo-osmotic shock [21]. In the current study, no exogenous activator was used. Platelet activation was accomplished through sonication, freezing and thawing of the preparations [19, 20], and potentially through the endogenous collagen of the AF cell matrix.

Under in vitro culture conditions, AF cells are fragile and tend to lose their specific markers when expanded and passaged [28]. Using freshly isolated cells, we observed a down-regulation in matrix gene expression within all groups during culture. It is likely that during culture the AF

Fig. 3 Relative mRNA expression of AF cells after 2 and 4 days of culture with platelet-rich plasma (25 and 50 % PRP), platelet-lysate preparation (25 and 50 % PL) and platelet-poor plasma (50 % PPP). (a) collagen type I (COL1), (b) collagen type II (COL2), (c) aggrecan (ACAN), (d) versican (VCAN), (e) biglycan (BGN), and (f) decorin

(DCN) are shown. Data were normalized to the values of freshly isolated cells (day 0). Asterisk indicates a significant difference ($P < 0.05$) as compared to the 50 % PPP group. Mean + SEM, $n = 4$, triplicates per group

cells reduced the expression of their native phenotype when compared with the freshly isolated cells (day 0). Culture under more specific conditions including mechanical load and low oxygen tension may be necessary to fully maintain the phenotype of the disc cells in vitro. Furthermore, there was no significant effect of PRP/PL on the regulation of the collagen gene expression. This finding is consistent with an in vitro study on tendon cells, which also showed no significant differences in collagen gene expression between cells treated with platelet-rich and platelet-poor clot releasates [29]. One reason might be that specific gene expression responses of the AF cells to the platelet preparation may depend on the concentration of the factors present in the formulation. It has been shown previously that only high concentrations of PRP (100 %) up-regulated the gene expression of collagens I and III in short-term tendon explant cultures [30]. Dose-dependent

effects of platelet-rich preparations have been reported [15, 27]. Excessive amounts of platelets may, on the other hand, lead to apoptosis, GF receptor down-regulation and desensitization, ultimately resulting in paradoxical inhibitory effects [27]. With our method, we were able to increase the platelet count within the undiluted PRP by 10 times. Even though the in vitro application showed increased cell proliferation rates and ECM molecule synthesis in all PRP and PL groups, gene expression of several matrix molecules was notably highest in the 25 % PL group, which confirms a dose-dependent response.

A recent study also suggests that a polymer of polyglycolic acid combined with hyaluronan is favorable for AF cell re-differentiation [31]. Oriented scaffolding structures have been designed with properties similar to native tissue with the aim to mimic the AF cell environment and therefore support the AF-specific phenotype [3]. An attractive

Fig. 4 Representative images of the whole disc model after Safranin-O staining; scale bar 200 μm. (a) Cut through the AF without addition of PRP (control). (b) Injured AF and injection of PRP.

(c) The area adjacent to the cut; sample without PRP. (d) The area adjacent to the cut, after PRP injection

approach for AF repair would consist in the combination of an appropriate scaffold providing a structure and mechanical support and infiltrated PRP or PL to stimulate cell proliferation and new matrix formation. For larger AF defects and to prevent loss or leakage of PRP under load, an effective annulus closure method will be necessary to support and supplement the PRP application ([3] for review). Furthermore, NP replacement or regeneration techniques have been used to facilitate the restoration of the disc's biomechanical function after discectomy. However, these techniques will only be successful if the AF concurrently maintains its function to withstand the intradiscal pressure [32]. Different attempts have been described to close an AF defect, including sutures with and without sealants, such as fibrin and cyanoacrylate glues [33, 34]. Furthermore, various implants have been used to seal and reinforce the AF defect either by suturing them to the remaining AF or by anchoring them into the adjacent vertebral bone. However,

their safety and efficacy remain controversial. Effective AF repair strategies need to withstand the high tensile hoop stresses originating from NP pressurization and tensile and compressive stresses resulting from spinal motion. Recent data have demonstrated the potential and limitations of in vitro biomechanical tests for evaluation of AF closure devices [35], and it is suggested that purely mechanical repair may often not be sufficient. Currently, novel biomaterial-based approaches for AF repair are under investigation in in vitro, organ culture and biomechanical studies [3, 36–38]. Thus, combination of a biomaterial-based annular repair system or annular closure device with an anabolic stimulus, such as PRP or PL may represent a promising strategy for more effective AF repair.

This study demonstrates the feasibility of PRP/PL delivery in vitro and in a short-term organ culture. Immediate effects of PRP or PL have previously been described in alginate cultures of IVD cells [10], in fibroblasts and

osteoblasts [39], tenocytes [30], and co-cultures of endothelial progenitor cells and mesenchymal stem cells [40]. Taken together, these results suggest a positive influence of PRP/PL on the initial healing response of an AF defect. Nevertheless, the data do not allow drawing conclusions with respect to a sustained repair effect. Long-term experiments, carried out in a bioreactor system under relevant mechanical loading conditions [41], will reveal continued effects of PRP/PL on AF regeneration. Ultimately, while in vitro and organ culture experiments provide important insight into the principal effects of PRP and PL preparations, large animal models with biomechanical properties similar to the human will be required to assess their performance in a preclinical situation. Annular defect studies have been described in sheep [42–44], mini-pig [45, 46], and goat [47] models. Such investigations will be supportive to evaluate the effectiveness of platelet preparations, and their combinations with annular closure procedures, for sustained AF restoration and prevention of disc herniation.

Conclusions

The present findings indicate that both PRP and PL have proliferative effects on AF cells and are able to increase ECM production in vitro. PRP supplementation created a gel-like structure, which affected the morphology of the AF cells, but had no major influence on the cell phenotype in short time culture. Platelet-rich preparations may therefore be considered to promote AF repair. For direct delivery to the defect site, the immediate formation of a stable gel is a clear advantage of PRP compared to PL, minimizing the risk of leakage from the defect site. PL may be considered for AF repair in combination with an AF closure device or material. Long term and preclinical studies will be needed to assess the therapeutic potential of platelet preparations to restore functional AF tissue.

Conflict of interest None.

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