Increasing Platelet Concentration in Platelet-Rich Plasma Inhibits Anterior Cruciate Ligament Cell Function in Three-Dimensional Culture

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ABSTRACT: Tissue engineering is one new strategy being developed to treat ACL ruptures. One such approach is bio-enhanced ACL repair, where a suture repair is supplemented with a bio-active scaffold containing platelets. However, the optimal concentration of platelets to stimulate ACL healing is not known. We hypothesized that increasing platelet concentrations in the scaffold would enhance critical cell behaviors. Porcine ACL fibroblasts were obtained from explant culture and suspended in platelet poor plasma (PPP), 1× platelet-rich plasma (PRP), 3× PRP, 5× PRP, or phosphate buffered saline (PBS). The cell suspensions were cultured in a 3D collagen scaffold. Cellular metabolism (MTT assay), apoptosis (TUNEL assay), and gene expression for type I and type III collagen were measured. 1× PRP significantly outperformed 5× PRP in all parameters studied: Type I and III collagen gene expression, apoptosis prevention, and cell metabolism stimulation. ACL fibroblasts cultured with 1× PRP had the highest type I and type III collagen gene expression. 1× PRP and PPP groups had the highest cell metabolism and lowest apoptosis rates. Concentration of platelets had significant effects on the behavior of ACL fibroblasts; thus, it is an important parameter that should be specified in clinical or basic science studies. © 2013 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 32:291–295, 2014.

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The anterior cruciate ligament (ACL) is a key stabilizer of the knee that is prone to injury, especially in young, active women. The annual incidence of ACL rupture in the U.S. alone is 400,000.[1] Unlike other ligaments, such as the medial collateral ligament, the ACL fails to heal even after suture repair. Therefore, the current standard of treatment for ACL rupture is either rehabilitation alone or ACL reconstruction (removal of the torn ligament and replacement with a tendon graft).[2] However, neither of these two treatments can prevent the development of premature osteoarthritis.[3,4] Conservative treatment leads to an increasing number of future meniscal tears over time in young, active patients[5] and reconstruction carries additional risks related to graft morbidity. Therefore, new treatment options are sought which could potentially limit the development of post-traumatic osteoarthritis seen with conservative treatment and could be less invasive than ACL reconstruction.

Tissue engineering is a potential approach to improve ACL treatment. In particular, platelet-rich plasma (PRP) has received much interest for various orthopedic applications. PRP is plasma with high concentrations of platelets. Platelets are rich sources of various growth factors including platelet-derived growth factor (PDGF-AB), transforming growth factor beta (TGF-B), and vascular endothelial growth factor (VEGF).[6] These cytokines are known to encourage wound healing by serving as mitogens, chemoattractants, and stimulators of cell proliferation.[7] Furthermore, a recent study has shown that PRP activation by collagen leads to sustained release of these factors.[8]

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Studies in large animal models have yielded promising results. While PRP alone did not improve primary ACL repair,[9] the combination of collagen scaffold and PRP did enhance ACL healing at early time points.[10–15] In vitro experiments have also confirmed that the addition of PRP to ACL fibroblasts cultured in three-dimensional collagen scaffold stimulates cell proliferation and collagen expression.[16]

One key consideration when using PRP is platelet concentration. The concentration of platelets in PRP can be easily modulated by centrifugation or dilution. Prior studies have reported benefits of higher concentration of platelets in PRP used to stimulate bone regeneration,[17] and that a higher concentration of platelets leads to greater bone marrow stromal cell stimulation.[18] One could similarly hypothesize that PRP with a higher concentration of platelets would supply more growth factors, thus leading to increased ACL cell stimulation. To test this hypothesis, we studied the behavior of ACL cells in a well-defined in vitro environment to evaluate the effect of platelet concentrations in PRP. We hypothesized that increasing platelet concentration would significantly enhance ACL fibroblast proliferation and collagen production.

METHODS

Collagen Preparation

Bovine knee capsular tissue was harvested and solubilized in acidic pepsin solution to make collagen slurry as previously described.[10] The collagen concentration was neutralized with 0.1 M HEPES, five times Phosphate buffered saline (PBS), and 7.5% sodium bicarbonate and kept on ice until use.

PRP and Platelet Poor Plasma (PPP) Preparation

Porcine whole blood was centrifuged for 6 min at 150*g*. The supernatant was collected and centrifuged at 1,000*g* for 10 min. The supernatant from this second spin was saved as PPP. The platelet pellet was resuspended in measured volumes of PPP to make 1×, 3×, and 5× PRP preparations.

The platelet concentration of the whole blood was 122×10^6 platelets/ml, 1× PRP was 129×10^6 platelets/ml, 3× PRP was 370×10^6 platelets/ml, 5× PRP was 615×10^6 platelets/ml, and PPP was 8×10^6 platelets/ml. The WBC concentrations in all samples were 0.03×10^6 cells/ml or less, and the RBC concentrations were 0.01×10^6 cells/ml or less. Thus, the 1× and 3× PRP were Type 3B of the Mishra classification and the 5× PRP was Type 3A of the Mishra classification for PRP.[19]

ACL Fibroblast Preparation

ACL explants were obtained from five pig knees using sterile technique. The explants were cultured separately for each animal in complete media made with Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% antibiotic/antimycotic (Invitrogen). Once the primary outgrowth cells were 80% confluent, they were trypsinized and frozen. The first passage cells were thawed, expanded, and passaged. Fifth passage cells were used for the studies.

Construct Preparation

Three dimensional scaffolds were created and seeded with the ACL fibroblasts in an established wound surrogate model as previously reported.[16,20] In brief, ACL fibroblasts were suspended in PBS, PPP, 1× PRP, 3× PRP, or 5× PRP at a concentration of 1.3×10^6 fibroblasts/ml for all groups. Eight milliliter of cell suspension was mixed with 13 ml of neutralized collagen slurry. The final collagen density in all groups was 3 mg/ml, and the final ACL fibroblast concentration in all groups was 5.0×10^5 fibroblasts/ml. The collagen-cell mixture was placed in 3-cm-long semicylindrical molds with a polyester mesh at each end to anchor the gels. Each construct was incubated in a humidified 5% CO_2 incubator at 37°C for 1 h to achieve gelation. Thereafter, the constructs were cultured in completed DMEM. Medium was changed every 3 days.

MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay was performed to determine the rates of cellular metabolism ($n = 5$ per group, each specimen from a different animal, each run in triplicate). Constructs were rinsed with PBS and incubated in a solution of 0.5 mg/ml MTT (Invitrogen) in DMEM for 4 h in a 5% CO_2, 37°C incubator. Constructs were incubated in a solution of 0.1 N HCl (Sigma, St. Louis, MO) in isopropyl alcohol (Sigma) for an additional 4 h, and the optical density of the resulting supernatant was measured at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Apoptosis Assay

After 14 days of culture, one set of constructs were rinsed in PBS, fixed for 24 h in 10% neutral-buffered formalin, and embedded in paraffin. Serial longitudinal sections (6 μm) of the constructs were cut, and sections at 150 μm intervals from the outer surface to the center were selected for staining. The sections were stained with terminal deoxynucleotidyl tranferase-mediated dUTP nick-end labeling (TUNEL) using a commercial available kit (Roche, Indianapolis, IN), according to the manufacturer's instructions. Subsequently, the sections were treated with 40,6- diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Carlsbad, CA) to quantify the total number of nuclei. The apoptotic (TUNEL positive) and total (DAPI positive) cells were manually counted for each group ($n = 4$ per group, each specimen from a different animal, each ran in triplicate), and the fraction of apoptotic cells were expressed as a percentage of the total cells.

Real-Time PCR

After 14 days, another set of constructs were harvested and washed with PBS ($n = 4$ per group, each specimen from a different animal, each ran in triplicate). RNA was extracted from the constructs using a RNeasy mini kit (Qiagen, Valencia, CA). The RNA samples were reverse-transcribed into cDNA with RETROscript kit (Ambion, Austin, TX). Real-time PCR was performed with StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using SYBRGreen PCR Master Mix Kit (Applied Biosystems). Types I and III pro-collagen genes (*COL1A1* and *COL3A1*) were the targets, and GAPDH was used as the reference gene. The primer sequences were:

COL1A1 forward 5′-CAGAACGGCCTCAGGTACCA-3′;
COL1A1 reverse 5′-CAGATCACGTCATCGCACAAC-3′;
COL3A1 forward 5′-CCTGGACTTCCTGGTATAGC-3′;
COL3A1 reverse 5′-TCCTCCTTCACCTTTCTCAC-3′;
GAPDH forward 5′-GGGCATGAACCATGAGAAGT-3′;
GAPDH reverse 5′-GTCTTCTGGGTGGCAGTGAT-3′.

The transcript levels of *COL1A1* and *COL3A1* normalized to *GAPDH* were calculated using the $2^{-\Delta\Delta Ct}$ formula.

Statistical Analyses

All results are presented as mean ± SD with 95% confidence intervals. Data were analyzed using one-way ANOVA with subgroup analyses using Bonferroni correction for multiple testing. A p-value of less than 0.05 was considered significant.

RESULTS

MTT Assay

Cell metabolic activity in the groups containing platelets was greatest in the 1× PRP and PPP groups (Fig. 1). The 1× PRP group had significantly higher cell metabolic activity than the PBS group ($p < 0.001$), 3× PRP group ($p < 0.05$), and 5× PRP group ($p < 0.001$), and PPP was significantly higher than PBS ($p < 0.001$) or 5× PRP ($p < 0.01$), but not significantly different from the other groups. 3× PRP had higher activity than PBS ($p < 0.05$). All other comparisons were not statistically significant.

Cell Apoptosis

The lowest rate of apoptosis was seen in the groups cultured with 1× PRP and 3× PRP (Fig. 2). The rate of apoptosis was significantly lower in the 1× PRP group than in the PBS group ($p < 0.001$), PPP group ($p < 0.05$), and 5× PRP group ($p < 0.01$). The density of cells undergoing apoptosis was significantly lower in the 3× PRP group than in the PBS group ($p < 0.05$). The highest rate of apoptosis was seen in the group

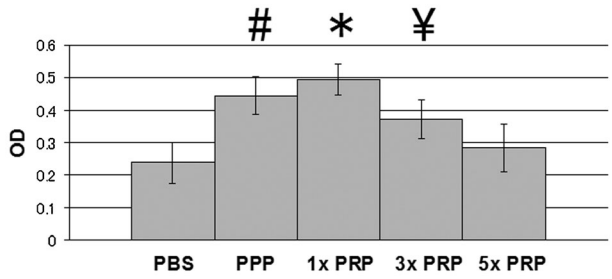

Figure 1. ACL fibroblast metabolic activity as measured by the MTT assay. The results are shown as mean ± SD ($n = 5$ per group). *MTT activity was significantly higher in the 1× group than in the 3× group ($p < 0.05$), 5× group ($p < 0.001$), and PBS group ($p < 0.001$). #MTT activity in the PPP group was significantly higher than in the 5× PRP group ($p < 0.01$) and the PBS group ($p < 0.001$). ¥MTT activity in the 3× group was higher than PBS ($p < 0.05$). There were no other statistically significant differences between groups.

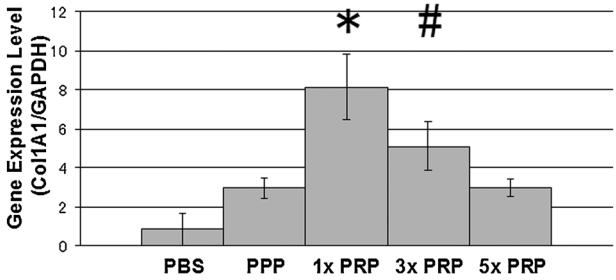

Figure 3. Type I pro-collagen gene expression by ACL fibroblasts as measured by RT-PCR. The results are shown as mean ± SD ($n = 4$ per group). *Type I pro-collagen gene expression was significantly higher in the 1× PRP group than in the PBS group ($p < 0.001$), PPP group ($p < 0.001$), 3× PRP group ($p < 0.01$), and the 5× PRP group ($p < 0.001$). #The Type I pro-collagen gene expression was significantly higher in the 3× PRP group than in the PBS group ($p < 0.001$). All other differences between groups were not statistically significant.

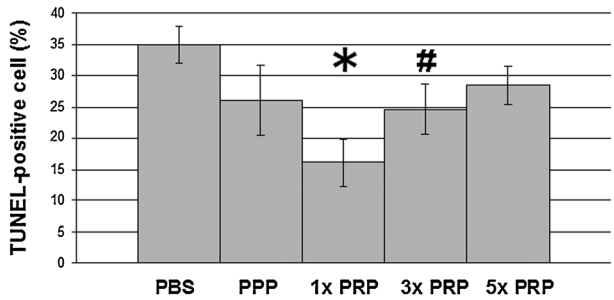

Figure 2. ACL cell apoptosis rate as measured by TUNEL staining. The results are shown as mean ± SD ($n = 4$ per group). *The density of cells undergoing apoptosis was significantly lower in the 1× PRP group than in the PBS group ($p < 0.001$), the PPP group ($p < 0.05$). and the 5× PRP group ($p < 0.01$). #The density of cells undergoing apoptosis was significantly lower in the 3× PRP group than in the PBS group ($p < 0.05$). All other differences between groups were not statistically significant.

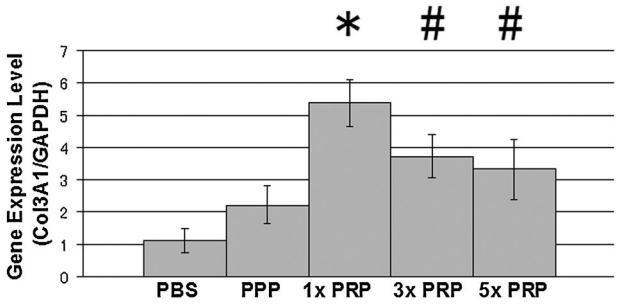

Figure 4. Type III pro-collagen gene expression by ACL fibroblasts as measured by RT-PCR. The results are shown as mean ± SD ($n = 4$ per group). *Type III pro-collagen gene expression was significantly higher in the 1× PRP group than in the PBS group ($p < 0.001$), PPP group ($p < 0.001$), 3× PRP group ($p < 0.05$) and the 5× PRP group ($p < 0.01$). #The Type III pro-collagen gene expression was significantly higher in the 3× PRP group and in the 5× PRP group than in the PBS group ($p < 0.001$, $p < 0.01$). All other differences between groups were not statistically significant.

cultured with PBS where 35% of cells were TUNEL positive. All other comparisons were not statistically significant.

Type I Pro-Collagen Expression

ACL fibroblasts cultured with 1× PRP had the highest Type I pro-collagen expression, which was significantly higher than groups with PBS, PPP, 3× PRP, and 5× PRP ($p < 0.001$, $p < 0.001$, $p < 0.01$, and $p < 0.001$, Fig. 3). Cells in the 3× PRP group expressed more Type I pro-collagen than cells in the PBS group ($p < 0.001$). All other comparisons were not statistically significant.

Type III Pro-Collagen Expression

ACL fibroblasts cultured with 1× PRP had significantly higher Type III pro-collagen gene expression when compared to cells cultured with PBS, PPP, 3× PRP and 5× PRP ($p < 0.001$, $p < 0.001$, $p < 0.05$, $p < 0.01$, Fig. 4). The Type III pro-collagen gene expression was significantly lower in the PBS group than in the 1×, 3× or 5× groups ($p < 0.001$, $p < 0.001$, and $p < 0.01$ respectively). All other comparisons were not statistically significant.

DISCUSSION

Platelets are rich reservoirs of various growth factors that can stimulate wound healing. PRP can be quickly prepared from autologous blood, which makes it a practical agent for use in the operating room and office setting. There are many protocols and kits to make PRP, but one key parameter of PRP is its platelet concentration. The purpose of our study was to investigate how this parameter influences the effect of PRP on ACL fibroblasts.

The concentration of platelets in PRP is typically described using the platelet concentration of whole blood as a reference. Our data showed that 1× PRP, which has the same concentration of platelets as the original whole blood, was the best stimulator of ACL cell metabolism and pro-collagen gene expression. Specifically, the use of 1× concentration of platelets

resulted in higher ACL cell metabolism and production of Type I and III procollagen than the use of a 3× or 5× concentration of platelets. Use of a 1× concentration was also more effective in preventing cell apoptosis than use of a 5× platelet concentration. There was no statistically significant difference between the effects of 1× and 3× PRP on apoptosis, but there was a trend toward 1× group having a greater effect.

While higher concentrations of platelets had diminishing effects, complete removal of platelets was also unfavorable. PPP, which contains almost no platelets, did not stimulate type I or type III pro-collagen gene expression or prevent apoptosis to the extent that 1× PRP did. Thus, platelets seem to play a key role in the stimulatory effect of PRP. It should be noted however, that PPP did have some effect on its own; it increased the metabolic activity of the ACL fibroblasts like 1× PRP (there was no statistically significant difference between 1× PRP and PPP), and significantly more than the group cultured with PBS. This may be due to plasma proteins such as fibronectin, which are known to increase cell proliferation.[21]

There are several in vitro studies that have investigated the effect of PRP concentrations. The results are mixed. Kawasumi et al.[17] observed a greater effect of 10.6× PRP on bone regeneration compared to 3.5× and 1.2× PRP. They cultured rat bone marrow cells in PRP gels for 6 days. The group with the highest concentration of PRP (10.6×) showed significant increase in cell proliferation at days 2, 4, and 6, but lower PRP concentrations had no significant effect. They also used rat limb lengthening model to evaluate in vivo effects of PRP. After osteotomy of femur and 10 days of lengthening, PRP gels with rat bone marrow cells were injected into the distraction callus. After 4 weeks, the groups were evaluated by radiography and microCT. The 10.6× PRP group showed significantly more bone mineralization and had completely healed. Central radiolucent areas were present in the other groups.

Arpornmaeklong et al.[18] also saw a dose-dependent stimulation of bone marrow stromal cells by PRP with no significant ceiling effect. They cultured rat osteoblastic bone marrow cells in 3D collagen scaffolds with different concentrations of PRP, and found that higher PRP concentrations stimulated more cell proliferation. The discrepancy between our results and these studies may be due to several different factors. For example, the difference in cell type may play a pivotal role. Osteoblasts and fibroblasts typically live in different environments, both in terms of oxygen, nutrition, and surrounding vasculature. A bony injury often occurs within a well-vascularized bed, whereas an ACL injury typically occurs in the synovial environment within fluid that has no vasculature. Thus, the cells may be programmed to respond to different platelet concentrations.

On the other hand, Graziani et al.[22] found that lower PRP concentrations had greater effects. They exposed human osteoblasts and gingival fibroblasts to 1×, 2.5×, 3.5×, and 4.2–5.5× PRP. At the 24 h time point, both fibroblasts and osteoblasts proliferated the most with 1× PRP, and at the 72 h time point, 2.5× had the greatest effect. Choi et al.[23] also found that low PRP concentrations better stimulated cells. They measured cellular outgrowth from alveolar bone chips cultured in media supplemented with different concentrations of PRP. The cell viability and proliferation decreased in a concentration-dependent manner. Our results are in greater accordance with these previous studies in that we also found that increasing the platelet concentration above that found in whole blood resulted in lower cell metabolism, higher cell apoptosis, and decreased gene expression for collagen—Three major factors important in wound healing.

One of the limitations in this study is the use of the porcine model, rather than the human model. Animal models allow for the use of obtaining ACL cells from young, healthy, normal ligaments, but may have species-specific differences. When designing a study like this, one must decide whether it is better to use healthy non-human ACL tissue as a source of tissue from intact ACLs or to use intact human ACLs which are typically either obtained from older patients undergoing knee replacement surgery for osteoarthritis, or from younger patients who have lost a limb secondary to cancer (and whom have often received radiation or chemotherapy). While the pig is not a perfect substitute for the human condition (the knee does not fully extend and the pig is a quadruped rather than a biped), it has become a more commonly used model for wound healing and ACL treatment, given the similarities between porcine and human hematologic systems[24,25] and knee and ACL biomechanics.[26]

In addition, the use of porcine age-matched tissues and blood provides the additional benefit of improved reproducibility of testing these cells and tissues. The pigs are genotypically similar, providing less inter-animal variability, and allowing for more repeatable assays than in studies of human tissue. However, caution must be observed when attempting to interpret results from animal models for the human condition, and clinical trials are certainly an important step prior to adoption of any of the findings demonstrated here.

Our results are also consistent with two in vivo studies in animal models evaluating the effect of PRP concentration on ACL healing in vivo. A study in pigs comparing 3× and 5× PRP showed no significant difference in functional ACL healing in terms of maximum load or stiffness of the repaired ligament.[27] A histological study in rabbits showed that intermediate platelet concentrations (2–6×) also had the optimal biological effect on bone regeneration. Low platelet concentrations had minimal effect, while highly concentrated PRP (6–11×) had an inhibitory effect.[28] Interestingly, the ceiling effect for the rabbit bone was

higher than we found in this study, where concentrations from 2× to 6× were effective for rabbit bone, but in our study, 5× was detrimental. While the species and cell type differences may be responsible, both our study and that in the rabbit bone regeneration study showed a nonlinear effect of increasing platelet concentration and that more platelets are not necessarily better for connective tissue healing.

The results from our studies suggest that the results of clinical studies that do not carefully control for platelet concentration should be interpreted with caution. Mazzocca et al.[29] have recently shown that the platelet concentration in PRP prepared using the same method from three repetitive blood draws in the same individuals varied greatly. The variation in platelet concentration may significantly affect the results of clinical studies. Thus, studies of PRP should report the platelet concentrations used.

A limitation of this study is that it does not necessary predict the optimal PRP concentration for clinical application. If PRP is delivered in vivo to ACL rupture site, platelets may die or become diluted in the local environment. The optimal amount and concentration of PRP may also be a function of the number of viable ACL fibroblasts near the injury site. Nevertheless, our conclusion is that more platelets may not always be better, and platelet concentration should be specified when describing treatments using PRP.

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