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What is This?

The Positive Effects of Different Platelet-Rich Plasma Methods on Human Muscle, Bone, and Tendon Cells

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Investigation performed at the Department of Orthopaedic Surgery, University of Connecticut Health Center, Farmington, Connecticut

Background: Clinical application of platelet-rich plasma (PRP) in the realm of orthopaedic sports medicine has yielded variable results. Differences in separation methods and variability of the individual may contribute to these variable results.

Purpose: To compare the effects of different PRP separation methods on human bone, muscle, and tendon cells in an in vitro model.

Study Design: Controlled laboratory study.

Methods: Blood collected from 8 participants (mean $\pm$ SD age 31.6 $\pm$ 10.9 years) was used to obtain PRP preparations. Three different PRP separation methods were used: a single-spin process yielding a lower platelet concentration (PRP_{LP}), a single-spin process yielding high platelet and white blood cell concentrations (PRP_{HP}), and a double-spin that produces a higher platelet concentration and lower white blood cell concentration (PRP_{DS}). Human bone, muscle, and tendon cells obtained from discarded tissue samples during shoulder surgery were placed into culture and treated with the 3 PRP preparations, control media (2% fetal bovine serum [FBS] and 10% FBS), and native blood. Radioactive thymidine assays were obtained to examine cell proliferation, and testing with enzyme-linked immunosorbent assay was used to determine growth factor concentrations.

Results: Addition of PRP_{LP} to osteocytes, myocytes, and tenocytes significantly increased cell proliferation ($P \leq .05$) compared with the controls. Adding PRP_{DS} to osteoblasts and tenocytes increased cell proliferation significantly ($P \leq .05$), but no significance was shown for its addition to myocytes. The addition of PRP_{HP} significantly increased cell proliferation compared with the controls only when added to tenocytes ($P \leq .05$). *Osteoblasts*: Proliferation was significantly increased by addition of PRP_{LP} compared with all controls (2% FBS, 10% FBS, native blood) ($P \leq .05$). Addition of PRP_{DS} led to significantly increased proliferation compared with all controls, native blood, and PRP_{HP} ($P \leq .05$). Proliferation was significantly less when PRP_{HP} was added compared with PRP_{DS} ($P \leq .05$). *Myocytes*: Proliferation was significantly increased by addition of PRP_{LP} compared with native blood ($P \leq .05$). Adding PRP_{HP} or PRP_{DS} to myocytes showed no significant increase in proliferation compared with the controls or the other separations. *Tenocytes*: Proliferation was significantly increased by addition of PRP_{LP} compared with all controls (2% FBS, 10% FBS, native blood) ($P \leq .05$). Addition of PRP_{DS} showed a significant increase compared with the controls and native blood. For tenocytes, there was a significant increase ($P \leq .05$) seen when PRP_{HP} was added compared with the controls and native blood but not compared with the other separations.

Conclusion: The primary findings of this study suggest the application of different PRP separations may result in a potential beneficial effect on the clinically relevant target cells in vitro. However, it is unclear which platelet concentration or PRP preparation may be optimal for the treatment of various cell types. In addition, a ''more is better'' theory for the use of higher platelet concentrations cannot be supported. This study was not intended to prove efficacy but to provide a platform for future research to be built upon.

Clinical Relevance: The utilization of different PRP separations may result in a potentially beneficial effect on the clinically relevant target cells in vitro, but it is unclear which platelet concentration or PRP preparation may be optimal for the treatment of various cell types.

Keywords: PRP; bone; muscle; tendon

Platelet-rich plasma (PRP) as a clinical treatment for bone, muscle, tendon, and cartilage injury has gained popularity in the field of orthopaedic sports medicine. Multiple PRP preparations are used in clinical practice, but individual differences between methods of procurement have resulted in preparations with varying volumes and concentrations of platelets and white and red blood cells. These differences as well as variability of the patient may contribute to the variable results seen in recently published studies examining the use of PRP in a clinical setting.[‖] Although in vitro studies in animal models have predominately shown positive results for PRP with regard to soft tissue and bone

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‖References 1, 6, 9, 11, 18, 20, 22, 29, 30.

healing, clinical data have yet to produce evidence of a consistent clinical effect.[1,17,25]

Investigation of the dose response of various plasma preparations on human tissue may assist in advancing the understanding of PRP and its potential as a treatment for various musculoskeletal injuries. Several studies have employed in vitro animal cell culture models to examine the effects of PRP and its associated growth factors.[4,8,14,16,20,23] Consistent data evaluating the effects of various clinically used PRP preparations on specific human target cells (bone, muscle, and tendon) may be helpful in determining optimal clinical dosages and methods of application. To our knowledge, there are currently no studies that compare the effects of plasma preparations obtained from various commercially available and clinically used isolation procedures in vitro on specific target cells.[1,17,25]

The purpose for this study was to compare the effects of PRP obtained from 3 commercially available, clinically relevant protocols on human target cells. Specifically, the effect of these PRP preparations on primary human tenocyte, osteoblast, and myocyte cell cultures obtained from surgical cases was evaluated. The entire spectrum of methods for PRP procurement was considered in an effort to provide results that were applicable to the clinical setting. The selected methods included a single-spin protocol (PRP_{LP}), a single-spin protocol with a higher amount of platelets (PRP_{HP}), and a literature-based double-spin protocol (PRP_{DS}). This human in vitro model may assist in predicting complex in vivo reactions to PRP as well as provide meaningful information to assist clinicians in decision making. Our hypothesis was that human cells treated with different PRP preparations results in increased cell proliferation when compared with cells treated only with control media and native blood.

METHODS

Participants

Venous blood was collected from 8 healthy volunteers included in this study (mean ± SD age, 31.6 ± 10.9 years). Inclusion criteria were healthy adults who agreed to participate in the study. Exclusion criteria were a medical history of blood-derived illness or consumption of any medications known to affect platelet function or concentration for a minimum of 2 weeks previous to testing. A 60-mL syringe prefilled with 5 mL acid citrate dextrose (ACD-A) was used for the standardized blood draw. Blood was then transferred directly to each of the 3 different separation systems. This group of participants had been used in a previous study demonstrating the variability of different PRP products and the individual variability of different blood draws from the same donor.[27] However, fresh blood draws have been used in the current study.

Preparation of PRP Products

The University of Connecticut Institutional Review Board (IRB) provided approval of the study protocols (IRB No. 10-204-2) used to collect blood samples and discarded tissue and allowed their combined use for scientific experiments. Three different separation methods for preparing the PRPs were used, and each was prepared from fresh venous blood samples from each individual: a single-spin process that is quicker (PRP_{LP}), a single-spin process that produces high platelet and white blood cell (WBC) concentrations (PRP_{HP}), and a double-spin process that produces a higher platelet concentration and lower WBC concentration (PRP_{DS}).[12,27] We chose to use these 3 commonly employed but basically different methods that represent both ends of the spectrum of what is being performed in the orthopaedic community.[1,13,18,27]

1. *PRP_{LP}*. The Arthrex Double-Syringe (Arthrex, Inc, Naples, Florida) for the production of autologous conditioned plasma: 10 mL of blood was filled into the double syringe to produce 3 mL of PRP_{LP}. Syringes were centrifuged with 1500 rpm for 5 minutes. This separated the blood into 2 distinct layers: a plasma layer and a red and WBC layer containing erythrocytes and leukocytes. The plasma containing the platelets was then isolated with the inner syringe.
2. *PRP_{DS}*. It was separated according to a literature-based double-spin method.[12] A volume of 10 mL of peripheral blood was used, and after a first centrifugation of 1500 rpm for 5 minutes, the plasma containing the platelets was drawn up and centrifuged a second time (20 minutes at 6300 rpm). Finally, half of the superficial plasma layer was removed, and the platelet pellet was suspended in the remaining half of the plasma volume.
3. *PRP_{HP}*. The Biomet GPS III Platelet Concentrate System was used to produce approximately 3 mL of PRP_{HP} out of 27 mL of blood. The tubes were centrifuged for 15 minutes at 3200 rpm according to the manufacturer's protocol. The specific construction of the tubes, using a fixed dual-buoy mechanism, enabled the separation of platelet-poor plasma (PPP), a buffy coat containing platelets and WBCs (PRP_{HP}), and the red blood cells on the bottom. The buffy coat (PRP_{HP}) separated between the 2 buoys was finally aspirated and drawn into a syringe.

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A 1-mL sample of each native blood specimen and each preparation was analyzed by the University of Connecticut's blood laboratory. The platelet number, number of red blood cells, and WBC differentiation was determined by a complete blood counter (Gen-S System 2 Hematology Analyzer; Coulter Corp, Miami, Florida).[28]

Isolation of Cells

Discarded samples of bone, muscle, and tendon were collected at the surgical centers of the University of Connecticut (IRB No. 10-204-2). The IRB decision clearly stated that cells gained from discarded tissue could be used in combination with donated blood products for scientific reasons.

Human bone cells were prepared from bone fragments obtained from shoulder arthroplastic surgeries. Bone samples were immediately cleaned of connective tissue, minced, and cultured for 2 weeks to allow osteoblast outgrowth. Bone chips were removed and cells were allowed to become confluent. Assessment of alkaline phosphatase activity and cell proliferation in the first 3 passages demonstrated highly consistent results within each age and sex group as previously shown by Zhang et al.[37]

For muscle cell cultures, fresh muscle samples were obtained during latissimus dorsi transfer procedures. Discarded muscle was cleaned of connective tissue, minced, and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, California) with 15% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, Georgia) and penicillin/streptomycin (PS; Invitrogen) for 2 weeks to allow for myocyte outgrowth. Minced muscle pieces were removed and cells were allowed to become confluent. Assessment of specific muscle markers nestin and caveolin-3 in the first 3 passages demonstrated highly consistent results within each age and sex group for the first 3 passages. There was no evidence of myotubual formation as assessed by routine daily microscopic review of the cell cultures.

Human tendon cells were isolated from pieces of proximal biceps tendons, which were extracted in the process of tenodesis of tendons in shoulder surgery. Pieces were included if they appeared without degenerative changes. Tendons were cut and placed into a 2% collagenase solution. The resulting tenocyte cell suspension was filtered, collagenase was removed, and the tenocytes were cultured in DMEM with 10% FBS and PS. The tenocyte genotype was confirmed by quantitative real-time polymerase chain reaction (qPCR) for the tenocyte markers tenascin-C, decorin, and collagen types I and III. Furthermore, normal tenocyte morphologic characteristics were confirmed by microscopy.

Polymerase Chain Reaction

RNA was isolated from cultured cells using TRIzol reagent (Invitrogen). RNA quantity and purity were measured using uQuant software (Life Technologies, Grand Island, New York). RNA was reverse transcribed into cDNA using 1 μg mRNA and a High Capacity Reverse Transcription kit (Invitrogen). The qPCR was performed using 10 to 100 ng cDNA as template and the StepOne Real-Time PCR System (Applied Biosystems, Foster City, California). Resultant cycle threshold (Ct) values were normalized to the endogenous control, GAPDH, and analyzed using the standard curve method.

Cell Culture Conditions

Cells were plated into Falcon Primaria dishes (BD Biosciences, Franklin Lakes, New Jersey) at a density of 2500 cells/cm^2 containing DMEM supplemented with 2% FBS and PS. The PRPs were added to 2% FBS before the cells were finally exposed to a resulting concentration of 10% PRP in each well. Two control groups were used: 2% FBS (FBS low) served as the negative control, as this provides enough nourishment to support cell viability but not to stimulate proliferation, and 10% FBS (FBS high) was the positive control because it is known to stimulate cellular proliferation. For osteoblast culture, 5% FBS was used as negative and 15% FBS as positive control. Experiments were repeated 3 times with each of the 3 cell types (myocytes, osteoblasts, and tenocytes). For all cell types, experiments were conducted using cells within the first and second passage. To each cell culture dish, 100 μL was added of each PRP treatment. Cells were exposed to each treatment for 96 hours before the final assays were performed.

Cell Proliferation Analysis

A radioactive thymidine assay was used to measure the separation effects on cell proliferation.[34] Such assays have been used in numerous studies as a reliable procedure for the specific evaluation of cell proliferation.[2,7,19,33] The radioactive thymidine is incorporated into the DNA of dividing cells, and therefore cellular proliferation will correlate with radioactivity. After 4 days of culture, cells were labeled with radioactive thymidine by adding 5 μCi [^{3}H] thymidine/mL to the wells during the last 24 hours of culture. The thin floating layer of fibrin, formed upon the addition of PRP, was removed from each well at the end of 24 hours, and the remaining attached cells were washed twice with 10% trichloroacetic acid (TCA) to remove unbound thymidine. Adding 0.5 M NaOH to each well, lysed cells and the number of proliferating cells were assayed by measuring the number of disintegrations per minute (dpm) with a scintillation counter. Each well was measured in triplicate, and results were normalized to cell number (per 1000 cells) in each well. To ensure reproducibly of the results, complete testing was repeated with 4 different wells for each group.

Growth Factor Analysis

Growth factors were quantified in media at the initiation of culture time 0 and at 96 hours, which was the conclusion of culture. Before the beginning of the assay, the fibrin clot was removed from each well containing PRP to ensure an adequate measurement of the diluted growth factors. The growth factors EGF, FGF2, HGF, IGF-1, PDGF, TGF-β, and VEGF were chosen for analysis because of their specific roles in tissue healing and regeneration.[1,17] Active TGF-β, FGF2, HGF, EGF, IGF-1, VEGF, and PDGF

TABLE 1
Mean Concentrations of White and Red Blood Cells and Platelets for Each Separation Method[a]

	White Blood Cells, $10^3/\mu L$	Red Blood Cells, $10^6/\mu L$	Platelets, $10^3/\mu L$
Peripheral blood	5.2 ± 1.3	4.2 ± 0.3	121.7 ± 69.5
PRP_{LP}	0.6 ± 0.3[b]	0.0 ± 0.0[b]	382.0 ± 111.6
PRP_{HP}	17.0 ± 5.2[b]	1.5 ± 2.4[b]	940.1 ± 425.8[b]
PRP_{DS}	1.5 ± 0.6	0.0 ± 0.1[b]	472.6 ± 224.2[b]

[a]Data are expressed as mean ± standard deviation.
[b]$P < .05$ compared with the native blood sample.

concentrations were determined using enzyme-linked immunosorbent assay (ELISA) in duplicate aliquots of all samples with the Quantikine1 Human Immunoassay kits (R&D Systems, Minneapolis, Minnesota).

Statistical Analysis

Means were compared within each experiment. One-way analysis of variance (ANOVA) was used to compare group means for experiments with normally distributed data followed by Bonferroni post hoc tests for experiments with a statistically significant difference in means. The Kruskal-Wallis test was used to compare group means for experiments with a data distribution that was not normal, even after performing appropriate data transformations. Experiments with a statistically significant difference in means were followed by post hoc tests. A P value of $\leq .05$ was used to determine statistical significance. All statistical analyses were performed using SPSS software (IBM Corp, Armonk, New York). A power analysis was performed using previously collected data from an examination of the effects of control treatments on human tenocytes. A difference of 6000 dpm and a standard deviation of 2000 dpm were representative of our previous data. Using a probability value of .05 (α) and power of .80 ($1 - \beta$), the estimated sample size for participants was 2.

RESULTS

Platelet Concentration

The results of the different preparation methods demonstrated the results expected. The mean concentrations of platelets, white blood cells, and red blood cells of each separation method are demonstrated in Table 1.

Isolation of Cells

Human bone cells were successfully obtained from discarded bone (humeral head) fragments at a concentration of approximately 4 million cells/sample. Positive confirmation of an osteoblastic phenotype using gene-specific markers was obtained by qPCR. Tendon cells were successfully obtained via a primary digest at a concentration of 8 million cells/sample. Positive confirmation of a tendon phenotype using gene-specific markers was obtained by qPCR. Human muscle cells were obtained through cellular outgrowths at a concentration of 12 million cells/sample. Positive confirmation of a muscle phenotype, using gene-specific markers, was obtained by qPCR (see the Appendix, available online at http://ajs.sagepub.com/supplemental/).

Cell Proliferation Analysis

There were no significant differences ($P \leq .05$) shown between the negative (FBS low) and positive (FBS high) controls and the native blood (Blood) for all cell types. Overall results of proliferation according to cell type and separation are shown in Figures 1 to 3.

Osteoblasts. Proliferation was significantly increased by addition of PRP_{LP} compared with all controls (FBS low, FBS high, Blood) ($P \leq .05$) (Figure 1). Addition of PRP_{DS} to osteoblasts led to significantly increased proliferation compared with all controls, native blood, and PRP_{HP} ($P \leq .05$). Proliferation was less when PRP_{HP} was compared with PRP_{LP} but did not reach statistical significance ($P > .05$). PRP_{HP} showed significantly less proliferation compared with PRP_{DS} ($P \leq .05$).

Myocytes. Proliferation was significantly increased by addition of PRP_{LP} compared with native blood ($P \leq .05$) (Figure 2). Adding PRP_{DS} or PRP_{HP} to myocytes showed no significant increase in proliferation compared with the controls or the other separations.

Tenocytes. Proliferation was significantly increased by addition of PRP_{LP} compared with all controls (FBS low, FBS high, Blood) ($P \leq .05$) (Figure 3). There was no difference in the proliferation of tenocytes comparing all 3 PRP types. Addition of PRP_{DS} showed a significant increase compared with the controls and native blood. For tenocytes, there was a significant increase ($P \leq .05$) seen when PRP_{HP} was added compared with the controls and native blood but not compared with the other separations.

Growth Factor Analysis

Table 2 shows mean results and SD of selected growth factor concentration at 96 hours according to cell type and added separation. There were no significant differences ($P > .05$) seen in the addition of FBS low and FBS high on each cell type except for a significant increase ($P \leq .05$) of VEGF and IGF-1 when FBS high was added to myocytes.

Addition of native blood to osteoblasts resulted in increased concentration only for VEGF compared with the controls ($P \leq .05$). For myocytes, all growth factor

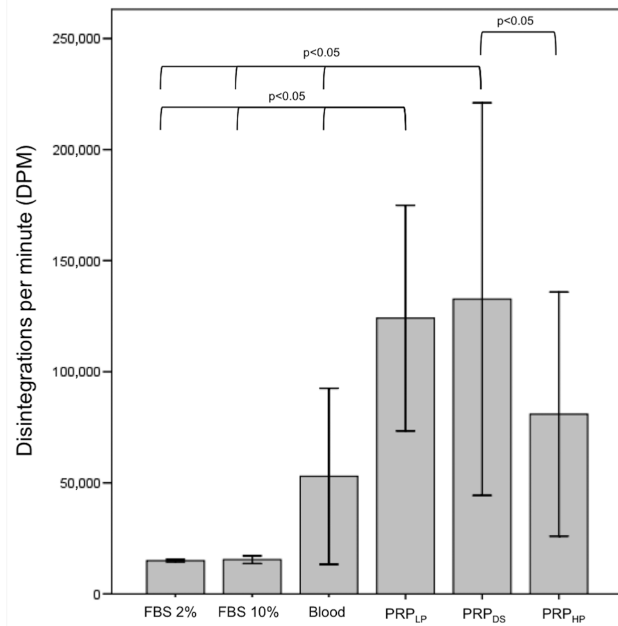

Figure 1. Osteoblast proliferation (disintegrations per minute, mean and standard deviation) according to separation methods.

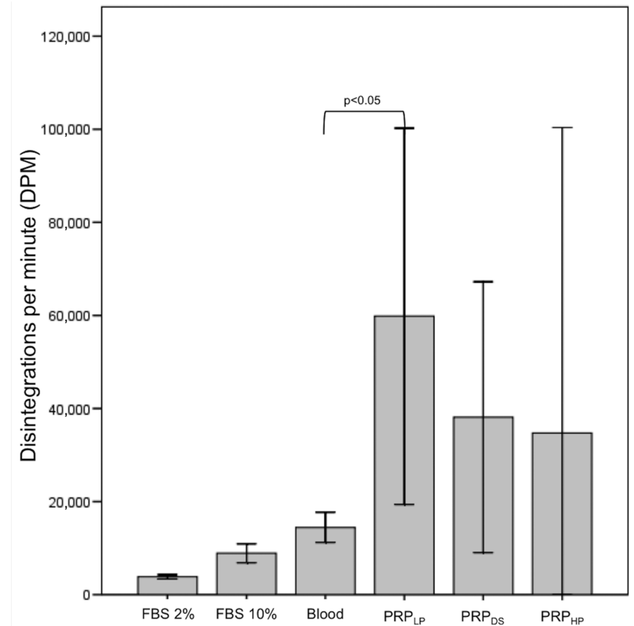

Figure 2. Myocyte proliferation (disintegrations per minute, mean and standard deviation) according to separation methods.

concentrations increased significantly ($P \leq .05$), except for FGF. Native blood added to tenocytes significantly increased concentrations only of PDGF and FGF ($P \leq .05$).

When treating osteoblasts and myocytes with PRP_{LP}, growth factor concentrations compared with the controls and native blood increased significantly ($P \leq .05$) for all, except HGF and FGF compared with negative and positive control and HGF, FGF, and EGF compared with native blood. No significant increase of growth factor concentration ($P > .05$) was found for VEGF, HGF, and FGF compared with the controls and native blood. Compared with native blood, no significant differences were also seen for PDGF and EGF. Adding PRP_{LP} to tenocytes significantly ($P \leq .05$) increased the concentration of TGF-β, PDGF, EGF, and IGF-1 compared with the controls and for PDGF compared with native blood. Addition of PRP_{LP} to tenocytes significantly increased TGF-β, VEGF, and HGF compared with the addition of PRP_{HP}.

Treatment of osteoblasts and myocytes with PRP_{DS} led to significantly increased concentrations ($P \leq .05$) of all measured growth factors except for FGF and HGF. Treatment of tenocytes showed no significant changes for VEGF, HGF, and FGF compared with low and high FBS and also for IGF-1 compared with high FBS ($P > .05$). There were no significant differences seen between treatment with PRP_{LP} and PRP_{DS} in all cell types.

Application of PRP_{HP} significantly ($P \leq .05$) increased growth factor concentrations compared with negative and positive controls and native blood for all cell types and growth factors except for FGF in all types and IGF-1 in tenocytes. No significant differences were seen between PRP_{LP} and PRP_{HP} for IGF-1 in all cell types, VEGF in osteoblasts, FGF in myocytes, and PDGF, FGF, and EGF in tenocytes. Comparing the effects of PRP_{HP} and PRP_{DS} on growth factor concentrations, IGF-1 showed no significant differences ($P > .05$) for all cell types, VEGF and EGF for osteoblasts, and PDGF, FGF, and EGF for tenocytes.

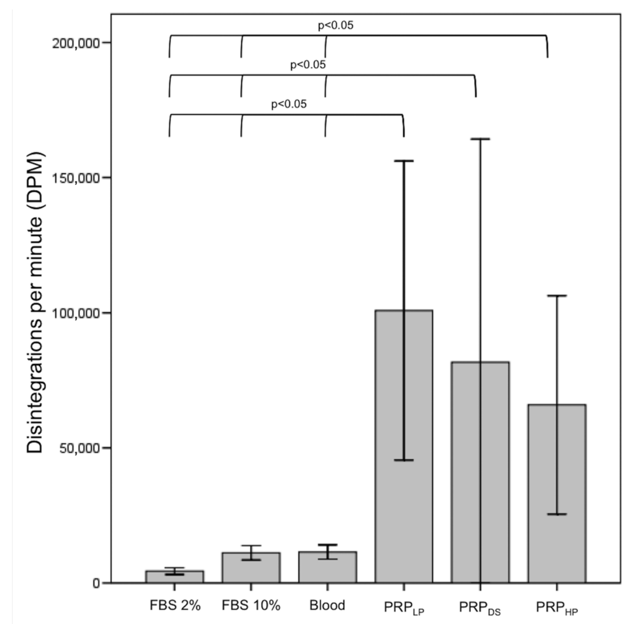

Figure 3. Tenocyte proliferation (disintegrations per minute, mean and standard deviation) according to separation methods.

TABLE 2
Concentrations of TGF-β, PDGF, VEGF, HGF, FGF, EGF, and IGF1 at 96 Hours According to Cell Type and Added Separation

Cell Type	Growth Factor	FBS Low	FBS High	Blood	PRP_{LP}	PRP_{DS}	PRP_{HP}
Osteoblasts							
	TGF-β	699.01 ± 134.95	1151.51 ± 15.81	3077.74 ± 1347.73	4553.79 ± 532.10	4902.24 ± 862.32	6098.50 ± 363.36
	PDGF	0.08 ± 0.11	ND	955.60 ± 607.38	1823.71 ± 499.02	2227.00 ± 850.26	3833.05 ± 399.28
	VEGF	84.39 ± 8.63	75.11 ± 3.78	159.43 ± 62.77	178.77 ± 47.82	246.39 ± 76.16	671.96 ± 285.69
	HGF	ND	ND	17.73 ± 32.62	58.23 ± 224.63	ND	436.36 ± 119.61
	FGF	7.89 ± 0.87	6.51 ± 1.08	3.67 ± 1.86	2.81 ± 1.88	3.23 ± 2.19	32.58 ± 33.20
	EGF	ND	ND	66.86 ± 50.15	118.66 ± 53.45	156.32 ± 59.80	276.65 ± 101.80
	IGF1	0.12 ± 0.02	0.03 ± 0.00	1.95 ± 1.54	6.63 ± 2.77	7.27 ± 1.82	7.26 ± 1.25
Myocytes							
	TGF-β	300.17 ± 47.44	1419.89 ± 85.40	4332.39 ± 226.01	5078.24 ± 267.20	5262.02 ± 308.87	5988.99 ± 364.68
	PDGF	ND	ND	1931.36 ± 405.59	2936.54 ± 647.90	3334.80 ± 501.34	4343.75 ± 239.36
	VEGF	74.63 ± 6.56	136.63 ± 1.03	601.93 ± 67.09	258.75 ± 70.58	277.97 ± 51.03	750.29 ± 112.00
	HGF	ND	ND	124.68 ± 48.80	66.19 ± 54.08	46.30 ± 42.30	1020.43 ± 299.10
	FGF	1.93 ± 0.22	3.30 ± 0.00	4.76 ± 2.64	5.56 ± 4.37	5.56 ± 6.34	11.29 ± 7.89
	EGF	ND	ND	100.41 ± 34.01	137.54 ± 36.62	147.28 ± 29.66	254.41 ± 66.29
	IGF1	0.36 ± 0.23	2.64 ± 0.36	6.03 ± 1.43	8.28 ± 0.75	7.75 ± 0.80	7.86 ± 1.88
Tenocytes							
	TGF-β	611.13 ± 88.16	1323.35 ± 53.83	1912.15 ± 1164.86	3201.88 ± 816.36	2596.50 ± 435.10	5475.68 ± 2001.05
	PDGF	44.22 ± 7.51	42.59 ± 2.89	703.02 ± 677.26	1662.50 ± 557.70	1526.73 ± 857.90	2490.03 ± 1346.74
	VEGF	149.56 ± 2.92	158.93 ± 2.20	800.03 ± 511.16	592.89 ± 207.16	905.85 ± 490.10	1496.87 ± 227.27
	HGF	294.11 ± 4.28	264.80 ± 7.01	460.77 ± 353.19	600.21 ± 125.90	639.83 ± 143.71	1676.87 ± 836.90
	FGF	5.17 ± 0.12	5.21 ± 0.11	0.36 ± 1.44	3.17 ± 4.91	2.69 ± 3.23	7.70 ± 5.72
	EGF	ND	ND	42.35 ± 30.77	69.38 ± 34.91	116.90 ± 82.39	130.99 ± 58.24
	IGF1	0.06 ± 0.033	0.36 ± 0.13	1.06 ± 0.86	3.03 ± 1.32	2.18 ± 1.29	2.70 ± 1.27

[a]Data are expressed as mean ± standard deviation. FBS Low, 2% fetal bovine serum controls; FBS High, 10% fetal bovine serum controls; Blood, native blood sample; ND, nondetectable.

DISCUSSION

This study was designed to examine the effects of different PRP preparations on human muscle, bone, and tendon cells. Three different isolation procedures (low, medium, and high platelet number) were chosen as they represent the spectrum of commercially available and clinically relevant protocols for obtaining PRP. The use of human cells was incorporated into the study design to identify any potential negative effects of PRP. We observed increases in cell proliferation for each target cell (bone, muscle, tendon) across all tested methods of PRP procurement.

Literature regarding the efficacy of PRP application is variable. To date, clinical application of PRP for tendon healing in surgical tendon repair has failed to show positive results.[6,30,32] Concurrent data have highlighted the positive effects of in vitro and in vivo PRP application on animal and human cells as well as its effectiveness in the treatment of various injuries.[1,16,17,22,25] The variability present in published data along with the attractiveness of PRP treatment due to ease of clinical use and proposed benefits warranted further investigation of different separation methods and their relative effects on specific target cells.[27] This type of evaluation seems necessary as PRP preparations are not consistently defined and are obtained through numerous methods of procurement, and basic effects may be dependent on platelet concentrations or different cell types that are not yet understood.[1,15,17,25] We have recently published a study demonstrating such variability of different PRP preparations, even if produced from the same donor.[27] The purpose of the current study was in contrast to this previous study to demonstrate the variable effects of different PRP products on human cells.

The effect of different PRP preparations, specifically their platelet concentrations, on cell proliferation of osteoblasts may represent a dose-dependent or nonlinear relationship where the amount of cell proliferation continues to rise with increasing platelet numbers to a point before falling off and decreasing despite continued increases in platelets. In the present study, adding PRP_{LP} and PRP_{DS} significantly increased the proliferation of osteoblasts. Kanno et al[24] observed a beneficial effect of PRP on human osteoblast-like cells as demonstrated by its assisting in bone regeneration and serving as an initiator in wound healing. Similarly, Markopoulou et al[26] reported a significant increase in cell proliferation of human osteoblasts after the addition of PRP. Conversely, Slapnicka et al[31] did not show positive effects of activated and nonactivated PRP on human osteoblasts, which were obtained from the American Type Culture Collection (ATCC; Manassas, Virginia). Of note, they used an automated separation system and relatively high concentrations of platelets (mean: 665 $\times 10^9$/L).[31] The results described in these studies may reflect the platelet concentration-dependent effect of PRP on osteoblasts reported by previous investigators.[10,21,35] Our experimental data agree with these findings as a dramatic and

significant increase in osteoblast cell proliferation was observed after the introduction of PRP_{LP} and PRP_{DS} (low platelet and WBC concentration), whereas the addition of PRP_{HP} (high platelet and WBC concentration) to osteoblasts did not result in a significant increase in cell proliferation.

We observed significantly higher rates of myoblast cell proliferation over the controls with the application of the 2 lower-level platelet-rich plasmas (PRP_{LP} and PRP_{DS}). The addition of high platelet and WBC-concentrated PRP_{HP} did not show a significant increase in cell proliferation. This was similar to the dose-dependent or nonlinear effect observed with osteoblasts. Very little literature exists on the effects of PRP on human muscle cells. Foster et al[17] demonstrated that 2 growth factors, IGF-1 and FGF2, both produced beneficial effects on the healing of injured muscle in mice. After injection with PRP, the muscle cells in a gastrocnemius contusion of a mouse responded with regenerating myofibers of increased diameter.

In the present study, PRP_{HP}, PRP_{DS}, and PRP_{LP} all significantly increased the proliferation of tendon cells. Few studies examining the effects of PRP on human tendon cells exist. Anitua et al[3] reported that tenocytes subjected to PRP responded with increased cell proliferation as well as increased production of endogenous growth factors, VEGF and HGF. Recently, Fallouh et al[16] showed increased overall cell proliferation and collagen production in anterior cruciate ligament (ACL) cells treated with PRP produced in a 2-step procedure.

The number of growth factors and their respective concentrations prohibit confident conclusions regarding which factors are the most appropriate for specific cell types. Various growth factors are involved in both soft tissue (muscle and tendon) and bone healing. All cell types showed a significant increased expression of TGF-β after treatment with each preparation (PRP_{LP}, PRP_{DS}, and PRP_{HP}), with PRP_{HP} demonstrating the highest expression of TGF-β. Although all PRP preparations produced significant increases of PDGF concentration for all cell types, the highest concentrations were observed in cells treated with PRP_{HP}. VEGF as a modulator protein for angiogenesis and FGF demonstrated a trend toward increased levels in all 3 groups (PRP_{LP}, PRP_{DS}, and PRP_{HP}). The existing literature has demonstrated a correlation between high TGF-β concentration and platelet numbers but not a correlation for PDGF and WBC counts.[36]

There are several limitations of this study. The in vitro behavior of the different cell types may not mimic correctly the in vivo environment. We chose this experimental setup to have a reproducible and comparable environment. Our study was not intended to simulate different biological dilutions or the effects of different application techniques (single vs repetitive dose). The heterogeneous nature of the blood and tissue samples may have affected the study. The individuals undergoing blood draws used to obtain PRP differed from those individuals who donated the target cells. This situation raises concern over a potential graft versus host reaction from incidental exposure of WBCs from 1 individual on the target cells from a different individual. This potential effect was likely minimized as up to 98% of WBCs were eliminated with the PRP_{LP} and PRP_{DS} separations, whereas negative effects with the PRP_{HP}, which produces significantly higher numbers of WBCs, could not be excluded. Last, dose-dependent or nonlinear effects were observed in our study. These effects could be the result of several factors, including the processing aspects of the production methods used to obtain PRP as well as the differing cell type and concentration present in each preparation. Our study design does not permit isolation of one specific factor, although existing literature seems to suggest that cell proliferation is dependent on platelet concentration. Furthermore, these interactions between preparations with varying platelet concentrations and human cells appear to be specific to each targeted cell type.

Thymidine incorporation is a longstanding and accurate assay for the measurement of cell proliferation. A literature search revealed that more than 4000 articles have been published using this evaluation within the last 25 years. Since this assay was first published, numerous accomplished scientists have published their research using thymidine incorporation as a stand-alone assay for determining cell proliferation.[2,7,19,33] Another potential threat to the validity of the thymidine assay is the lack of statistical significance between the positive and negative control groups (2% and 10% FBS). Comparisons of these groups, both in our laboratory and in the literature, have historically produced statistically significant differences.[5] Although our data did not reach statistical significance, the observed differences (Figure 1-3) suggest that proliferation values for 2% and 10% FBS come from separate distributions. In this regard, the lack of statistical significance may be the result of insufficient numbers (low power) as the sample size was calculated for differences between treatment groups as opposed to differences between control groups.

In this current submission, platelets from the blood of 8 patients and their effect on primary human cells were evaluated. For the proliferation assay, 4 wells/patient/group were assayed. This was repeated 3 times for accuracy and, as demonstrated by the small standard deviation, did not substantially vary. Given the reproducible nature of this assay along with its well-published history as a method for the measurement of cell proliferation, we believe we are justified in reporting results obtained with thymidine incorporation without validation of the data with another assay.[2,7,19,33] Our intention was to examine whether the application of PRP resulted in proliferation. As such, we chose the 5-day mark as it was thought that this time point would allow for the determination of whether significant proliferation occurred as opposed to its rate or time course.

The purpose of this study was to quantify cell proliferation after the application of different plasma preparations to human myocytes, tenocytes, and osteoblasts. Evaluation of cell-specific matrix production via gene expression or immunohistochemistry was not performed as these methods are used to qualify as opposed to quantify cell proliferation. Although examination of cell-specific matrix production would produce valuable data, this is a separate question to be addressed by another study, specifically one whose intent is to qualify cell proliferation.

CONCLUSION

The primary findings of this study suggest that the application of different PRP separations may result in a potential beneficial effect on the clinically relevant target cells in vitro. However, it is unclear which platelet concentration or PRP preparation may be optimal for the treatment of various cell types. In addition, we cannot support a “more is better” theory for the use of higher platelet concentrations. This study was not intended to prove efficacy but to provide a platform for future research to be built upon.

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