

## **PLATELET-POOR PLASMA OF ATHLETES IS A POTENT INDUCER OF MYOGENIC DIFFERENTIATION OF C2C12 MYOBLASTS**

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### Abstract

*Introduction.* Blood products, i.e. platelet rich plasma (PRP), leukocyte-poor plasma (PPP) and platelet poor plasma (PPP), have previously been used to improve muscle regeneration. In this study, six months' frozen-stored PPP of individuals who practiced different types of physical exercise was analysed; it could steer mouse C2C12 myoblast cells towards proliferation, migration and myogenic differentiation, and it could affect the morphology/shape of myotubes.

*Materials and Methods.* PPP of male Olympic weightlifters, football players and professional folk dancers, aged 15-19, was collected 12 h post-training and stored for 6 months at -20°C. C2C12 cell proliferation was assessed by MTT test, motility by scratch assay, myogenic differentiation by myotube formation and gelatinase activity by gel-zymography.

*Results and Conclusions.* PPP induced proliferation and migration of C2C12 cells. Proliferative capacity was as follows: weightlifters > dancers > football players; mean migratory capacity was: weightlifters = dancers > football players. PPP induced formation of myotubes; significant inter-individual variations were detected: PPP from

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weightlifters induced formation of round myotubes, and PPP from football players and dancers induced formation of elongated myotubes. The mean myotube area was as follows: football players > dancers > weightlifters. PPP gelatinolytic activity was observed; it was negatively correlated with C2C12 myoblast proliferation. These results provide general but distinct evidence that PPP of individuals practicing certain types of exercise can specifically modify myoblast morphology/function. This is significant for explaining physiological responses and adaptations to exercise. In conclusion, long-term, frozen-stored PPP preserves its potential to modify myoblast morphology and function.

**Keywords:** C2C12 myoblasts, Exercise, Myogenic differentiation, Myotube morphology

## INTRODUCTION

Exercise itself is a form of physiological stress known to induce an adaptation response (Mooren & Krüger 2015). Specifically, different factors, including growth factors, mechanical strain, hypoxia, inflammation and extracellular matrix composition, are altered by exercise and strongly influence the cell niches and behaviour of mesenchymal stem cells (MSCs) (Boppart et al., 2015; Diaz et al., 2017).

Besides the traditional approach of studying stem cells in the context of embryonic development, stem cells are known to play an essential role in regeneration of skeletal muscle following exercise-induced muscle damage by responding to tissue microenvironment specific signals (Boppart et al., 2015). This fact suggests multipotent MSCs as possible powerful therapeutic agents in tissue regeneration. For instance, pre-clinical studies of MSC therapy have demonstrated enhanced wound healing (Dantzer et al., 2003). Muscular injuries cover one-third of sports injuries, leading to muscle fatigue and pain, and sometimes to injury recurrences (Ekstrand et al., 2011; Woods et al., 2004), which are then associated with long absences from training and competition (Maffulli et al., 2015). Besides the established approach of managing sports injuries (protection, rest, ice, compression and elevation (PRICE principle), nonsteroidal anti-inflammatory drugs, physical therapy modalities and corticosteroid injections (Sheth et al., 2018), more attention has been recently focused on platelet-derived products.

MSC activities *in vivo* are known to be regulated by molecular microenvironments. Furthermore, structural support, protection and maintenance of the functional integrity of skeletal muscle are provided through the extracellular matrix, which is strongly controlled by matrix metalloproteinase (MMP) activity (de Sousa Neto, 2018). Therefore, it is considered that plasma and platelet derived blood fractions could enhance tissue repair and regeneration, inducing the release of signalling factors that will create a pro-healing environment at sites of tissue injury (Rubio-Azpeitia et al., 2014). Plasma has been extensively studied and used in many different cases to speed up wound healing, producing better tissue adherence and homeostasis. Platelet rich plasma (PRP) and related blood products, platelet poor plasma (PPP) and leukocyte-poor PRP, have been used to improve muscle regeneration and repair by enhancing differentiation of skeletal myoblasts. PRP has been viewed as an attractive and safe

option, as it enables use of the patient's own growth factors and biomolecules to augment tissue repair in musculoskeletal injuries (Kon et al., 2011).

However, to avoid the loss of platelet bioactivity, some authors suggest injecting PRP within 3 h of preparation (Bausset et al., 2012). According to blood transfusion guidelines, the shelf-life of PPP (with its therapeutic potential preserved) is up to one year (Anđelić & Pantelić, 1995). Increased levels of growth factors were induced by stored PPP compared to stored PRP (Bausset et al., 2012), while the effect of the level of PPP from healthy blood donors on human myogenic differentiation has been clarified (Miroshnychenko et al., 2017). Therefore, following on from those studies, the aim of this study was to observe, using mouse C2C12 myoblasts, the difference in the capacity of six months' frozen-stored PPP from individuals who each practiced one of three different types of physical exercise to modulate myoblast morphology/function *in vitro*.

## MATERIALS AND METHODS

### Participants

Competitive Olympic weightlifters (n=6), competitive football players (n=6) and professional folk dancers (n=6), all males aged 15-19, participated in the study. This research was conducted according to the principles of the Declaration of Helsinki and in accordance with Good Clinical Practice. All participants, including the parents of the minors, were informed of the purpose of the study and consented to the research protocol and the taking of blood samples over the course of this study. Participants were free-living, and maintained their usual food intake. They were non-smokers and in apparent good health upon clinical examination. None of the participants reported any infectious episodes or any relevant condition, including musculoskeletal injuries, in the four weeks preceding the study. All participants had trained regularly for their sport in the month before the study. None of the participants involved in our study were using steroidal/non-steroidal anti-inflammatory drugs prior to or during the study.

### Platelet Poor Plasma aliquots

Blood was drawn 12 h post-training from the antecubital vein into 9 ml sterile tubes containing EDTA (B2 Vacutainer E2E (EDTA), Becton Dickinson, Plymouth, UK). Cells were removed from plasma by centrifugation for 20 min, at 3500 rpm, and at 22°C. Plasma aliquots were stored for 6 months at -20°C.

### C2C12 cell viability - proliferation (MTT) assay

Mouse C2C12 myoblast cells (ATCC, Rockville, MD, USA) were grown in DMEM cell culture medium (Sigma-Aldrich) supplemented with 10% foetal bovine serum (FBS) (PAA Laboratories GmbH, Pasching, Austria) (DMEM-10%FBS). Viability

of C2C12 cells was analysed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (Kocić et al, 2103). The cells ( $5 \times 10^3$  in 100  $\mu\text{L}$  DMEM-10%FBS per well) were seeded into 96-well plates and cultivated overnight. The following day DMEM/10%FBS was replaced with DMEM medium supplemented with 2% FBS (control for the baseline proliferation level) or DMEM supplemented with 2% PPP, and the cultivation was continued for 48 h. After this period, 10  $\mu\text{L}$  of MTT (Sigma-Aldrich) solution (5 mg/mL in PBS) was added, and after 3 h, the formazan crystals were dissolved with 100  $\mu\text{L}$  of 10% SDS containing 10 mM HCl. The absorbance was read at 540 nm. Results are presented as a relative viability index, where the viability of C2C12 cells grown in DMEM medium supplemented with 2% FBS was set at 100%.

### **C2C12 motility – scratch assay**

The motility of C2C12 cells was analysed by *in vitro* scratch assay (Kocić et al, 2103). The cells ( $2.5 \times 10^4$  in 500  $\mu\text{L}$  DMEM-10%FBS per well) were seeded in a 24-well tissue culture plate. When the cells reached confluence, a scratch in the monolayer over the total diameter of each well was made using a sterile pipette tip, and cells were further cultivated over the next 24 h in DMEM supplemented with 2% PPP. Then, the cells were fixed with ice-cold methanol and stained with 0.1% crystal violet. Migration of cells into the scratch area was documented by light microscopy and quantified by TScratch software (Computational Science and Engineering Laboratory, Swiss Federal Institute of Technology, ETH Zurich, Zurich, Switzerland).

### **Myogenic differentiation**

Myogenic differentiation was confirmed by the test as described in Kocić et al. (2013). C2C12 cells ( $2.5 \times 10^4$  in 500  $\mu\text{L}$  DMEM-10%FBS per well) were seeded in a 24-well tissue culture plate. When the cells reached 80% confluence, myogenic differentiation was induced with myogenic differentiation medium consisting of DMEM with 2% horse serum (PAA Laboratories GmbH, Pasching, Austria) (positive control) and DMEM with 2% PPP. Cultures of C2C12 cells grown in DMEM-10%FBS were used as a negative control. The media were changed every 48 h, and six days later, myogenic differentiation was confirmed based on myotube formation. For every participant in this study, up to 30 myotubes were visualized by crystal violet staining and documented by light microscopy. The morphometric analysis of myotubes (determination of length, width and area parameters) was performed with ImageJ software (National Institutes of Health, USA). The myotube sphericity index (SI) is the quotient of the length and width of a cell.

### **Osteogenic differentiation**

C2C12 cells ( $2.5 \times 10^4$  in 500  $\mu\text{L}$  DMEM-10%FBS per well) were seeded in a 24-well tissue culture plate. When cells reached 80% confluence, osteogenic differentiation was induced with osteogenic differentiation medium consisting of 50  $\mu\text{M}$  ascorbic acid

(Sigma-Aldrich), 10 nM dexamethasone, 10 mM beta-glycerophosphate (Applichem, Darmstadt, Germany), and DMEM-10%FBS (positive control) or DMEM with 2% PPP. Cultures of C2C12 cells grown in DMEM-10%FBS were used as a negative control. The media were changed every 48 h, and six days later, osteogenic differentiation was confirmed by ALP activity test as described in Kocić et al. (2013).

### **Evaluation of relative MMP gelatinase activity in PPP**

MMP activity was determined in the blood plasma of the examined athletes. Plasma was exposed to a zymography assay on non-reducing 8% polyacrylamide gels containing 0.2% gelatine as the MMP substrate. After electrophoretic separation, proteolytic activity was revealed by staining gels with Coomassie brilliant blue, and clear degraded band areas were quantified by densitometry (Kocić et al, 2103). The MMP activity of each sample was normalized to a control serum sample (5% foetal calf serum), which was set as 1 (or 100%). The relative activity of MMPs in the samples was expressed as magnitude of difference compared to the control.

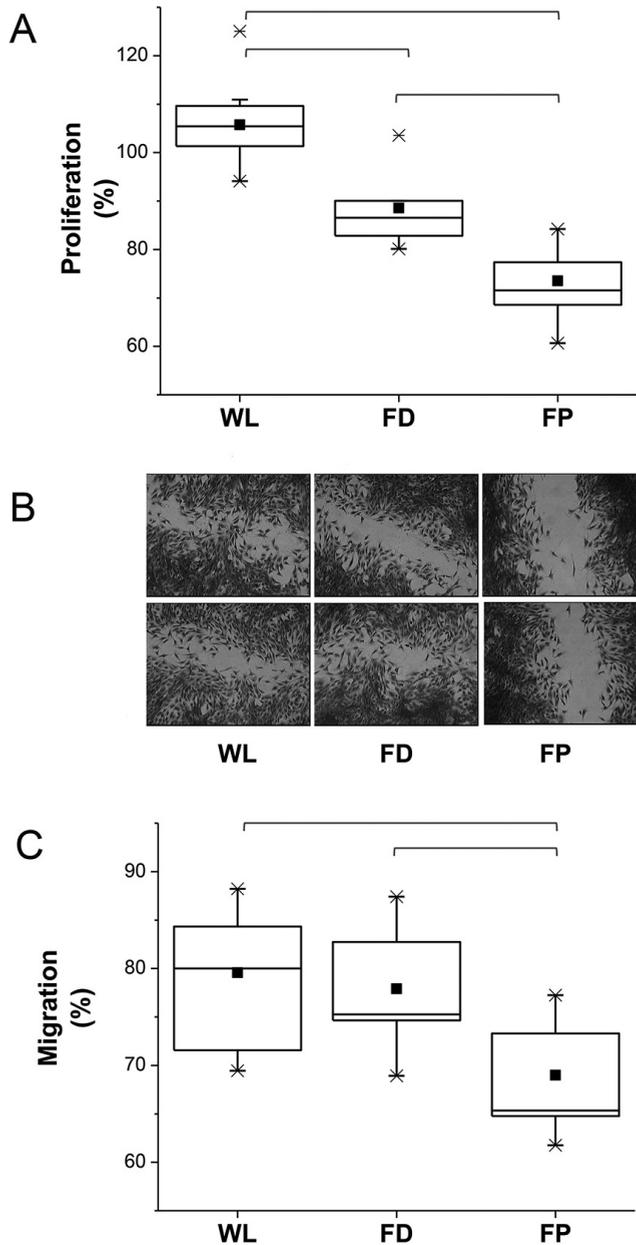
### **Statistical analysis**

The results are expressed as means  $\pm$  SD. Statistically significant differences between the groups were determined by Student's t-test. Correlation between the analyzed parameters was tested by linear regression. Differences with p-values of  $<0.05$  were considered significant. All statistical analyses were carried out in the OriginPro8 program.

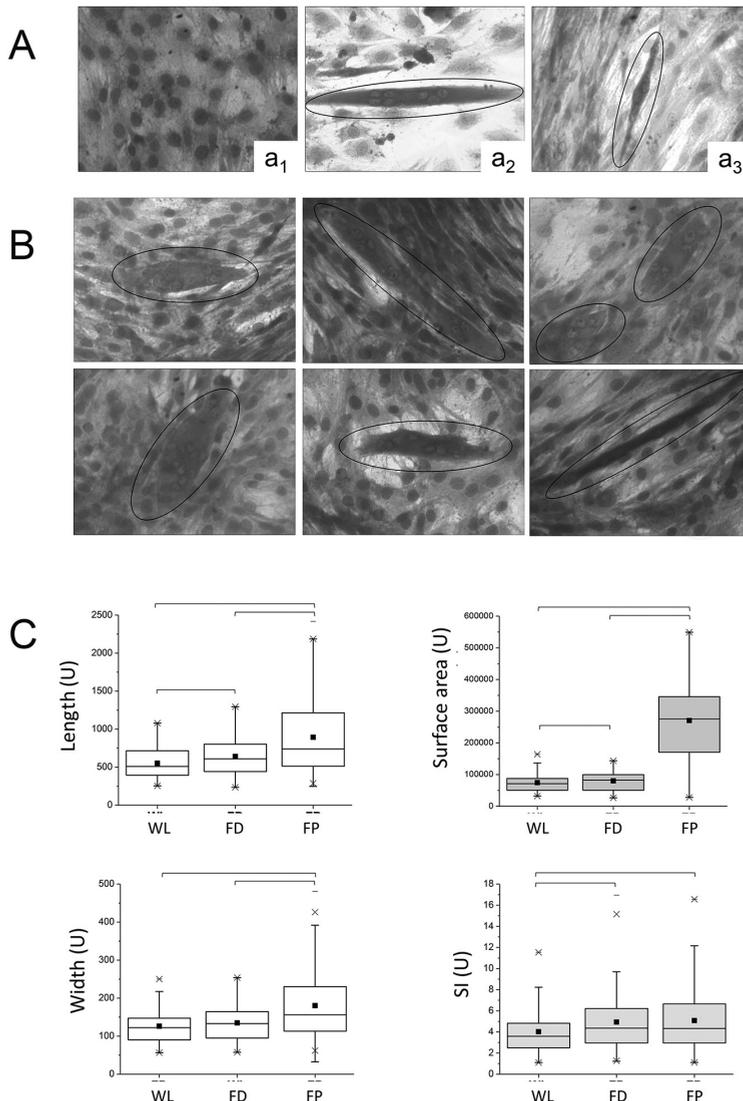
## **RESULTS**

The effect of PPP from weightlifters (WL), football players (FP) and professional folk dancers (FD) on proliferative and migratory characteristics and differentiation potential of mesenchymal stem cells was studied. The C2C12 myoblast cell line, the behaviour of which corresponds to that of the progenitor lineage, was used as a model to study the commitment of mesenchymal cells to myogenic (of skeletal muscle development) and osteogenic lineages.

Firstly, we analysed if the soluble factors present in athletes' plasma fraction with reduced platelet count could steer C2C12 cells towards proliferation and migration. The results of the MTT proliferation assay (Figure 1A) showed that all PPP (2% concentration) samples analysed in this study induced proliferation of C2C12 cells, and, among the three groups of athletes, the mean capacities of PPP to induce proliferation were as follows: WL > FD > FP. Additionally, we found the PPP, regardless of the type of athletic activity, induced migration of C2C12 cells, and the mean induction capacities were as follows: WL = FD > FP (Figures 1B and C). Further, cell proliferation and motility were positively correlated ( $p=0.03$ ;  $r=0.46$ ).



**Figure 1.** Platelet poor blood plasma (PPP) of Olympic weightlifters, professional folk dancers and football players modulates proliferation and migration of myoblast C2C12 cells. WL: Olympic weight lifters, FD: professional folk dancers and FP: football players. **A)** Cell proliferation; **B)** Microscopic images of the stretch motility assay; **C)** Cell migration. The plots show the mean value (■) median (line within box), 25%-75% i.e. interquartile range (box), outliers (whiskers), 1%-99% range (\*), and minimum to maximum range (-). Horizontal bar indicates a significant difference between groups. Proliferation: WL vs FD  $p=0.001$ ; WL vs FP  $p=0.001$ ; FD vs FP  $p=0.004$ ; Migration: WL vs FD  $p=0.8$ ; WL vs FP  $p=0.007$ ; FD vs FP  $p=0.03$ .



**Figure 2.** Platelet poor blood plasma (PPP) of Olympic weightlifters, professional folk dancers and football players is a potent inducer of myogenic differentiation of myoblast C2C12 cells. WL: Olympic weight lifters, FD: professional folk dancers and FP: football players. A) Cell proliferation; A) Representative micrographs of undifferentiated C2C12 cells grown in DMEM-10%FBS cell culture medium (a1) and C2C12 cell myotubes induced with DMEM supplemented with 2% horse serum (a2 and a3). B) Representative micrographs of C2C12 cells myotubes induced with DMEM supplemented with 2% of athletes' or dancers' blood plasma. C) Morphometric characteristics of myotubes induced by blood plasma of Olympic weight lifters (WL), professional dancers (FD) and football players (FP). The plots show the mean value (■) median (line within box), 25%-75% i.e. interquartile range (box), outliers (whiskers), 1%-99% range (\*), and minimum to maximum range (-). Horizontal bar indicates a significant difference between groups. Length: WL vs FD  $p=0.008$ ; WL vs FP  $p=0.001$ ; FD vs FP  $p=0.001$ . Width: WL vs FD  $p=0.2$ ; WL vs FP  $p=0.001$ ; FD vs FP  $p=0.001$ . Area: WL vs FD  $p=0.014$ ; WL vs FP  $p=0.001$ ; FD vs FP  $p=0.001$ . Sphericity index (SI): WL vs FD  $p=0.008$ ; WL vs FP  $p=0.013$ ; FD vs FP  $p=0.7$ .

Next, we analysed if PPP had the capacity to induce myogenic differentiation of C2C12 cells. The myogenic differentiation potential of C2C12 cells was first confirmed by formation of long multinucleated myotubes after six days of cultivation in the myogenic differentiation medium i.e. 2% horse serum (Figure 2A, Table 1). Then, we confirmed that all PPP samples analysed induced massive myogenic differentiation of C2C12 cells (Figure 2B). In computer-assisted morphometric analysis, significant individual variations in myotube morphometric parameters (area, width, length and sphericity index; SI), even between individuals engaged in the same type of physical activity, were detected (Table 1). When myotubes from individuals engaged in different types of physical exercise were compared, their morphology/shape was strongly affected by the type of physical exercise (Figure 2B). Morphometric analysis of mean myotube area for the different groups of athletes (Figure 2C) showed the following increase: WL < FD < FP. Myotubes of the different groups of athletes were calculated and arranged in order of decreasing mean SI: WL > FP = FD. These results showed that PPP of FD and FP induced formation of elongated myotubes compared to PPP of WL.

**Table 1.** Morphometric parameters of myotubes induced by platelet-poor blood plasma (PPP) of weightlifters (WL), professional dancers (FD) and football players.

		Surface area (x10 <sup>3</sup> )	Length (U)	Width (U)	SI
HS		67 ± 25	549 ± 224	144 ± 47	3.9 ± 2.7
		62	499	143	3.2
		(30-144)	(247-1030)	(68-285)	(1.3 - 12.6)
WL	WL 1	102 ± 49	664 ± 183	165 ± 57	4.0 ± 2.2
		94	573	167	3.6
		(57-218)	(445-978)	(80-254)	(1.6 - 8.2)
	WL 2	70 ± 31	483 ± 176	156 ± 37	2.9 ± 1.2
		63	491	162	2.9
		(26-139)	(260-876)	(80-196)	(1.3 - 5.7)
WL 3	56 ± 19	507 ± 152	122 ± 44	4.2 ± 2.5	
	50	504	125	3.9	
	(30-98)	(283-777)	(60-238)	(1.1 - 11.5)	
WL 4	65 ± 39	491 ± 152	140 ± 43	3.4 ± 1.6	
	49	403	148	3.3	
	(18-151)	(254-828)	(58-205)	(1.3 - 7.0)	
WL 5	48 ± 20	406 ± 83	125 ± 44	3.2 ± 1.2	
	39	411	122	3.4	
	(28-87)	(298-544)	(74-222)	(1.4 - 5.2)	
WL 6	78 ± 34	664 ± 216	125 ± 38	5.1 ± 2.2	
	75	649	118	4.7	
	(32-163)	(265-1079)	(76-209)	(1.7 - 10.5)	
FD	FD 1	82 ± 28	698 ± 161	119 ± 47	6.0 ± 3.1
		82	704	119	5.9
		(23-132)	(442-1063)	(63-209)	(2.1 - 13.6)

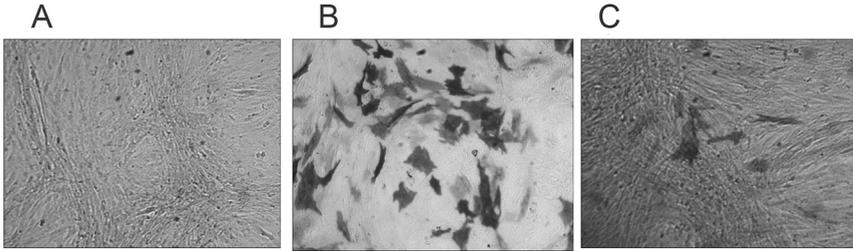
cont. Table 1.

	FD 2	85 ± 26 84 (42-141)	640 ± 303 570 (285-1095)	141 ± 52 130 (76-250)	4.7 ± 3.7 3.2 (1.3 - 12.2)
	FD3	93 ± 44 82 (24-164)	798 ± 284 813 (312-1294)	110 ± 31 114 (57-173)	6.9 ± 3.5 6.8 (2.6 - 16.6)
	FD 4	67 ± 34 45 (32-133)	506 ± 214 423 (234-878)	135 ± 41 126 (76-212)	3.5 ± 1.6 3.7 (1.5 - 6.6)
	FD 5	82 ± 35 82 (27-144)	653 ± 251 630 (267-1229)	128 ± 40 130 (57-190)	5.0 ± 2.9 4.1 (1.9 - 10.3)
	FD 6	75 ± 32 67 (31-126)	566 ± 185 573 (268-911)	128 ± 50 125 (63-218)	4.4 ± 2.1 4.4 (1.1 - 9.0)
FP	FP 1	64 ± 19 64 (39-94)	497 ± 122 499 (248-741)	126 ± 40 124 (68-209)	3.6 ± 2.7 3.7 (1.2 - 7.4)
	FP 2	95 ± 47 85 (25-212)	650 ± 307 583 (289-1214)	149 ± 49 146 (69-238)	4.4 ± 2.9 3.8 (1.4 - 12.6)
	FP 3	76 ± 18 75 (51-109)	552 ± 145 548 (378-883)	134 ± 42 142 (78-198)	4.1 ± 2.5 3.3 (2.2 - 9.7)
	FP 4	62 ± 21 66 (15-249)	590 ± 172 557 (324-1864)	100 ± 41 92 (33-193)	6.1 ± 3.7 4.8 (1.9 - 15.2)
	FP 5	321 ± 119 310 (143-549)	1024 ± 324 1198 (692-2047)	258 ± 90 249 (113-481)	4.8 ± 1.9 4.6 (1.7 - 9.5)
	FP 6	306 ± 112 304 (144-640)	1364 ± 469 1198 (453-2417)	243 ± 89 229 (103-426)	5.8 ± 3.5 5.5 (1.3 - 17.0)

NOTE: Data for each athlete's PPP sample are presented as mean ± SD, median and minimum through maximum values of given range. HS: Positive control, 2% horse serum.

As was expected, strong osteogenic differentiation of C2C12 cells was induced in osteogenic differentiation medium. However, for every athlete analysed, 2% PPP in the cell culture medium was only a weak inducer of osteogenic differentiation (Figure 3). The low capacity to induce osteogenic differentiation was not influenced by the type of physical activity and showed no inter-individual variation within groups of athletes.

When blood plasma from athletes executing different types of physical activity was evaluated for total MMP gelatinase activity, no statistically significant difference between the groups emerged (Table 2, Figure 4). A negative correlation between cell proliferation/viability of C2C12 myoblasts and gelatinase activity of PPP was noted.

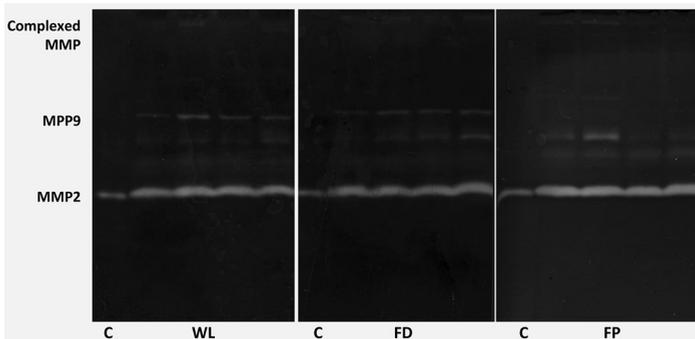


**Figure 3.** Platelet poor blood plasma (PPP) of Olympic weightlifters, professional folk dancers and football players is a weak inducer of osteogenic differentiation of myoblast C2C12 cells. **A)** negative control: absence of osteogenic differentiation in the cells grown in DMEM-10% FBS cell culture media; **B)** positive control: strong osteogenic differentiation induced by osteogenic differentiation medium; **C)** weak osteogenic differentiation induced by blood plasma of examined athletes.

**Table 2.** Relative gelatinase activity of MMP in platelet-poor blood plasma (PPP) of Olympic weight lifters (WL), folk dancers (FD) and football players (FP)

	WL	FD	FP	p#	Proliferation*	Migration†
MMP activity	3.7 ± 1.3 3.8 (2.0 - 5.4)	3.3 ± 0.6 3.2 (2.7 - 4.4)	5.3 ± 1.7 5.0 (3.6 - 8.2)	>0.05	r = -0.50 p = 0.02	r = -0.40 p = 0.10
MMP2 %	73 ± 9 73 (60 - 87)	67 ± 4 66 (61 - 75)	75 ± 6 74 (66 - 84)	>0.05		
MMP9 %	15 ± 3 14 (12 - 20)	17 ± 4 19 (11 - 23)	20 ± 9 19 (9 - 32)	>0.05		
MMP complexed %	14 ± 6 9 (9 - 24)	15 ± 3 14 (11 - 20)	7 ± 3 7 (2 - 11)	>0.05		

#Significance of difference in gelatinolytic activity between the groups analysed; \*The correlation between gelatinolytic activity of PPP and viability/proliferation of C2C12 cells; r – correlation coefficient, p – probability; †The correlation between gelatinolytic activity of PPP and migration of C2C12 cells; r – correlation coefficient, p – probability.



**Figure 4.** Gelatinolytic activity of platelet poor blood plasma (PPP) of Olympic weightlifters, professional folk dancers, and football players: Representative zymograms. C: Control, foetal calf serum; WL: Olympic weight lifters, FD: professional folk dancers, FP: football players.

## DISCUSSION

Despite the increasing popularity of PRP use for treatment of muscle injuries, conflicting results on the effect of this biologic on muscle regeneration (Grassi et al, 2018; Sheth et al., 2018) indicate that modifications in present formulations of PRP are needed (Miroshnychenko et al. 2017). On the other hand, the use of PPP is more convenient as it can be frozen for prolonged times (Miroshnychenko et al., 2017, Bausset et al. 2012).

The evidence presented in our research shows that long-term, frozen-stored PPP from individuals practicing certain types of exercise could specifically modify myoblast function. Therefore, this frozen plasma retains the capacity to stimulate proliferation, migration and myogenic differentiation of C2C12 myoblasts, which is significant for explaining the physiological response and adaptation to exercise. Additionally, morphometric analysis showed that myotube morphology/shape was significantly affected by the type of physical exercise. Indicative inter-individual variability in myotube area within the exercise groups indicates the potential therapeutic use of autologous, long-term, frozen-stored PPP in muscular tissue regeneration. To the best of our knowledge, this study is the first to show the distinct effect of PPP obtained from athletes training in different types of physical activity on morphology/shape of myotubes.

MSCs express, on various elastic matrices, high basal levels of myoblast determination protein 1 transcription factor on induction by myoblast induction media, and these levels are akin to the constitutive expression levels of C2C12 myoblasts (Engler et al., 2006). Additionally, a clear peak for MSCs induced by myoblast induction media, showed by Engler et al. (2006), suggest a statistically similar level of lineage commitment for MSCs and C2C12 cells, which justifies the use of C2C12 myoblasts in our study as an *ex vivo* model of myogenesis.

A positive effect of PPP at low concentration (2%) on myogenic differentiation of C2C12 myoblasts was observed in our study. Observations provided by Martínez et al. (2016) suggest a positive effect of PPP on bone differentiation of human periodontal ligament stem cells even at low concentrations (2.5%) of this platelet-derived fraction. Recently published results by Miroshnychenko et al. (2017) indicate the *in vitro* dose range of PPP (i.e. soluble factors of blood plasma) of healthy blood donors for myogenic differentiation or formation of multinucleated myotubes is likely between 1-4%. Miroshnychenko et al. (2017) showed that application of PPP to cultures of human skeletal muscle myoblast induced myoblast differentiation with visible multinucleated myotube formation. Furthermore, the authors noted that only platelet removal from PRP and modified-PRP types (i.e., neutrophil-, TGF- $\beta$ 1- or myostatin-depleted) led to induction of myoblast differentiation by these preparations at the level achieved by the PPP-2% horse serum positive control. Conversely, PRP modification with no platelet removal led to increased myoblast proliferation compared to PPP and no evidence of myogenic differentiation.

The following analysis supports our research data associated with PPP use presented in our study. Bausset et al. (2012) evaluated the growth factor-releasing capacity of PRP and PPP after 3, 6, and 24 h storage at 20°C. They measured vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF-AB) that act mainly on mesenchymal stem/stromal cells and play key roles in wound healing. Mean growth factor levels induced by PPP between 3 and 6 h were unchanged but significantly lower compared to levels induced by PRP. Conversely, prolonged 24 h storage brought about a significantly greater, approximately four-fold, reduction in VEGF release by PRP and a significant, approximately five-fold, increase in VEGF levels by PPP. To summarize, over an extended (24 h) period, the concentration of VEGF induced by PRP and PPP were  $479\pm 82$  pg/mL and  $281\pm 278$ , respectively. Furthermore, Matsakas (2018) recently presented supporting evidence that platelet factors such as VEGF and PDGF could produce positive effects on myoblast proliferation and their commitment to differentiation into muscle fibres.

In the current study, we have provided evidence that morphology/shape of the analysed myotubes was influenced by the type of physical exercise. Recently, for treatment of sport-related musculoskeletal conditions, autologous platelet-derived fractions have been used as a source of growth factors, i.e., platelets have been used as therapeutic tools (Anitua & Orive, 2012; Wroblewski et al., 2010). Furthermore, clinical reports that PRP and related blood products do not show a consistent clinical effect are on the rise (Miroshnychenko et al., 2017). There is no standardization, defined concentrations or protocols for preparing platelet-derived products (PPP and platelet rich fibrin) from PRP, which makes the final products prone to variability. Some authors, to avoid inter-individual variation in platelet and leukocyte count, use allogeneic pooled platelets for preparation of PRP to potentially acquire more consistent quality (Lange et al., 2007; Sonker et al., 2015). By performing morphometric analysis, we showed inter-individual variability between myotube parameters within and between the exercise groups. In this type of study, inter-individual variations in morphometric parameters within the groups could be due either to variation in residual platelet and leukocyte content and/or to factors pertaining to each athlete's status, i.e. hydration status, inflammation, circadian rhythms or diet among others (Boswell et al., 2012). Overall, our study provides evidence that PPP could be applied as a suitable treatment adjunct for tissue regeneration in an autologous manner.

MMPs participate in the adaptive modifications, remodelling and maintenance of musculoskeletal tissues induced by physical exercise, but elevated concentrations of MMPs can create a catabolic environment which can compromise healing (Arnoczky et al., 2007; Bramono et al., 2004). Because of the anabolic versus catabolic potential of PRP, we measured the total relative gelatinase activity/response of MMP-2 and -9 in 6 months' stored PPP obtained from the different groups of athletes. We found that relative gelatinase activity of MMP was not influenced by the type of physical exercise, yet total relative MMP activity was negatively correlated with C2C12 myoblast proliferation. There is evidence that gelatinase determination in serum compared to

plasma tends to overestimate enzyme concentrations because of the release from leukocytes and platelets during coagulation (Jung et al., 2001). Conversely, it is not clear whether MMP concentrations in various PRP formulations depend on platelet count, leukocyte concentration, or both. Also, the enzymatic state, active or inactive, of MMPs in PRP has not been determined (Pifer et al., 2014). Pifer et al. (2014) hypothesized that both PRP formulations (low-platelet, low-leukocyte and high-platelet, high-leukocyte PRP) release MMP-2, -3, and -9 for several days, and they concluded that MMP activity is dependent on platelet count, i.e. higher MMP-2, -3, and -9 concentrations in the PRP system was found with higher platelet and leukocyte concentrations. Future studies are recommended to clarify the spectrum of MMP activity in different formulations of PRP, since little is known about their role in PRP (or PPP) and how they affect tissue healing.

## CONCLUSIONS

This study provides the data that platelet poor plasma frozen-stored for six months has preserved biological activity that induces myotube formation *in vitro*. Even more interesting is the fact that PPP obtained from three groups of athletes training in different sports has a distinct effect on myogenic differentiation and morphology/shape of newly formed myotubes. This strongly suggests sport-dependent specific factors should be considered in the future design of these muscle regeneration therapies.

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## Author contribution

All authors contributed equally to this work.

## Conflict of interest disclosure

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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## PLATELET-POOR PLAZMA SPORTISTA KAO POTENCIJALNI INDUKTOR MIOGENE DIFERENCIJACIJE C2C12 MIOBLASTA

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### Kratak sadržaj

*Uvod.* Krvna plazma obogaćena leukocitima, plazma sa niskim sadržajem leukocita i plazma sa niskim sadržajem trombocita (platelet poor plasma; PPP) su produkti krvi koji se koriste za stimulaciju regeneracije mišića. U ovom radu smo ispitivali da li zamrzavana PPP osoba koje se bave različitim tipovima fizičke aktivnosti, usmerava C2C12 myoblaste u pravcu povećane proliferacije, migracije i miogene diferencijacije, i da li utiče na morfologiju/izgled miotuba.

*Materijal i metode.* PPP osoba muškog pola starih 15-19 godina je izolovana iz krvi dizača tegova, fudbalera i profesionalnih igrača folklor, 12 sati nakon treninga. Uzorci PPP su čuvani šest meseci na -20°C. Uticaj PPP na proliferaciju C2C12 ćelija je analiziran MTT testom, na migraciju „scratch” testom, a uticaj na miogenu diferencijaciju je analiziran na osnovu sposobnosti PPP da indukuju formiranje miotuba. Želatinolitička aktivnost PPP je analizirana gel-zimografijom.

*Rezultati i zaključak.* Uzorci PPP su indukovali proliferaciju i migraciju C2C12 ćelija, a kapacitet da stimulišu proliferaciju je bio: dizači tegova > igrači > fudbaleri. Kapacitet PPP da utiču na migraciju C2C12 ćelija je bio: dizači tegova = igrači > fudbaleri. Svi uzorci PPP su indukovali formiranje miotuba, ali su zapažene značajne interindividuelne varijacije. PPP dizača tegova su indukovali formiranje okruglih miotuba, dok su miotube formirane u prisustvu PPP igrača i fudbalera bile izdužene. Površina miotuba se, zavisno od tipa fizičke aktivnosti, menjala po sledećem rasporedu: fudbaleri > igrači > dizači tegova. Želatinolitička aktivnost PPP je negativno korelirala sa proliferacijom C2C12 ćelija. Rezultati ove studije pokazuju da PPP osoba koje se bave određenim tipom fizičke aktivnosti mogu da na specifičan način modulišu morfologiju/funkciju mioblasta. Ovaj rezultat je od značaja za objašnjenje fiziološkog odgovora i adaptacije na vežbanje. On pokazuje i da PPP nakon dugotrajnog zamrzavanja imaju očuvanu sposobnost modifikovanja morfologije i funkcije mioblasta.

**Ključne reči:** C2C12 mioblasti, fizičko vežbanje, miogena diferencijacija, morfologija miotuba