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From Cell Biology to Tissue Engineering

PRP and MSCs on tenocytes artificial wound healing: an *in vitro* study comparing fresh and frozen PRP

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Summary. Tendon tissue has poor regenerative capacity due to its low vascularization, cell density and extracellular matrix (ECM) production. Therefore, tendon injuries are an increasing clinical problem because of the formation of scar tissue with traditional therapies. Regenerative medicine aims at triggering a healing response through the use of biological treatments such as mesenchymal stromal cells (MSCs) and growth factors (GFs). MSCs show several advantages in tendon clinical setting, while platelet rich plasma (PRP) has gained popularity because of its high GF concentration, although its applications in the tendon clinical setting are still controversial.

The aim of the present study was to evaluate a combined treatment of MSCs and PRP in an *in vitro* microwound model of tendon injuries. In addition, fresh and frozen PRP were compared.

Single human tenocytes cultures or co-cultures with bone marrow derived MSCs (BMSCs) were set up with or without human PRP, fresh or frozen.

After 24 hours of culture, it was observed that MSCs alone significantly increased tenocyte migration speed, microwound healing rate, fibronectin, collagen I and aggrecan production. These effects were enhanced by the combination with PRP, fresh being more effective than frozen PRP. In addition, the number of MSCs and tenocytes inside the microwound was significantly increased, especially with fresh PRP.

In conclusion, the combination of MSCs and PRP, especially the fresh one, increases tenocytes and MSC migration speed, as well as ECM protein production compared to the use of MSCs alone.

Key words: Tenocytes, Mesenchymal stem cells, Tendon injury, Tendon healing, Platelet rich plasma

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Introduction

Tendon injuries are increasing in both young and aged active populations, and their treatment is challenging because of the poor ability of tendon tissue to regenerate itself. Tendons low vascularization, cell density and extracellular matrix (ECM) production (Veronesi et al., 2015a) are known as the main causes of its scarce regenerative potential. For these reasons, in spontaneous tendon healing it is common to observe the formation of a scar tissue with low collagen (COLL) content and consequent poor mechanical properties, with the consequent high possibility of reinjury (Veronesi et al., 2015b).

Available treatments, either conservative or surgical, often fail to provide satisfactory results (Bagnaninchi et al., 2007; Docheva et al., 2015). Regenerative medicine, through the use of biological treatments, such as mesenchymal stromal cells (MSCs) and growth factors (GFs), aim at reversing the degenerative process induced by tissue injuries triggering a healing response (Salamanna et al., 2015; Kobolak et al., 2016).

MSCs, directly injected into the lesion site or seeded onto a scaffold, show several advantages over the use of

mature tenocytes or fibroblasts because of their differentiation ability, low immunogenicity and trophic effect due to the release of several GFs (Veronesi et al., 2011). Several clinical studies on the use of MSCs from placenta and bone marrow for Achilles tendinopathy, rotator cuff acute injuries, chronic patellar or elbow tendinopathies and lateral epicondylitis have recently been published (Veronesi et al., 2017). Some ongoing clinical trials employ autologous bone marrow mesenchymal stromal cells (BMSCs) or allogenic MSCs from adipose tissue for the treatment of chronic rotator cuff lesions, Achilles tendinopathy and lateral epicondylitis. Several different MSC sources are currently used *in vivo*, even if the most common are still bone marrow and adipose tissue, and all *in vivo* studies showed promising findings after the use of BMSCs (Veronesi et al., 2017).

In literature, some *in vitro* studies showed tenocytes in co-culture models with other differentiated cells, such as mononuclear cells (Stolk et al., 2017), mast cells (Behzad et al., 2013), leukocytes (Al-Sadi et al., 2012) or with MSCs, such as adipose-derived MSCs (ADSCs) (Kraus et al., 2013; Yu et al., 2016), BMSCs (Schneider et al., 2011; Ekwueme et al., 2016) or amniotic epithelial cells (AECs) (Barboni et al., 2012). Tenocytes have been known to increase the release of pro-inflammatory cytokines, Cyclooxygenase-2 (COX-2), Prostaglandin E2 (PGE2), Metalloproteinases 1 and 7 (MMP1, MMP7) and decrease COLL I when co-cultured with mononuclear cells, mast cells or leukocytes (Al-Sadi et al., 2012; Behzad et al., 2013; Stolk et al., 2017). In addition, tenocytes influenced tenogenic differentiation of ADSCs and AECs (Barboni et al., 2012; Kraus et al., 2013; Yu et al., 2016) while ADSCs increased tenocyte proliferation (Kraus et al., 2013) and BMSCs increased tenocyte metabolic activity (Ekwueme et al., 2016).

However, the use of different biological products, able to enhance cell therapies by supplying GFs, has been recently advocated. Among the emerging options to provide a regenerative stimulus in a tissue characterized by a poor healing capacity such as tendon, platelet-rich plasma (PRP) injection is currently the most exploited strategy in the clinical practice (Filardo et al., 2013). Platelet (PLT) concentration is nearly 2 to 5 fold higher than in whole blood in several PRP formulations, which allows to concentrate all GFs included in the platelet α-granules (Masuki et al., 2016). The increasing interest gained by PRP relies on the high concentration of these autologous GFs, which can be administered in a minimally invasive way and at low cost. The amount of PRP content is related to the characteristics of the patient and the procedures used.

Among GFs found in PRP are Platelet-Derived Growth Factor-BB (PDGF-BB), Transforming Growth Factor-β1 (TGF-β1), Epidermal Growth Factor (EGF), Insulin-Like Growth Factor (IGF), Vascular Endothelial Growth Factor (VEGF), Basic Fibroblastic Growth Factor (bFGF) and Hepatocyte Growth Factor (HGF). These GFs are able to increase neovascularization, cell differentiation and proliferation and to stimulate the production of ECM components (Masuki et al., 2016). Preclinical studies in animal models confirmed the potential of this biological treatment, with an increased tendon callus strength and stiffness after PRP application (Aspenberg and Virchenko, 2004; Virchenko and Aspenberg, 2006). However, despite the promising preclinical results, literature findings on the clinical application of PRP for tendinopathies are controversial (Filardo et al., 2018). In some studies no or negative effects on tendon healing were observed. For example, in some clinical trials, no or worse healing rates were obtained with the addition of platelet rich fibrin matrix (PRFM) in comparison to empty defects in the ambit of rotator cuff tears (Abtahi et al., 2015). PRP also seems to exert positive effects in some tendons, but not in others (Filardo et al., 2013; Patruno and Martinello, 2014) and the role of single PRP growth factor is not completely revealed (Patruno and Martinello, 2014). This may be explained by the heterogeneity of the procedures used, differing in terms of formulation and application modality, such as number and timing of injections and activation (Tschon et al., 2011; Cavallo et al., 2016; Filardo et al., 2018). Among these variables, some studies report the storage of frozen PRP units for multiple injection cycles, which may affect the properties and effects of PRP concentrates (Filardo et al., 2013; Roffi et al., 2014).

Some *in vitro* studies showed that the addition of PRP alone, in different formulations and concentrations, stimulated cell proliferation, metabolism and migration of ovine tenocytes (Kelly et al., 2016) and matrix production of human or rat tenocytes (de Mos et al., 2008; Tohidnezhad et al., 2011; Jo et al., 2012; Wang et al., 2012; Giusti et al., 2014). PRP also counteracted the inhibition of tenocyte proliferation induced by osteoblast co-cultures (Zhai et al., 2012), ciprofloxacin, dexamethasone (Zargar Baboldashti et al., 2011) or prednisolone (Hilber et al., 2017). Finally, PRP was also tested as an adjuvant to other therapies, such as electric field (Chiu et al., 2017), corticosteroids (Jo et al., 2017), ketorolac tromethamine (Beitzel et al., 2013) or tenocyte culture medium (Rubio-Azpeitia et al., 2017). In another study, tenocytes were also co-cultured with BMSCs in presence of exogenous GFs (IGF-1 and TGF-β1), showing an up-regulation of COLL I, Decorin, Tenomodulin and Scleraxis expression (Schneider et al., 2011). However, until now, no *in vitro* studies evaluated the adjuvant effects of PRP on MSCs in tenocytes co-cultures.

If the available literature offers overall evidence of good *in vitro* potential of both MSCs and PRP, less satisfactory outcomes have been highlighted in a clinical setting. Thus, the aim of this study was to evaluate if MSCs potential could be optimized by the addition of PRP, providing the rational for a combined treatment approach for tendon healing. To this purpose, the regenerative ability of BMSCs was tested in an *in vitro* model of tendon injury (Torricelli et al., 2013), with or

without the presence of PRP. In particular, healing rate was evaluated together with the assessment of tenocytes migration speed and tendon ECM protein production. Moreover, in order to understand if the anabolic and healing potential was hampered by platelets freeze-storing, the effects of both fresh and frozen PRPs were compared in this wound tendon healing model. This provided clinicians with a relevant element for an easier and more efficient use of PRP, which is still not very clear.

Materials and methods

PRP preparation

PRPs were obtained starting from 180 mL blood sample from 3 healthy male volunteers (mean age 36±4 years old), who signed a research donation consent from ethical committee approval. PRPs were prepared with the Pall Celeris system (Pall Medical Corporation) according to the Manufacturer's instructions (Fig. 1). Briefly, peripheral blood was loaded into the input bag where 9 mL of DDW (Double Distilled Water) had previously been injected. Then, the blood sample was passed through a filter by gravity until the filter had no remaining blood. During filtration, platelets were trapped and concentrated in the filter, while plasma, red blood cells and part of the nuclear cells were released. Enriched platelets were recovered by back-flushing the filter with 8 mL of 2% sterile saline and collected in the cell collection bag (Fig. 1).

Samples of each PRP were analyzed with an automated blood cell counter (COULTER LH 750 Haematology Analyzer) in order to determine the number of platelet and nucleated cells (linearity was 5-1000x10^3/µl for platelet count and 0.1-100x10^3/µl for white blood cell count). Each sample was successively divided in three aliquots, one was used immediately for cell culture experiments (fresh PRP), one was frozen at -80°C and thawed before use (frozen PRP) and the other was utilized for GFs evaluations. Both fresh and frozen PRP were activated with 10% $CaCl_2$ before being used.

GFs and cytokines evaluation in PRP

PRP was evaluated for the content of interleukin (IL)-1β, IL4, IL6, IL8, IL10, EGF, VEGF, TGF-β1, PDGF-BB, HGF, IGF-1 and FGF-b. First, PRP was activated with 10% $CaCl_2$ (final concentration: 22.8 mM), incubated at 37°C for 1 hour or 24 hours and centrifuged at 2800xg for 15 minutes at 20°C. Supernatants were collected and immediately frozen at -30°C until GF and cytokine evaluation. Samples were assayed in duplicate by using a commercially available immunoassay kit (Bio-Rad Laboratories, Hercules, California) and quantified by Bio-Plex Protein Array System (Bio-Rad). Standard levels between 70% and 130% of the expected values were considered accurate.

Experimental design

Human primary tenocytes (Tebu-Bio Srl, Magenta, Milan, Italy) were obtained from a Caucasian male 61 years old and were used at the 4th passage. Human

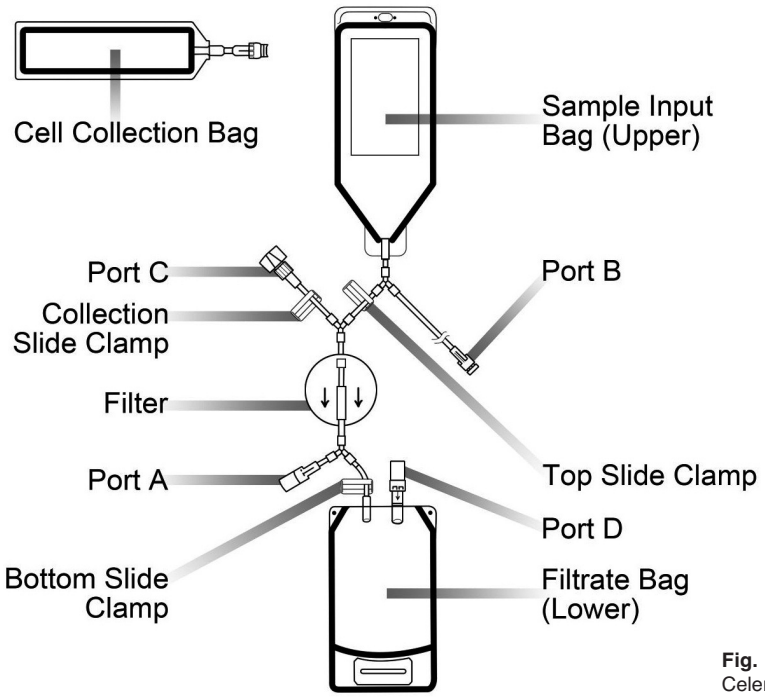

Fig. 1. Schematic representation of PRPs preparation with the Pall Celeris system.

primary BMSCs (Lonza, Walkersville, MD, USA) were also used at the 4th passage. Tenocytes were cultured and expanded in a growth medium composed of a 1:1 mixture of HAM's F12 (Sigma Aldrich, St. Louis, Mo, USA) and Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich), with 25 μg/mL ascorbic acid (Sigma Aldrich). hMSCs were cultured and expanded in DMEM (Sigma Aldrich). Both culture media were supplemented with 10% Fetal Bovine Serum (FBS; Lonza), 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO, Invitrogen), and cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. Before setting the cultures, both cell types were labeled with fluorescent markers. 25 μM Red CellTracker™ CMTPX (Molecular Probes, Life Technologies, Eugene, Oregon, USA) in DMSO was used to label MSCs and 25 μM Green CellTracker™ CMFDA (Molecular Probes) in DMSO to label tenocytes. After the incubation at 37°C for 45', cells were washed with PBS and fresh medium was added. One day after labeling, each cell type was seeded in 24-well multiplate at the following density: $1x10^4$ cells/cm^2 tenocytes in single cultures and $5x10^3$ cells/cm^2 tenocytes and MSCs in the direct co-cultures:

- Culture of tenocytes in monolayer (TEN) (Control);
- Direct co-cultures of tenocytes and hMSCs (TEN+MSC);
- Direct co-cultures of tenocytes and hMSCs with fresh PRP [TEN+MSC (fresh PRP)];
- Direct co-cultures of tenocytes and hMSCs with frozen PRP [TEN+MSC (frozen PRP)].

In the TEN Group the medium was composed of 100% tenocyte growth medium, while in the other group (TEN + MSC) the medium was composed of 50% tenocyte medium and 50% MSCs medium.

In the PRP groups [TEN+MSC (fresh PRP) and TEN+MSC (frozen PRP)], 10% (vol/vol) of PRP, previously activated with 10% $CaCl_2$ (22.8 mM final concentration) to produce a platelet gel, was added in 24-well 0.4-μm pore size transwells (COSTAR, Corning Incorporated, ME, USA), after having set up the microwound (Fig. 2).

All groups were maintained for up to 24 hours at 37°C in a 5% CO_2 humidified atmosphere and all experiments were performed in triplicate.

Microwound-healing model

For all experimental groups, the *in vitro* microwound healing model was performed as previously described (Maffulli et al., 2000; Torricelli et al., 2013) (Fig. 2). Briefly, once the tenocytes alone or co-cultured with hMSCs had reached confluence in a 24-well plate, a sterile micropipette tip was used to produce an artificial 1000 μm wide wound by scraping the cell layer (time zero: T0). The medium was changed, the PRP-containing transwells were transferred to the respective wells and the evaluations were performed after 4 (T4), 8 (T8) and 24 (T24) hours of incubation in standard conditions.

At different experimental times, the microwound healing potential was evaluated with an inverted microscope (NIKON Eclipse Ti-U) equipped with a digital camera, comparing the images captured at different time points with the images captured at T0. Three images per well were analyzed at 10x magnification to measure the whole length of the wound. Image analysis was performed with the use of the Nis Elements AR4 software (Nikon) and the width of the artificial wounds was measured by a blinded investigator (10 measures per image).

The healing rate was calculated according to the

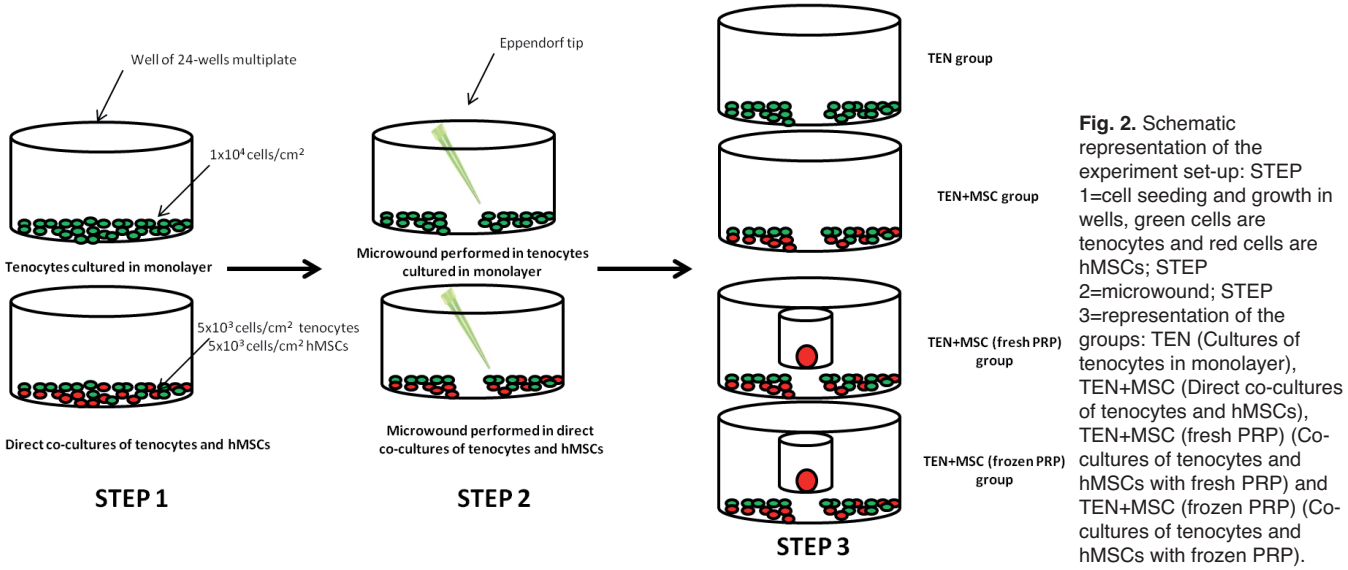

Fig. 2. Schematic representation of the experiment set-up: STEP 1=cell seeding and growth in wells, green cells are tenocytes and red cells are hMSCs; STEP 2=microwound; STEP 3=representation of the groups: TEN (Cultures of tenocytes in monolayer), TEN+MSC (Direct co-cultures of tenocytes and hMSCs), TEN+MSC (fresh PRP) (Co-cultures of tenocytes and hMSCs with fresh PRP) and TEN+MSC (frozen PRP) (Co-cultures of tenocytes and hMSCs with frozen PRP).

formula: [(T0 width- final time width)x100] / T0 width (Torricelli et al., 2013). In addition, to improve the quality and the reliability of the measure, the parameter was also evaluated with AIM software tool, which automatically quantifies cell-free regions in scratch wound healing assays (Cortesi et al., 2017). Using http://www.mcbeng.it/en/category/software.html website, the microwound healing rate was evaluated as (area at T4/T8/T24)/time lapse.

Cell migration speed was not evaluated by a time-lapse automated system, but it was calculated by dividing the average width values between different time points by time-point hours (Torricelli et al., 2013). Furthermore, the number of tenocytes and MSCs was evaluated inside the microwound at each experimental time in the co-culture systems. More precisely, by using the inverted microscope and the image analysis software, the precise number of the different cell types has been obtained, counting the number of red cells (MSCs) and the green ones (tenocytes). This cell count was performed inside the distance of 1000 μm at each experimental time.

Tenocyte synthetic activity

The supernatants were collected, centrifuged to remove cellular debris, if any, and maintained at -80°C until evaluation. The levels of collagen type I (COLL I), fibronectin (FBN) and aggrecan (AGG) were quantified by immunoenzymatic assay (Cloud, USC Life Science, Wuhan, China), following the manufacturer's instructions. Tenocyte synthetic activity was evaluated at T0 and T24.

Statistical analysis

The statistical evaluation of data was performed with the use of the software package SPSS/PC+Statistics TM 23 (SPSS Inc., Chicago, IL, USA). The study is the result of three independent experiments, and data are reported as mean±standard deviation at a significance level of $p<0.05$. After verifying the normal distribution of data and the homogeneity of variance, the general linear model (GLM) for repeated measure (within-subject factor: 'experimental time'; between-subject factor: 'groups') was used to identify significant differences for the results of tenocyte migration speed and microwound rate. To analyze the counts of MSC and tenocyte directly co-cultured inside microwound, a GLM for repeated measure (within-subject factor: 'experimental time'; between-subject factor: 'PRP (3 levels: No PRP; Fresh PRP and Frozen PRP)') was used for each cell type. One-way ANOVA was used to assess significant differences between groups for ELISA results. Adjusted p-value Sidak test was used for pairwise comparisons. In particular, the following comparisons were taken into account within each 'experimental time': (1) TEN+MSC versus TEN; (2) TEN+MSC (Fresh PRP) versus TEN+MSC; (3) TEN+MSC (Frozen PRP) versus TEN+MSC (Fresh PRP); within each 'group': (a) 8 hours versus 4 hours; (b) 24 hours versus 8 hours.

Results

PRP characterization

PLT concentration in PRP had a mean value of 487.700/μl that was about 2.2-fold higher than in whole blood (mean value of 204.300/μl). White blood cells (WBCs) had a mean value of 23.600/μL, about 3.6-fold higher than whole blood (mean value of 6.700/μL). Further characterization of each PRP sample in terms of PLT and WBCs number, GFs and cytokine production is summarized in Tables 1, 2 and in Fig. 3.

Tenocyte migration speed

Results showed that at T4 and T8 TEN+MSC group

Table 1. Soluble factor concentrations in PRP 1 hour and 24 hours after activation.

FACTORS	1 hr	24 hrs
PDGF-BB	8244.2 (3694.13-11185.8)	13691 (7537.67-21419.5)
FGF-b	34.74 (16.85-60.58)	95.73 (89.2-102.77)
VEGF	7038.7 (77.6-11186)	11291.1 (336.65-21419.5)
HGF	262.24 (248.59-271.47)	727.27 (509.36-894.03)
EGF	865.97 (670.91-1089.67)	1208.42 (895.25-1559.87)
TGF-β1	108838.89 (30972.47-209618.5)	99966.08 (26716.53-239931.1)
IGF-1	29393.3 (25340-33720)	20853.3 (14350-24250)
IL-1β	1.13 (0.62-2.00)	13.69 (0.89-31.95)
IL-4	1.57 (0.08-4.23)	1.73 (0.50-3.93)
IL-6	2.95 (2.56-3.54)	30.96 (2.97-74.1)
IL-8	93.23 (68.05-126.50)	42655.5 (67781.89-30798.1)
IL-10	3.08 (1.97-5.17)	5.04 (3.41-7.01)

Concentrations are expressed as pg/mL and reported as mean values (Minimum-Maximum) of the 3 healthy volunteers.

Table 2. Number of platelets (PLTs) and white blood cells (WBCs) ($10^3/\mu$l).

Products	Donor sex	Donor age	PLTs, $10^3/\mu$l	WBCs, $10^3/\mu$l	N-fold change in PLT concentration in PRP (Mean±SD)	N-fold change in WBC concentration in PRP (Mean±SD)
Whole blood (Mean±S.D.)	3 young male	34±4 years old	204.30±50.93	6.73±1.36	2.2±1.7	3.6±0.9
PRP (Mean±S.D.)	3 young male	34±4 years old	487.67±378.81	23.6±3.32		

with fresh PRP increased tenocyte migration speed significantly more than TEN+MSC group (***, p<0.0005). In addition, at T8, TEN+MSC with fresh PRP increased the value significantly more than TEN+MSC with frozen PRP (***, p<0.0005) (Fig. 4a). Regarding experimental times, TEN+MSC with fresh and frozen PRP significantly decreased tenocyte migration speed at T8 compared to T4 (a, p<0.0005) and all groups significantly decreased tenocyte migration speed between T8 and T24 (b, p<0.0005) (Fig. 4a).

Tenocyte microwound healing rate

With both methods employed, as previously described in the "Materials and methods" section, at T8 and T24 tenocyte microwound healing rate was significantly higher in tenocytes with MSCs in comparison to tenocytes alone (***, p<0.0005). At all experimental times, TEN+MSC with fresh PRP increased significantly more than TEN+MSC (***, p<0.0005) and, at T8 and T24, TEN+MSC with fresh PRP also significantly increased tenocyte microwound healing rate more than TEN+MSC with frozen PRP (***, p<0.0005) (Fig. 4b). All groups significantly increased this value between T4 and T8 (a, p<0.0005) and between T8 and T24 (b, p<0.0005) (Fig. 4b).

MSCs and tenocytes morphology and number inside the microwound

The images obtained after double fluorescent labeling (red and green for mesenchymal cells and tenocytes, respectively), allowed to appreciate the number and the movement of the different cell types inside the artificial microwound, in addition to cell morphology. In particular, some peculiarities emerged from the observation of each captured image: the morphology of tenocytes alone was mainly elongated and a minor fraction was more compact and small. Over time elongated cells increased. In "TEN+MSCs" and "TEN+MSCs+PRP" conditions the morphology of tenocytes was confirmed to be typically elongated at T0, while MSCs were polygonal; at the subsequent times tenocytes became smaller, with dimensions similar to mesenchymal cells, and with an increasing number of almost triangular cells. Fig. 6 shows some significant examples of cultures at the beginning and at the end of experimental times.

At T4, MSC (a, p<0.0005) and tenocyte (b, p<0.05) number inside the microwound was significantly higher with fresh PRP compared to no PRP or frozen PRP (Fig. 5). At T8 and T24, fresh PRP significantly increased MSC (a, p<0.0005) and tenocyte (c, p<0.0005) number compared to no PRP or frozen PRP (Fig. 5).

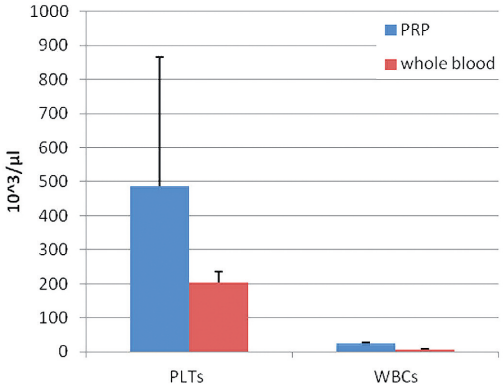

Fig. 3. Number of PLTs and WBCs (103/µl) in PRP (blue histogram) and in whole blood (red histogram).

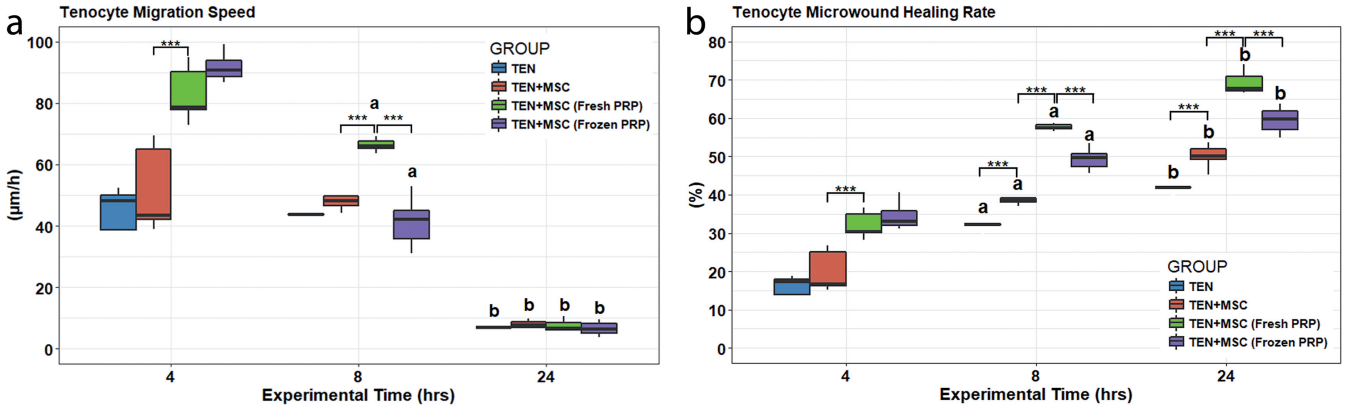

Fig. 4. Boxplots of tenocyte migration speed (µm/h) **(a)** and tenocyte microwound healing rate (%) **(b)** results at 4, 8 and 24 hours (Mean±SD, n=3 duplicates). Adjusted Sidak multiple comparison test: –within each 'experimental time' (***, p<0.0005): TEN+MSC versus TEN; TEN+MSC (Fresh PRP) versus TEN+MSC; TEN+MSC (fresh PRP) versus TEN+MSC (Frozen PRP); –within each 'group': 8 hours versus 4 hours (a, p<0.0005); 24 hours versus 8 hours (b, p<0.0005).

Synthetic activity

Regarding FBN values, TEN+MSC with fresh PRP significantly increased FBN in comparison to TEN+MSC (***, p<0.0005) and in comparison to TEN+MSC with frozen PRP (*, p<0.05) (Fig. 7a). COLL I was significantly higher in TEN+MSC than in TEN group, in TEN+MSC with fresh PRP in comparison to TEN+MSC and to MSC+TEN with frozen PRP (***, p<0.0005) (Fig. 7b). AGG was significantly higher in TEN+MSC with fresh PRP than in TEN+MSC and TEN+MSC with frozen PRP (***, p<0.0005) (Fig. 7c).

Discussion

The main finding of this study is that PRP has an adjuvant effect on MSCs in improving tenocytes responses, suggesting a faster microwound healing compared to the use of MSCs alone in an *in vitro* model of tendon injury.

The *in vitro* scratch model, employed in the present study, aims at mimicking in a simplified way the *in vivo* situation and it is usually employed to evaluate cell migration ability in several culture conditions to partially simulate what happens in an injured tissue (Maffulli et al., 2000; Torricelli et al., 2013; Giusti et al., 2014). As described by Sharma el al., in an *in vitro* tendon healing model, beside tenocytes metabolism, it is important to evaluate also their migration ability inside the wound (Sharma and Maffulli, 2005).

Data obtained from the model, used in the present study, documented several positive effects of MSCs. The addition of MSCs, harvested from bone marrow, increased tenocytes microwound healing rate and the production of COLL I protein. These results confirmed the findings of a previous *in vitro* study showing that COLL I production increased in tenocytes co-cultured with BMSCs at different ratio (90/10, 70/30, 50/50), and after 14 days of culture only in co-cultures 70/30 and 50/50 (Schneider et al., 2011). In addition, two of our previous *in vitro* studies showed an increase in microwound healing and COLL I expression in rat tenocytes indirectly co-cultured with rat ADSCs (Veronesi et al., 2015b) also when ADSCs were harvested from osteopenic rats even if to a lesser extent than those harvested from healthy rats (Veronesi et al., 2015a).

A recent review found 90 studies that evaluated the effects of MSCs on tendon healing, with a prevalence of BMSCs over other sources, and documented an enhancement of regeneration after MSC employment *in vivo* and in clinical studies (Veronesi et al., 2017).

The results of the present research also underlined another important aspect on the use of MSCs for tendon healing, that is the benefit of PRP combination. An adjuvant effect of PRP on MSCs was found for all the parameters evaluated.

Even if after 24 hours of culture it was not possible to observe a complete healing, with or without PRP, the percentage of microwound healing and tenocyte migration speed was significantly higher in presence of PRP compared to MSCs alone. Similar results were also found by Giusti et al. that analyzed the effects of PRP with three different platelet concentrations ($0.5x10^6$, $1x10^6$ and $5x10^6$ plt/μl) in the same *in vitro* model of microwound used in our study. They showed that microwound healing was achieved with lower platelet concentration (Giusti et al., 2014).

Concurrently, with the increase of the healing percentage over time, we observed a reduction in the cell migration speed as well. However, this inverse correlation between cell migration speed and healing

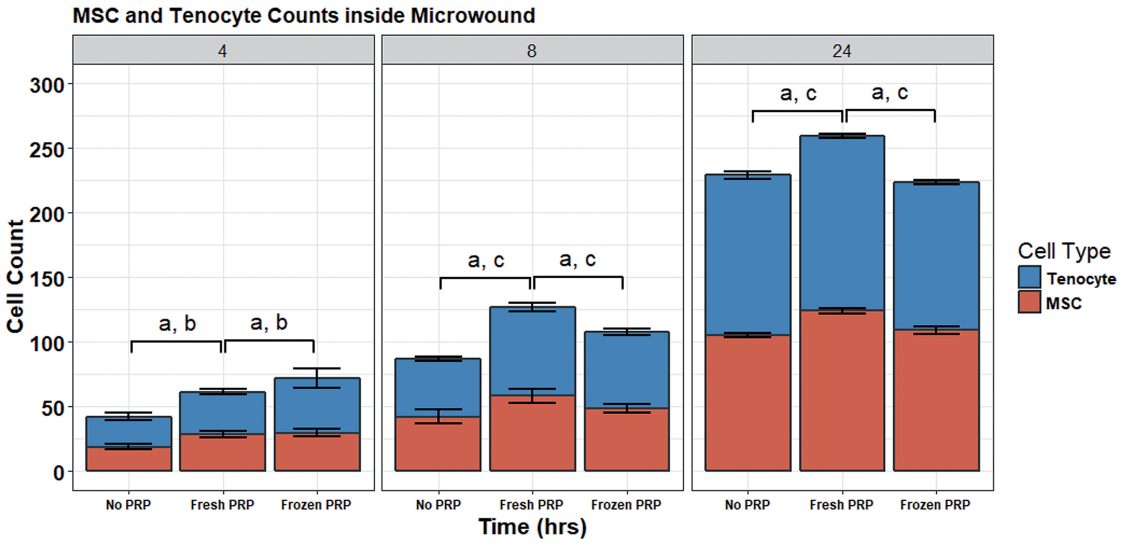

Fig. 5. Stacked histograms of count results of directly co-cultured tenocytes and MSCs inside the microwound at 4, 8 and 24 hours (Mean±SD, n=3 duplicates). Adjusted Sidak multiple comparison test between Fresh PRP versus No PRP and between Fresh PRP versus Frozen PRP within each 'experimental time' for MSCs (a, p<0.0005) and tenocytes (b, p<0.05; c, p<0.0005). Basal count values were: Tenocytes 11±1; MSCs 14±1.

stages was not related to treatment and may be related to the model itself, as previously observed by other authors in a similar model (Sharma and Maffulli, 2005; Gross and Hoffmann, 2013; Juneja and Veillette, 2013; Torricelli et al., 2013).

The analysis of the migration effect in this co-culture system unraveled interesting aspects of tenocyte behavior in a microwound healing model. It was observed that PRP increased the number of both MSCs and tenocytes during microwound healing. In this light, the further benefit of PRP and the interaction observed with MSCs may highlight two aspects: on one hand they explain a further mechanism of PRP effect on tendon healing due to its migration properties, able to recruit and guide MSCs to the healing site, and on the other hand they guide and stimulate tenocytes. Kajikawa also demonstrated that the *in vivo* use of PRP had an effect on migration of other cell types, such as circulating cells (macrophages and fibroblasts) (Kajikawa et al., 2008). In addition, in the co-culture model, MSCs did not differentiate into osteogenic lineage, as evaluated with Alkaline Phosphatase (ALP) ELISA test (data not shown), because tenogenic medium drove and supported the correct maintenance of the tenogenic microenvironment as already observed in our previously published *in vitro* studies (Veronesi et al., 2015a,b).

Besides overall healing, further analysis focused on the production by tenocytes of the three main components of tendon ECM: COLL I, FBN, and AGG. The addition of PRP favored an even higher production of these components compared to the one obtained with the use of MSCs alone in tenocytes culture, confirming the anabolic and anticatabolic properties of PRP documented in literature (Sharma and Maffulli, 2005; Morizaki et al., 2010; Wang et al., 2012; Filardo et al., 2013; Yu et al., 2015; Cavallo et al., 2016). Collagen I is produced by tenocytes and is the most abundant structural component of tendon ECM. About 60-85% of

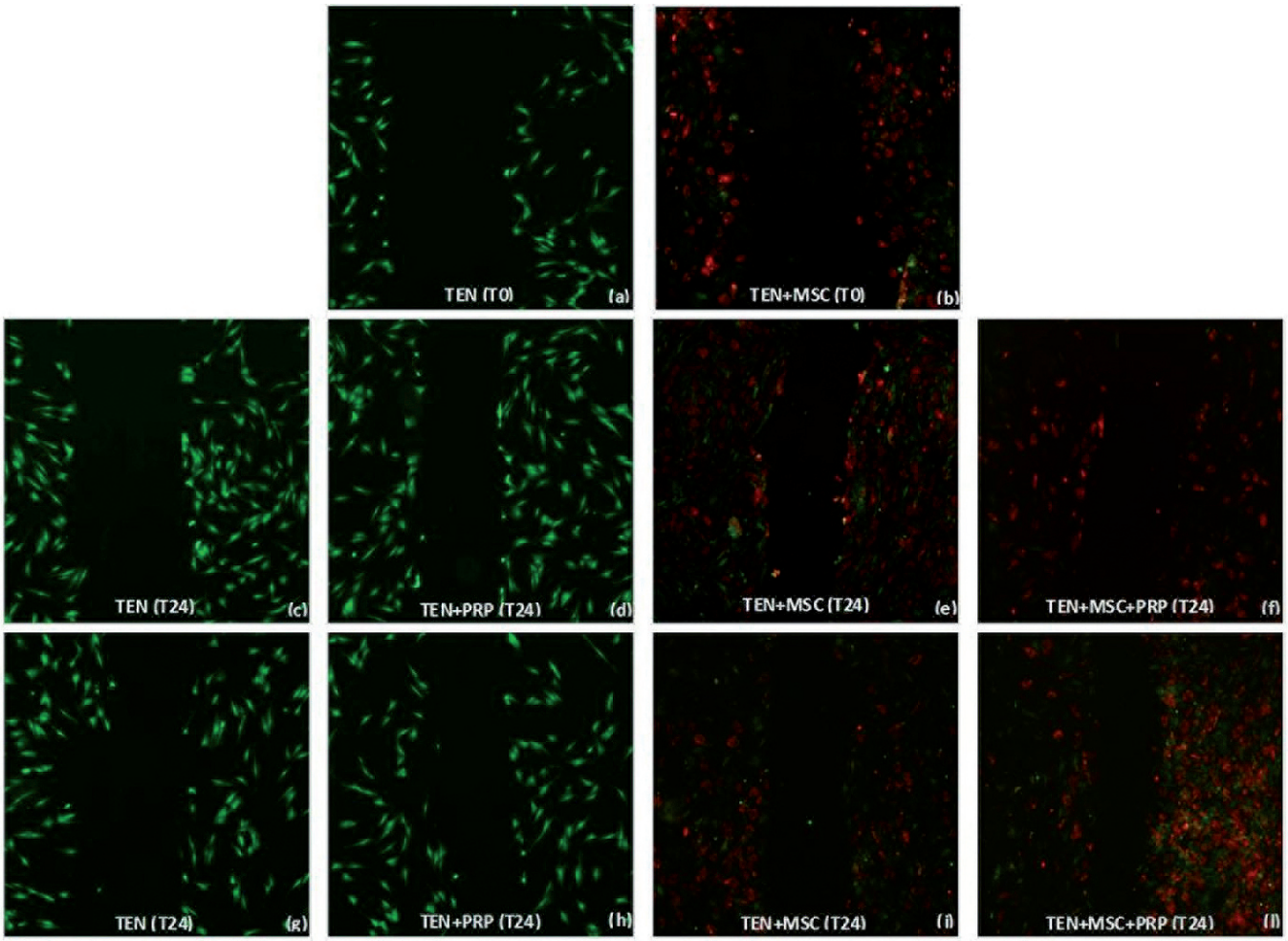

Fig. 6. Microscopic images of the microwound at T0 **(a, b)** and T24 **(c-l)**. TEN group **(a, c, d, g, h)** and TEN+MSC group **(b, e, f, i, l)**. Groups treated with PRP **(d, f, h, l)**: Frozen PRP **(d, f)** and Fresh PRP **(h, l)**. Green cells are tenocytes and red ones are MSCs. x 4.

tendon tissue dry mass is composed by collagens, 95% of which is collagen I (Nourissat et al., 2015; Leblanc et al., 2017). In degenerative tendon diseases, COLL I is abnormally low.

FBN has a multidomain structure and it is important in matrix assembly of integrins, collagen and proteoglycans with tenocytes (Halper and Kjaer, 2014).

It resists compressive and shear forces, facilitates ''sliding'' of collagen fiber bundles; it is found at increased levels in fibrocartilaginous region and it undergoes rapid, constitutive, aggrecanase-mediated turnover with ECM retention of resulting catabolites. Aggrecan is a proteoglycan implicated in tendon biomechanics because it favors the sliding of adjacent collagen fibers, resists compressive and shear forces and it is implicated in tendon matrix turnover (Rees et al., 2009).

However, as also observed in a previous ex vivo study, the presence of FBN fragments in cell supernatant should be an indicator of ECM degeneration and MMP activity increase, affecting its microwound healing ability (Dudhia et al., 2007). This is probably one of the reasons why in the present study a complete microwound closure was not observed within 24 hours.

A previous *in vitro* study already showed better biomechanical properties and cell viability of animal tendon explants with a collagen patch seeded with both PRP and BMSCs, compared to the same scaffold seeded with BMSCs alone (Morizaki et al., 2010), but the use of a scaffold was only appropriate as a model for a surgical application.

The present study also underlined an important methodological aspect for PRP use. In order to simplify multiple applications in the clinical practice, PRP has to be frozen (Kasjikawa et al., 2008), but the effects on PRP potential of this storage procedure have not been previously explored. Although many authors have focused on the best PRP formulation, in terms of red blood cells, leukocytes and GF concentration, on the best technique for PRP concentration and on the best dosage to be administered (Salamanna et al., 2015), no other previous study has compared fresh and frozen PRP in the same study for tenocytes metabolism, and in a tendon healing context with both tenocytes and MSCs. Only one recent *in vitro* study evaluated chondrocytes and synoviocytes metabolism in presence of fresh or frozen PRP. The authors did not find significant differences between the two PRPs in terms of the production of ECM cartilage, pro-inflammatory cytokines, anti-inflammatory and anticatabolic factors and of hyaluronan secretion in both chondrocytes and synoviocytes (Roffi et al., 2014). Even if the results of this study underlined that cryopreservation is a safe procedure, which maintains PRP ability to induce cell proliferation and ECM production in these knee joint cells (Roffi et al., 2014), the effects on tenocytes remains to be determined. Our data showed that fresh PRP increased cell migration speed, tenocyte and MSC migration, FBN, COLL I and AGG production and, in general, induced an overall higher healing rate than frozen PRP. Thus, while the actual meaning of these findings in the *in vivo* setting remains to be determined,

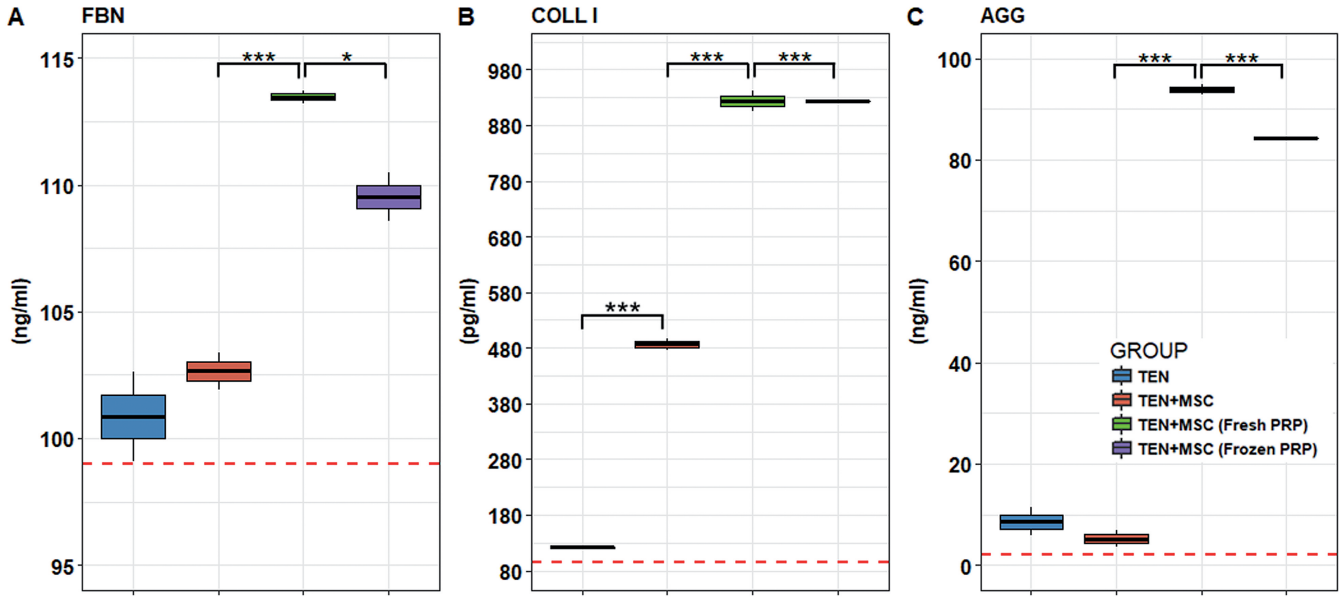

Fig. 7. Boxplots of ELISA results of Fibronectin (FBN) **(A)**, Collagen I (COLL I) **(B)** and Aggrecan (AGG) **(C)** at 24 hours (Mean±SD, n=3 duplicates). Red dashed lines represent culture basal values. Adjusted Sidak multiple comparison test (***, p<0.0005): TEN+MSC versus TEN; TEN+MSC (Fresh PRP) versus TEN+MSC; TEN+MSC (Fresh PRP) versus TEN+MSC (Frozen PRP).

these results suggest the possible benefits of applying fresh PRP in a clinical setting to take advantage of its healing potential for the treatment of tendon injuries.

The study presents some limitations, for example it only used healthy tenocytes in a healthy micro-environment and the microwound evaluations were performed only for up to 24 hours. Therefore, further studies, using pathological human tenocytes in an *in vitro* inflammatory model, for a longer experimental period, will be useful to further understand the extent of these findings in a model better representing the clinical scenery. In addition, the use of only one patient-derived tenocyte or one BMSC cell line limits our results because of the absence of physiological variability in terms of cell response to treatments. However, referring to the novelty of the study (comparison between fresh and frozen PRP in combination with MSCs), this study is considered preliminary. In future studies it will be necessary to increase MSCs lots and patient-derived tenocytes.

To summarize, even if our findings have to be further explored in preclinical *in vivo* studies, the use of BMSCs could be useful to treat tendon lesions. Moreover, the combination with PRP may further increase tenocytes and BMSCs migration speed and ECM production, leading to better results in terms of wound healing and extending the indications of this combined treatment, even for recalcitrant tendinopathy (Randelli et al., 2014). The additive effects of PRP on BMSCs are better exploited by using fresh PRP.

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