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Platelet rich plasma-derived microvesicles increased *in vitro* wound healing

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Abstract. – OBJECTIVE: Platelet rich plasma (PRP) is a haemoderivative used in clinical practice for the treatment of hard-to-heal wounds. Platelet (PLT) activation is a key factor in the wound healing process leading to the production of extracellular vesicles. We obtained PRP and PRP-derived microvesicles (PLT-MVs) from healthy donors and compared their pro-healing efficacy in an *in vitro* wound model using human keratinocytes.

MATERIALS AND METHODS: We evaluated PLT-MVs' direct effect on an *in vitro* model of wound healing. PRP, PRP activated using calcimycin, and PLT-MVs separated by high speed centrifugation were added to scratched keratinocyte monolayers. Fluorescein diacetate was used in flow cytometry to distinguish PLTs and PLT-MVs from debris, and then, PLT-MVs were quantified on the basis of relative dimensions (Forward Scatter signals).

RESULTS: Wound areas were measured at time 0 and after 24 hours and they were healed by 24.80 ± 4.28% in control conditions, while PRP, activated PRP, and PLT-MVs increased closure of 62.94 ± 0.96%, 52.69 ± 17.20% and 52.76 ± 9.44%, respectively.

CONCLUSIONS: Our results indicate that PRP pro-healing effects were fully replicable by PLT-MVs, suggesting a key role of microvesicles in the healing process and a possible clinical use as an alternative to PRP.

Key Words:
Wound healing, PRP, Microvesicles, Flow cytometry.

Introduction

Haemoderivatives are defined as blood components, such red blood cells, platelets, granulocytes, plasma, clotting factors, etc., used in therapeutic treatments[1]. Platelet-rich plasma (PRP) is the plasma fraction of autologous blood enriched in platelets obtained by centrifugation and used for the treatment of chronic ulcers[2]. Chronic ulcers are spontaneous or traumatic lesions with a delayed or incomplete healing process[3].

Wound healing is a dynamic process, leading to tissue repair through three main steps: inflammation, proliferation and remodelling[4]. The inflammatory phase begins after hemostasis, lasts a few days[5], macrophages and neutrophil granulocytes clean microbial agents and foreign material from the wound bed and they secrete growth factors and cytokines for the following proliferation phase[6]. The proliferative phase is characterized by angiogenesis, reepithelialisation and extracellular matrix deposition[7]. The remodelling phase takes place after about 2 weeks from trauma, lasting for few days/weeks[6], during this period immature type III collagen is replaced by mature type I collagen through a complex signalling network, repairing the previously damaged structures[8].

Different clinical therapies are routinely applied to enhance wound repair, such as recombinant growth factors, factors acting in foetal wound-healing processes[6] and stem cell[7] administration or biological tissue replacement[9]. Recently, the use of Platelet-Rich Plasma (PRP) has become a simple and relevant therapy for the treatment of hard-to-heal wounds[10] even if its effectiveness and possible adverse effects need more extensive investigation along with its action mechanisms[11].

Platelets are able to release microvesicles from their plasma membranes by exocytosis[12] triggered by mechanical and pharmacological stress, hypoxia[13], or intracellular calcium variations, followed by cytoskeleton changes and lipid membrane asymmetry[14].

Platelet-derived microvesicles (PLT-MVs) are the most abundant microvesicles in human blood,

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particularly during inflammation, during which they support the coagulation cascade through their cargo components (functional enzymes, transcription factors, receptors, cytokines, nucleic acids and mitochondria). Moreover, levels of circulatory PLT-MVs result altered during several disease manifestations, such as coagulation disorders, rheumatoid arthritis, systemic lupus erythematosus, cancers, cardiovascular diseases, and infections, pointing to their potential contribution to different diseases[15].

To our knowledge, no studies have focused on the role of PLT-MVs in wound healing. In this study we obtained PRP and PLT-MVs from healthy donors and compared their pro-healing efficacy in an *in vitro* wound model using human keratinocytes.

Materials and Methods

Cell Culture

Human spontaneously immortalized keratinocytes (HaCaT), isolated from human adult skin[16] are rather resistant to transformation *in vitro*. Immortalization has been achieved by SV40 but has resulted in cell lines with altered differentiation. We have established a spontaneously transformed human epithelial cell line from adult skin, which maintains full epidermal differentiation capacity. This HaCaT cell line is obviously immortal (greater than 140 passages were purchased from Cell Line Service GmbH (Eppelheim, Germany). Cells were grown in culture flask (75 cm^2) in Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose levels, supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (all from Immunological Science, Italy) in a humidified atmosphere containing 5% CO_2 at 37°C. HaCaT cells were seeded into a 12-multiwell plate and incubated in a humidified atmosphere containing 5% CO_2 at 37°C until confluence was reached.

PRP Preparation

Blood samples (16 ml) of 8 healthy subjects (2 males and 6 females) aged 20 to 50 years (33.2 ± 12.9 years old) were collected in vacuette centrifuge tubes with green safety cap containing sodium heparin (CGF K1 P50V, Silfradent S.r.l., Santa Sofia, Italy), after informed consent and after receiving the approval of the Ethic Committee (C.E. 61/10).

Blood small volume was used for complete blood count performed at the Immunotransfusion Service, "Maggiore della Carità" Hospital, Novara, and the average platelet concentration was 283.43±7.48 x 10^3 platelet/µL.

PRP has been obtained using the centrifuge MEDIFUGE CGF (Silfradent S.r.l., Santa Sofia, Italy) through a standardized separation PRP protocol performed by this specific centrifuge in an easy and fast way. In fact, after a single standardized centrifugation (PRP cycle) without the addiction of exogenous substances, 3 fractions were visible in each tubes: red blood cells in the lower part, Platelet Poor Plasma (PPP) in the upper part, and Platelet Rich Plasma (PRP) between these two fractions.

PRP underwent to a second centrifugation at 800 g for 10 minutes (Refrigerated Microfuge 1-14K, Sigma-Aldrich, Laborzentrifugen GmbH, Osterode am Harz, Germany) and it was suspended in 1 ml of 0.2 µm filtered Phillips Buffer (NaCl 96.5 mM, Glucose 87.5 mM, EDTA 1.1 mM, Tris Base 8.5 mM, $MgCl_2$ 2 mM, $CaCl_2$ 2 mM, KCl 3 mM. PH 7.4), a solution of Ca^{2+} that can induce platelet activation and degranulation, at a concentration of $4.0x10^6$ platelets/ml.

The platelet pellet was washed and centrifuged a last time to reduce cellular debris, (400 g x 5 minutes) and finally it was suspended in Phillips buffer. PRP samples (5 ml) were qualitatively checked after any separation using a flow cytometer (FACSCALIBUR, BD Bioscience, San Jose, CA, USA).

PRP Activation

Some PRP samples (4.0×10^6 platelets/mL) were stimulated with 5 µM calcium ionophore calcimycin (A-23187, Invitrogen by Thermo Fisher, Waltham, MA, USA) diluted in dimethyl sulfoxide (DMSO) at a final concentration of 0.1% for 5 hours at 37°C in an atmosphere at 5% CO_2 to induce PLT-MVs formation[17].

PLT-MVs Isolation

Microvesicles were isolated by centrifugation[18] (20000 g for 40 minutes, room temperature) after platelets activation using a Refrigerated Microfuge 1-14K centrifuge (SIGMA Laborzentrifugen GmbH, Osterode am Harz, Germany). Microvesicles pellet was suspended in 2 ml of 0.2 µm filtered Phillips Buffer. PLT-MVs presence was assessed in every sample using a flow cytometer (FACSCALIBUR, BD Bioscience, San Jose, CA, USA).

Flow Cytometry Analysis of PRP and PLT-MV

PRP, PRP stimulated with calcimycin and PLT-MVs were loaded with Fluorescein Diacetate (FDA, 1 µg/mL) (Sigma Aldrich, St. Louis, MO, USA), the dark for 30 minutes at 37°C in an atmosphere at 5% CO_2 in order to discriminate them from debris[19]. The apolar FDA compound was internalized and cleaved to polar Fluorescein by a specific esterase present in PLT and PLT-MVs.

After incubation, the samples were analysed by flow cytometry using a FACS CALIBUR cytometer (BD Bioscience, San Jose, CA, USA) equipped with a 15-mW air-cooled argon ion laser operating at 488 nm. A minimum of 10000 events was collected per sample at a low sample flow rate setting of 12 µl ± 3 µL/min for a good resolution and an precise discrimination between the non-fluorescent noise and fluorescent signals[20]. The forward (FSC) and side scatter light (SSC) values were collected using a logarithmic scale to display microvesicles' populations. Fluorescein green fluorescence was measured on the FL1 green channel after a 530 nm BP filter and displayed on a four-decade log scale. Data were collected using the CellQuest software (BD Bioscience, San Jose, CA, USA), and then, analysed using Flowing Software 2.5.2 (University of Turku, Finland).

In Vitro Scratch Assay

Cell migration has been evaluated by *in vitro* scratch assay[21]. HaCaT cells were cultured in a 12-wells plate in complete DMEM medium to reach a confluent monolayer. Cell monolayers were then mechanically scratched with a cut yellow tip (diameter = 2 mm), interrupting their contiguity and simulating a wound, and cell debris were washed away with sterile Phosphate-buffered saline solution (PBS). To fix the initial scratch width, some controls were immediately fixed (T-0) using 3.7% formaldehyde, 3% sucrose solution in PBS. A volume of 1 ml of fresh serum free DMEM was added to other samples untreated or a treated with 100 µl of PRP, PRP activated and PLT-MVs solution and incubated at 37°C in a humidified atmosphere containing 5% CO2 for 24 hours (T-24h).

At the end of the experiment, both control and treated samples were fixed using 3.7% formaldehyde, 3% sucrose solution in PBS, and then, stained with a 1% crystal violet solution. The samples images were recorded by optical microscopy (AE200 inverted microscopes, Motic, Honk Kong, China, equipped with an Eyepiece MD500-CK AmScope Camera, Irvine, CA, USA) with a 4X magnification.

In vitro wound healing response was evaluated determining the percentage of wound closure of stained samples, compared to samples fixed at time T-0. Wound width has been measured and quantified using Image J software (Wayne Rasband, National Institute of Health, Bethesda, MD, USA) and wound closure has been expressed as mean ± SD values.

Statistical Analysis

Statistical analysis was performed by one-way ANOVA test using R software 4.0.1. *p*-values <0.05 were considered statistically significant.

Results

PRP obtained by blood centrifugation was diluted in Phillips buffer and analyzed by flow cytometry after FDA incubation. Physical signals obtained (FSC and SSC) were collected using a logarithmic scale to observe events as small as 100-1000 nm. The heterogeneous population obtained was a mix of PLT, PLT-MVs and debris (Figure 1-A1).

Quantification of PLT and PLT-MVs detected by flow cytometry was optimize using FDA. In the presence of FDA, we could distinguish between non fluorescent events (debris = not able to convert fluorescein-diacetate in fluorescein because their lack of intact membrane and unspecific esterase) and fluorescent events (PLT and PLT-MVs). Using this strategy, we could also discriminate PLT and PLT-MVs based on their relative dimension (FSC signals) and fluorescence (FL1 signals) (Figure 1-A2).

Events presenting Fluorescein green fluorescence positivity (R-1 region) were used to quantify PLT-MVs on the basis of their FSC signals (R-2 region) (Figure 1-A2). In fact, PLT present in control samples were the 45.67 ± 0.72% of events present in R1 regions while PLT-MVs account for the 54.33 ± 0.79% (R2) (*p*-value < 0.0001).

PLTs stimulated by calcimycin (5 µg/ml) showed a shift towards lower FSC and SSC values (Figure 1 B1) suggesting the activation of PLT-MVs shedding. In fact, the PLT population in R1 region decreased from 45.67 ± 0.72% to

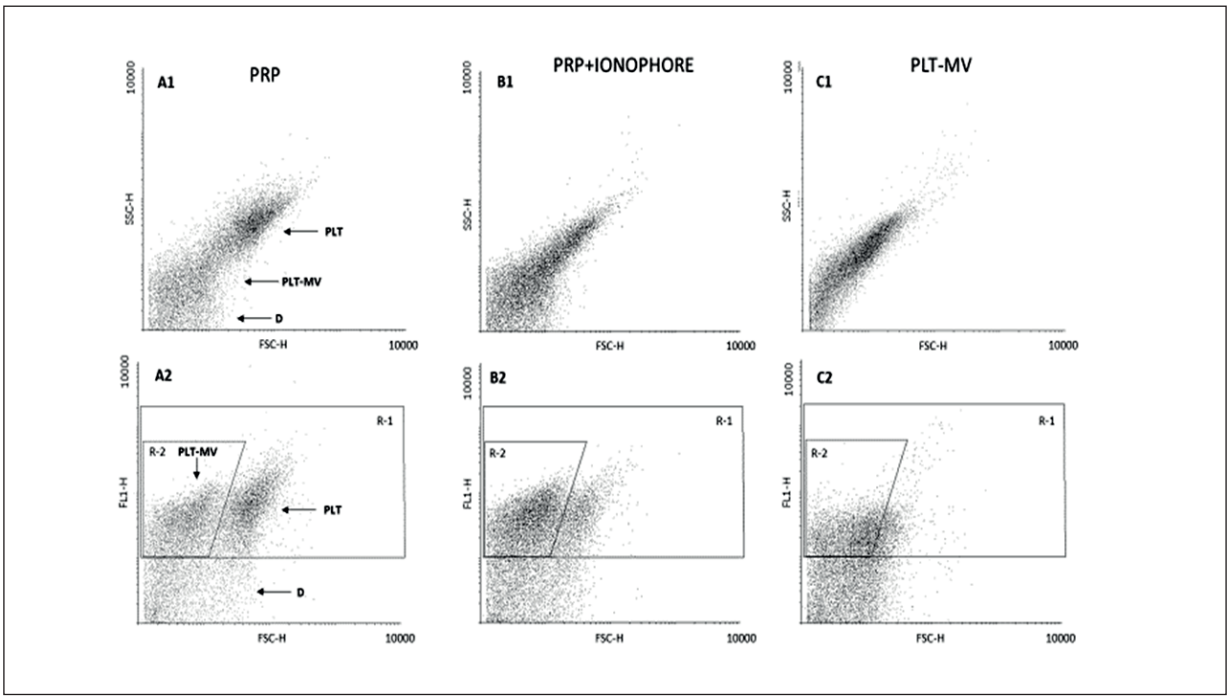

Figure 1. Dot plots FSC-SSC and FSC-FL1 of PRP, PRP activated and PLT-MVs. Representative dot plot FSC-SSC of (**A1**) untreated PRP, (**B1**) PRP activated with 5 µM ionophore calcimycin, (**C1**) PLT-MVs isolated from activated PRP; representative dot plot FSC-FL1 of (**A2**) untreated PRP, (**B2**) PRP activated with 5 µM ionophore calcimycin and (C2) PLT-MVs isolated from activated PRP. R-1 gate: Fluorescein-positive events (PLTs and MV-PLTs); R-2 gate: PLT-MVs.

19.47 ± 0.38% while the PLT-MV population reached the 80.53 ± 0.78% (Figure 1-B2) (p-value < 0.05).

Calcimycin-activated samples were centrifuged to isolate a PLT-MVs enriched population. As shown in Figure 1 C1, we obtained a population presenting low average FSC and SSC values compared to unstimulated and calcimycin-stimulated sample, conceivable with a population enriched in PLT-MVs. In fact, events present in the R2 region reached 89.63 ± 0.42 % (Figure 1-C2) (p-value < 0.001).

The data show that there is a major quantity of debris in the PLT-MVs analysis graph, due to the additional centrifugation and activation.

The effects of PRP, PRP activated and isolated PLT-MVs on migration ability of HaCaT cells were studied *in vitro* using a scratch assay (Figure 2A).

The scratch areas were measured using Image J software and expressed as % of closure compared to the initial scratch areas (T-0) (Figure 2B). After 24 hours the wound was healed by 24.80 ± 4.28 % in control conditions, while HaCaT treated with PRP showed an increased closure of 62.94± 0.96 % as expected. PRP activated with ionophore induced a similar closure percentage (52.69 ± 17.20%). HaCaT treated only with separated PLT-MVs were able to induce a percentage of closure of 52.76 ± 9.44%, comparable to that induced by both PRP and PRP activated.

Discussion

Platelet-rich plasma (PRP) is an hemocomponent rich in platelets and growth factors, widely used in clinical practice to accelerate the healing of damaged tissues, such as chronic wounds or ulcerations[22]. The several methods used to obtain PRP for wound healing therapies still need to be standardized[23] in order to broaden its use. The efficacy of PRP as therapy for hard-to-heal wounds is based on platelet activation during the hemostatic phase of wound healing: platelets in contact with the wound bed, exposed as a result of injury, release growth factors, such as platelet-derived growth factor (PDGF), transformative growth factor (TGF), vascular endothelium growth factor (VEGF), insulin-like growth factor (IGF) and epidermal growth factor (EGF), that modulate wound-healing phases[24]. Platelet activation has

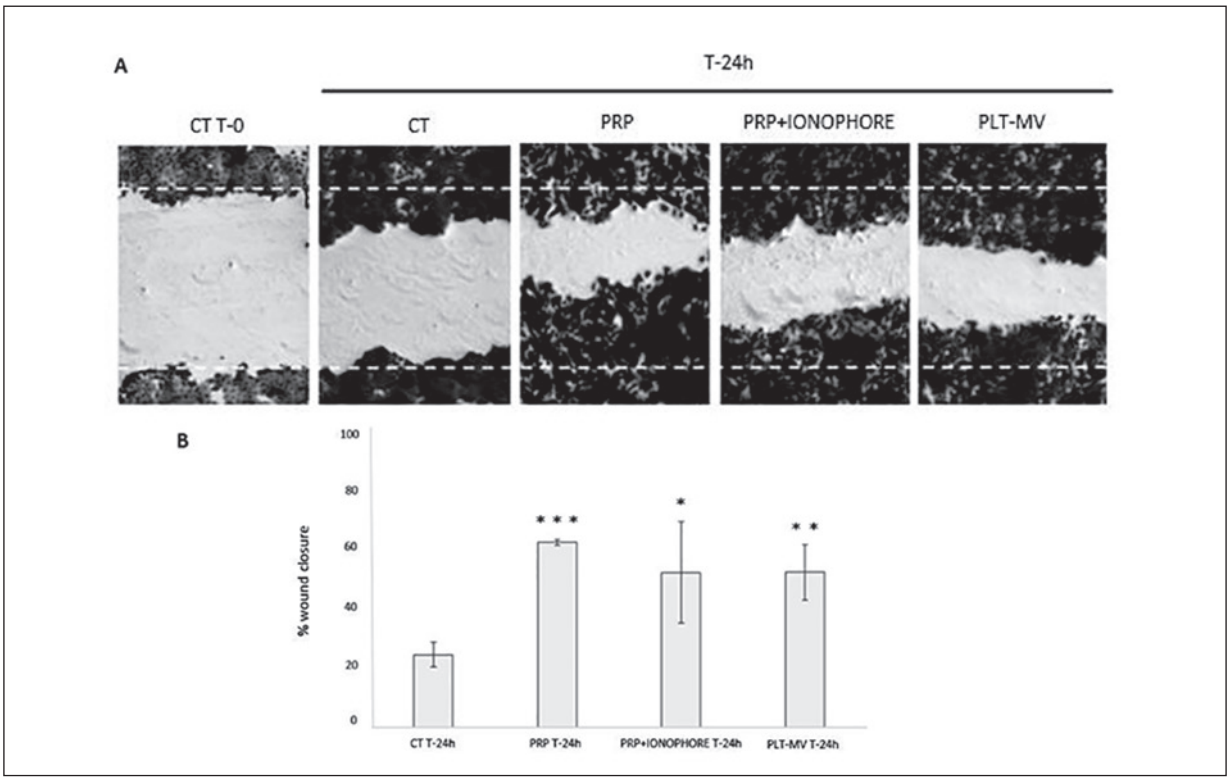

Figure 2. *In vitro* wound closure after 24-hours incubation. **A,** Representative optical microscopy images of *in vitro* wound healing (scratch) assay. Fixed cells were stained with 1% crystal violet. Magnification = 4×. Control samples fixed at time 0 as a reference for measurements (CT T-0). **B,** Quantification of wound closure. Results are expressed as % of wound closure after 24 hours, setting CT T-0 = 0%. **p*-value < 0.05, ** *p*-value < 0.001 and *** *p*-value < 0.0001.

been associated with the release of microparticles, and interest in their nature has increased in the last few years as they have been identified as microvesicles (MVs) released by shedding from platelets, and their content has been characterized. Platelet-derived microvesicles (PLT-MVs), with a diameter of 100-1000 nm, represent the most abundant group of MVs present in our blood and they are implicated in the transport and delivery of bioactive molecules participating in hemostasis, thrombosis, inflammation, tumorigenesis, angiogenesis, and immunity[25]. In fact, platelet-derived vesicles can transport cargo limited by plasma membrane, including membrane proteins, cytosolic proteins and RNA[26]. Moreover, they can activate intracellular signaling pathways and are implicated in wound healing and tissue regeneration[27]. Levels of PLT-MV circulating in the blood are altered in clotting disorders, rheumatoid arthritis, cancers, cardiovascular disease and infections, highlighting their potential contribution to different diseases and their possible development as a biomarker[15].

Even if platelets participate in wound healing, few or no information is available about the PLT-MVs direct or indirect role in this process, therefore we attempted to elucidate their action using a simple *in vitro* model (scratch assay) adding PRP, activated PRP, and a PLT-MV solution with a purity of more than 90%, to those *in vitro* wounds to assess their ability to stimulate human keratinocyte migration. The analysis of PLT-MV production was performed using a flow cytometry strategy based on the ability of a vital dye (FDA)[19].

Reepithelization is one of the main processes of wound healing caused by the so-called lateral migration of keratinocytes. Our data show that keratinocyte migration is induced in the presence of isolated PLT-MVs, with a potency comparable to that expressed by PRP and ionophore-activated PRP. In fact, the percentage of wound closure induced by PLT-MVs alone is similar to that measured for PRP and activated PRP.

This observation suggests that the PRP-positive effects on wound healing are mediated di-

rectly by PLT-MVs that are produced upon platelet activation by a plethora of stimuli[28]. From this point of view, an indirect confirmation for our observation has been provided by a study about PRP activation using electrical fields[29]. In that paper, electrical field application was able to induce a stronger shedding of Annexin V positive particles (PLT-MVs)[26] compared to activation mediated by thrombin and TRAP (thrombin receptor-activating peptides). Moreover, medium conditioned by PRP stimulated with the electric field was able to induce proliferation of MCFA10 (non-transformed mammary epithelial cell line) while medium conditioned by thrombin and TRAP failed in promoting cell growth. Based on these findings, PLT-MVs isolated from PRP could be used in a therapeutic approach, to treat chronic wounds as an alternative option instead of PRP. So far, the focus of this kind of research has been concentrated on exosomes (EXs), another vesicle that can be released by platelets and other cells. EXs are released by exocytosis, derive from multivesicular bodies and alpha-granules, present a diameter between 40 to 100 nm, and contain RNAs, miRNAs and bioactive proteins. EXs release is not induced by collagen, nor thrombin, nor calcium ionophore-mediated activation[30], moreover they are difficult to observe in flow cytometry because of their dimensions and difficult to eliminate during PLT-MVs preparation. EXs were able to promote wound healing similar to PRP, improve cell proliferation, migration and angiogenesis *via* phosphoinositide 3-kinase (PI3K)-Akt and mitogen-activated protein kinase (MAPK)-Erk signaling, transforming growth factor β (TGF-β) and yes-associated protein (YAP). In our preparations we cannot exclude EXs presence, but the exocytosis of exosomes is not induced by calcium flux[30], and moreover, they are a small percentage compared to PLT-MV[31].

Conclusions

This preliminary work is the first attempt to focus on the effects of PRP-derived PLT-MVs on *in vitro* wound healing to verify their real healing potential and to image a direct clinical use.

Even if further preclinical investigations are necessary to obtain stronger evidence of the PLT-MV potential, these initial data, obtained using standard, easy and fast procedures, seem to be encouraging. In order to fully understand the effects of PLT-MVs on wound healing, 3D-bioprinted skin model could be used. These complex *in vitro* models can show the cross-talk between different kinds of cells involved in wound healing, such as fibroblasts and keratinocytes, and matrix component deposition and maturation in the presence of PLT-MVs. However, our observations suggest that the positive effects of PRP on wound healing are mediated directly by PLT-MVs that are produced upon platelet activation.

Therefore, the use of highest-purity PLT-MVs, obtained by cell sorting or ultracentrifugation, could help to further test the PLT-MVs pro-healing effect also in animal models and to clarify if the effect could be modulated by age or general health status of patients, or in pathologies that cause wound-healing process alterations.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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