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ORIGINAL PAPER

Platelet-rich plasma (PRP) and adipose-derived mesenchymal stem cells: stimulatory effects on proliferation and migration of fibroblasts and keratinocytes in vitro

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Abstract The clinical use of tissue engineering associated with cell therapy is considered a new alternative therapy for the repair of chronic lesions with potential application in different medical areas, mostly in orthopedic and dermatological diseases. Platelet-rich plasma (PRP) is a rich source of growth factors and cytokines important for wound healing. Adipose-derived mesenchymal stem cells (ADSCs) have shown potential to accelerate the resolution of ulcers, to stimulate cell proliferation, and to benefit the quality of skin repair. This study aims to determine the effect of PRP and conditioned medium (CM) from ADSC on fibroblast and keratinocyte proliferation in vitro. Migration and proliferation assays were performed to evaluate the growth of fibroblasts and keratinocytes in the presence of PRP, CM, and CM + PRP. Significant proliferative stimulation was observed after 48 h of culture ($p < 0.05$) on mean absorbance of fibroblasts cultured with 10 and 25 % PRP, 100 % CM, and 25 % PRP + 25 % CM, if compared with control. Keratinocyte proliferation was stimulated after 48 h in cultures with 25, 50, and 100 % CM, and growth was compared with controls. The migration assay detected a significant migratory stimulus in fibroblasts cultured with 10 % PRP + 10 % CM after 48 h. These in vitro results suggest that PRP and ADSC have therapeutic potential for healing and re-epithelialization of chronic wounds in vivo.

Keywords Fibroblasts · Keratinocytes · Mesenchymal stem cells · Platelet-rich plasma

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Introduction

Chronic skin lesions are a serious worldwide public health problem. In the United States, approximately 6.5 million people suffer of skin lesions, and more than 25 billion dollars are spent each year treating complications related to chronic ulcers [25]. The etiology of leg ulcers consists of diabetes mellitus, dermatological, hematological, rheumatic, and vascular diseases (arterial and venous). It is estimated that 12–25 % of diabetic patients will develop cutaneous foot ulcers throughout their lives [1].

Wound resolution in diabetic patients is impaired by lesion-related physiological and biochemical defects, by abnormalities—in the angiogenic response, in collagen synthesis, in macrophage function, in granulation tissue formation, in growth factor production—and by a decline in fibroblast and keratinocyte proliferation and migration. The environment of the lesion is a chronic inflammatory state characterized by persistent pro-inflammatory cytokines [19]. In this scenario, topical anti-inflammatory strategies associated with growth factors have considerable therapeutic potential.

Wound healing is a complex biological event. Restoring skin integrity requires several cell types, extracellular matrix components, and cytokines [20, 28]. Platelet-rich plasma (PRP), the fraction of the blood plasma with high platelet concentration, has been used to accelerate healing

in dentistry, orthopedics, dermatology, and plastic surgery [16]. PRP is used for injury regeneration and for tissue repair, because several growth factors essential for healing, such as PDGF, TGF, IGF, and EGF, are concentrated in platelets [23]. In addition, PRP may act as a scaffold for other cell types, such as mesenchymal stem cells (MSCs) [27]. The need for developing therapeutic strategies with potential clinical efficacy, in the context of tissue engineering, has fostered the study of stem cells.

MSCs have immunomodulatory properties and reduce inflammation by secreting cytokines that promote tissue repair in the lesion [22, 29]. The rationale of this work is based on the existence of a possible beneficial interaction between the PRP and soluble factors from adipose-derived mesenchymal stem cells (ADSCs), on fibroblasts and keratinocytes. This interaction may open a new therapeutic avenue for the treatment of chronic skin ulcers.

Materials and methods

Biological materials

PRP was obtained from one unit of whole blood (450 mL) from a voluntary donation at the Hemocenter from Universidade Estadual de Campinas-UNICAMP (Campinas, São Paulo, Brazil). The volunteer was subjected to serology tests for HIV, HTLV I and II, Chagas, Syphilis, and Hepatitis B and C. Adipose tissue was collected from 30 to 45 (38.5 ± 6) year-old individuals with body mass index (BMI) over than 30 kg/m^2 and who underwent bariatric surgery at the Surgical Center from the Clinical Hospital of UNICAMP. Skin was obtained from patients over 60 undergoing a blepharoplasty procedure at the Ambulatory Surgical Center from the Clinical Hospital of UNICAMP. Prior to each procedure, donors were informed of the study, and invited to participate and to sign the consent form.

Production of platelet-rich plasma

PRP was obtained as proposed by Jo et al. [11]. Briefly, whole blood was centrifuged at 900 g for 5 min to separate the plasma. The supernatant plasma was collected and centrifuged at 1500 g for 15 min for platelet concentration [11]. Platelets, leukocytes, neutrophils, and erythrocytes were quantified by the automated hematology analyzer XE-500 Sysmex (Sysmex Corporation, Kobe, Japan).

Isolation and culture of ADSC

ADSCs were isolated following the protocol of Bunnell et al. [2]. Adipose tissue fragments were enzymatically digested with 0.075 % collagenase type I solution (Sigma-Aldrich, MS, USA) in a 37 °C shaking water bath for 30 min. Samples were centrifuged at 450 g for 5 min to obtain the stromal fraction. The oil and medium containing collagenase were removed. The pellet was resuspended in 1 mL lysis buffer (0.1 % $KHCO_3$, 0.82 % NH_4Cl, and 0.0037 % EDTA.4 Na) and incubated on ice for 10 min. Cells were washed with 20 mL phosphate buffer Dulbecco's phosphate-buffered saline (DPBS) and centrifuged at 450 g for 5 min. The cell suspension was filtered through a 70-μm cell strainer (Becton–Dickinson, NJ, USA). The filtrate was plated in T25 culture bottles (Sarstedt, Nümbrecht, Germany) at a ratio of 1 g of adipose tissue for each 6 cm^2 [2]. Cells were kept at 37 °C (in an incubator with 5 % CO_2).

Generation of conditioned medium

Third-passage ADSCs in 80–90 % confluence, cultured in T75 bottles (Sarstedt, Nümbrecht, Germany), were used to obtain the conditioned medium (CM). The cell surface was washed twice with DPBS phosphate buffer, pH 7.4, supplemented with 1 % penicillin, streptomycin, and L-glutamine (Gibco, New York, USA). 12 mL of M199 medium supplemented with 1 % penicillin, streptomycin, and L-glutamine was added to each T75 flask. The bottles were incubated at 37 °C (in an incubator with 5 % CO_2). After 48 h, the culture medium was aspirated and centrifuged at 300 g for 10 min to remove the debris. The supernatant was used as CM [13].

Immunophenotyping of ADSC

ADSC immunophenotyping was performed by flow cytometry (FACSCalibur-Becton Dickinson, NJ, USA). Third-passage ADSCs were labeled with monoclonal antibodies against CD73 and CD90 (Becton Dickinson, NJ, USA), CD105 (Biolegend, London, UK), the exclusion markers CD34 and CD45 (Becton Dickinson, NJ, USA), HLA-DR and CD19 (Biolegend, London, UK), and CD11b (Exbio, Vestec, Czech Republic). Viability was assessed with the Live and Dead Kit (Invitrogen, NY, USA).

Differentiation of ADSC

Third-passage ADSCs were cultured with medium provided by StemPro kits (Cibco, NY, USA), following the manufacturer's instructions, and stained with Oil Red O, Alcian Blue, and Alizarin Red (Sigma–Aldrich, MS, USA) to assess adipogenic, chondrogenic, and osteogenic differentiation, respectively.

Isolation and culture of fibroblasts and keratinocytes

Skin samples were placed in Petri plates with 0.025 % trypsin + 0.1 % EDTA and fractionated using scissors and clamps. The cell suspension containing dermal and epidermal cells was incubated at 37 °C and 5 % CO_2 for 3 h and filtered through a 40-μm cell strainer (Becton Dickinson, NJ, USA). The filtrate was centrifuged at 400 g for 10 min. Cells were counted in a Neubauer chamber, plated in T25 culture flasks at a density of 1.0×10^5 cells/cm^2, and incubated at 37 °C and 5 % CO_2. Cells were cultured in M199 medium supplemented with 10 % fetal bovine serum (FBS) (Gibco, NY, USA), 1 % penicillin, streptomycin, and L-glutamine for isolation of fibroblasts. Cells were cultured in keratinocyte-SFM medium (Gibco, NY, USA) supplemented with 10 % FBS and 1 % penicillin, streptomycin, and L-glutamine for isolation of keratinocytes.

ADSC proliferation in the presence of PRP

ADSCs were seeded ($n = 6$, technical replicates) in 96-well plates (Becton Dickinson, NJ, USA) at a concentration of 2×10^3 cells/well and cultured in M199 medium supplemented with 10 % FBS and 1 % penicillin, streptomycin, and L-glutamine. After 24 h, the medium was removed and replaced with culture medium M199 supplemented with 0 % (negative control), 10, 25, 50, and 100 % PRP (activated with 25 μL of 10 % calcium gluconate for each 1000 μL of PRP) or 10 % FBS and 1 % penicillin, streptomycin, and L-glutamine. Cell proliferation was assessed after 24 and 48 h of incubation by adding 10 μL of CCK-8 reagent, following the manufacturer's instructions. Absorbance was measured at 450 nm for analysis of cell growth.

Skin cells proliferation in the presence of PRP and CM

Fibroblasts and keratinocytes were seeded ($n = 6$, technical replicates) in 96-well plates (Becton Dickinson, NJ, USA) at a concentration of 2×10^3 cells/well. Cells were cultured in M199 medium supplemented with 10 % FBS and 1 % penicillin, streptomycin, and L-glutamine (fibroblasts) and keratinocyte-SFM medium supplemented with 10 % FBS and 1 % penicillin, streptomycin, and L-glutamine (keratinocytes). After 24 h, the medium was removed and replaced with M199 culture (fibroblasts) or keratinocyte-SFM medium (keratinocytes) supplemented with 0 % (negative control), 10, 25, 50, and 100 % PRP, 10, 25, 50, and 100 % CM, 10 % PRP + 10 % CM, 25 % PRP + 25 % CM, 50 % PRP + 50 % CM or 10 % FBS. All media was supplemented with 1 % penicillin, streptomycin, and L-glutamine. Cell proliferation was assessed after 24 and 48 h of incubation by adding 10 μL of CCK-8 reagent, following the manufacturer's instructions. Absorbance was measured at 450 nm for analysis of cell growth.

Migration assay

Cell migration was determined using a scratch assay, as proposed by Liang et al. [14]. Fibroblasts were seeded in 12-well plates at a concentration of 2×10^5 cells/well, in triplicates, and cultured in M199 medium with 0 % (negative control), 10 % PRP, 10 % CM, 10 % PRP + 10 % CM or 10 % FBS. ADSCs were plated in 12-well plates at a concentration of 2×10^5 cells/well, in triplicates, and cultured in M199 medium with 0 % (negative control), 10 % PRP or 10 % FBS. A line was drawn on the confluent cell monolayer using a P200 pipette. Images were acquired on the open space of the plastic. Cell migration was monitored after 24 and 48 h [14].

Statistical analysis

The Shapiro–Wilk test was used to analyze the normality of variances. Analysis of variance (ANOVA) followed by Kruska–Wallis and Student Newman–Keuls test for non-parametric data was used to determine significant differences between assays with more than two groups. Statistical significance was set at $p < 0.05$. The tests were performed using the *BioEstat* 5.3 software.

Ethical aspects

This study was approved by the Ethics Committee for Research of the Faculty of Medical Sciences, UNICAMP, and is registered at Platform Brazil (CAAE 11214513.6.0000.5404).

Results

Platelet-rich plasma analysis

The PRP had a platelet concentration factor of 2.050 ± 0.226 and a platelet recovery efficiency of 60.12 ± 1.77 %. A small fraction of leukocytes and neutrophils was obtained after the second centrifugation step (Table 1).

ADSC immunophenotyping

The immunophenotypic study of third-passage ADSCs showed expression (≤2 %) of exclusion markers, such as

Table 1 Concentration of platelets and cellular fractions pre- and post-centrifugation

Step	Red blood cells (10^6/μL)	Leukocytes (10^3/μL)	Neutrophils (10^3/μL)	Platelets (10^3/μL)
Pre-centrifugation	3.620	3.960	1.940	232.000
Post-centrifugation	0.020	0.260	0.120	476.667

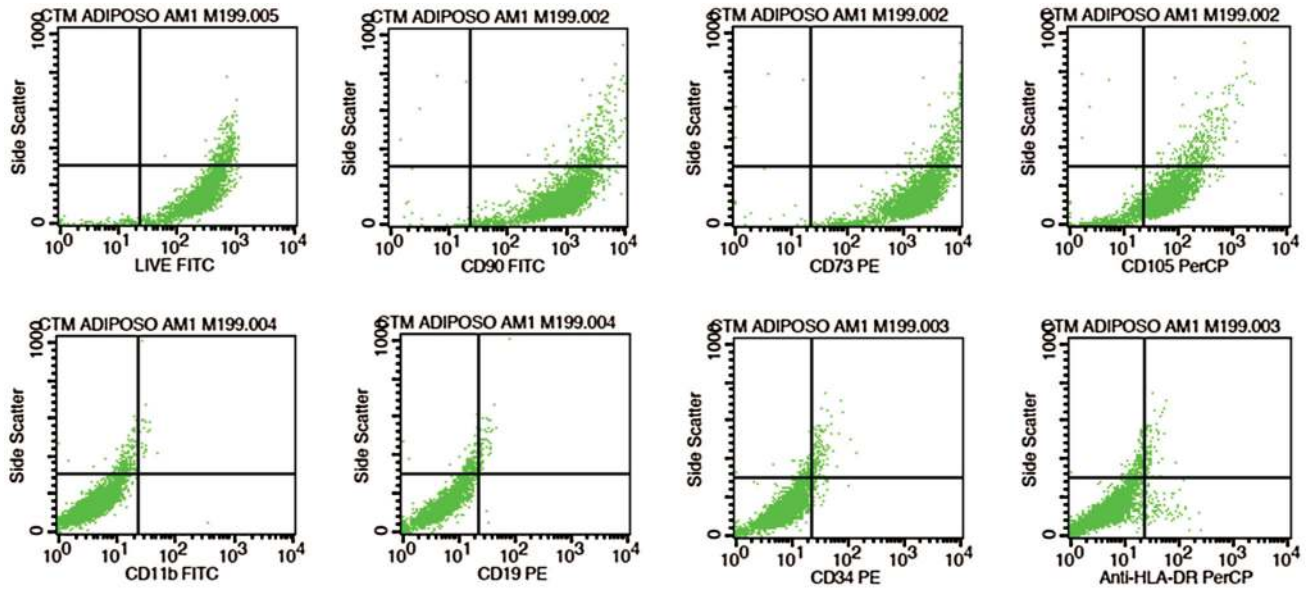

Fig. 1 Immunophenotypic characterization of ADSC by flow cytometry using different surface markers. Positive phenotype (≥95 %): CD90, CD73, and CD105. Negative phenotype (≤2 %): CD11b, CD19, CD34, and HLA-DR

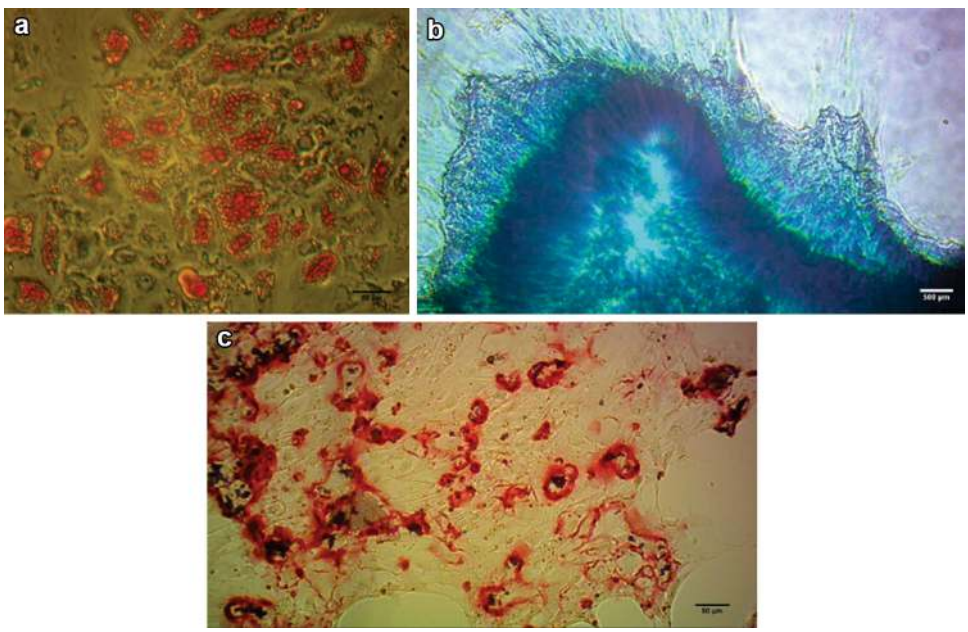

Fig. 2 Differentiation of ADSC in adipogenic (**a** *Oil red* O staining), chondrogenic (**b** *Alcian blue* staining), and osteogenic (**c** *Alizarin red* staining) lineages. 10× magnification (*scale bar* 50 μm)

CD34 (0.26 %), CD11b (0.04 %), HLA DR (0.73 %), and CD19 (0.02 %), typical of hematopoietic progenitor cells and leukocytes. In addition, significant positive expression (≥95 %) of ADSC characteristic markers was detected, including CD73 (99.78 %), CD90 (99.80 %), and CD105 (97.60 %).

Sample viability was 94.14 %. Figure 1 shows the flow cytometry results (graphical view).

ADSC differentiation

Adipogenic differentiation of ADSCs was observed after 7 days of culture with Oil Red O staining (used for visualization of lipid vacuoles). Chondrogenic differentiation was observed after 14 days of culture with Alcian Blue staining (used to show proteoglycans). Cells differentiated into osteoblasts were stained after 21 days with Alizarin Red (used for labeling calcium deposits), as shown in Fig. 2.

Proliferation of ADSCs under the influence of PRP

A 48-h proliferation assay was used to determine the influence of PRP on ADSC growth. As shown by the graph in Fig. 3, the concentration of PRP is inversely proportional to ADSC proliferation, i.e., lower PRP concentrations provide higher ADSC growths.

Significant differences could not be detected between ADSC treated with PRP and the negative control after 24 h of culture, except for the group cultured with 100 % PRP. This group showed a significant reduction in absorbance reading. Nevertheless, after 48 h, the mean for cells cultured with 10 % PRP was twice as high as in the group without stimulation (negative control), and this difference was statistically significant (Fig. 3).

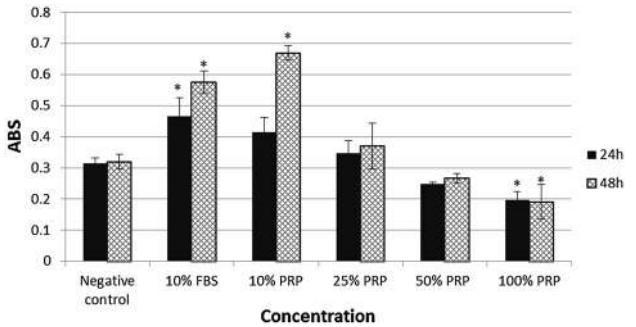

Fig. 3 Growth of ADSC cultured with different PRP concentrations, compared with negative control and FBS. *Statistically significant difference ($p < 0.05$) if compared with negative control for the same respective periods (24 or 48 h)

Proliferation of skin cells under the influence of PRP and CM

A 48-h proliferation assay (based on absorbance reading) was used to evaluate the influence of PRP and CM from ADSC on fibroblast and keratinocyte growth. As shown by the graphs in Figs. 4 and 5, lower PRP concentrations provide a greater stimulus for fibroblast proliferation and keratinocyte proliferation.

Significant differences could not be detected between cells cultured with PRP and the negative control after 24 h of culture, except for the group cultured with 100 % PRP. This group showed a significant reduction in absorbance reading for fibroblasts (Fig. 4a) and keratinocytes (Fig. 4b).

Nevertheless, after 48 h, the means for fibroblasts cultured with 10 and 25 % of PRP were 2.51 and 1.7 times higher than the group without stimulation (negative control), and these differences were statistically significant. Fibroblasts cultured with 100 % PRP maintained a difference even after 48 h (Fig. 4a). Significant differences could not be detected between keratinocytes treated with PRP and without stimulation after 48 h (Fig. 4b).

The graphs in Fig. 5 show that higher CM concentrations provide a greater stimulus for fibroblast proliferation and, particularly, for keratinocyte proliferation.

Statistically significant differences were observed between fibroblasts cultured with 50 % CM, keratinocytes

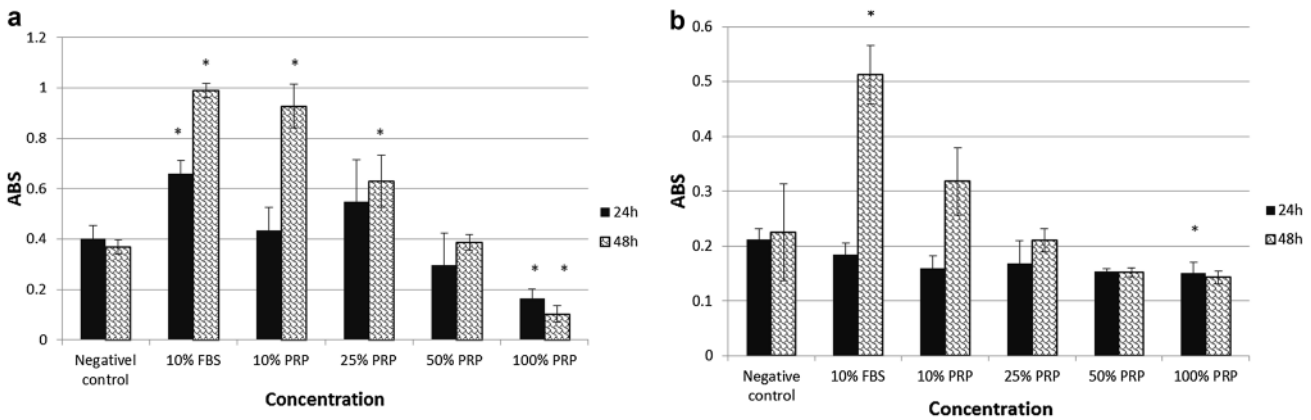

Fig. 4 Growth of **a** fibroblasts and **b** keratinocytes cultured with different PRP concentrations, compared with negative control and FBS. *Statistically significant difference ($p < 0.05$) compared with negative control for the same respective periods (24 or 48 h)

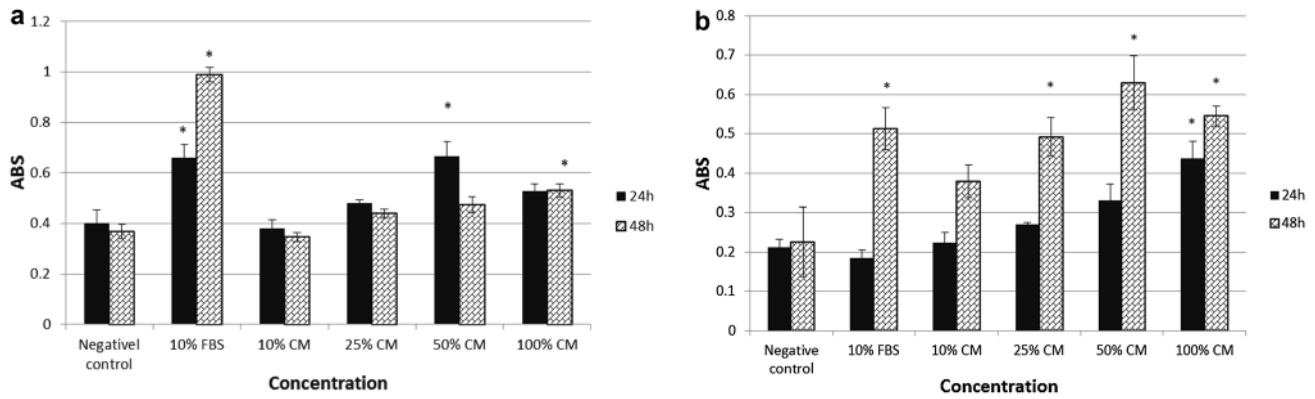

Fig. 5 Growth of **a** fibroblasts and **b** keratinocytes cultured with different CM concentrations, compared with negative control and FBS. *Statistically significant difference ($p < 0.05$) compared with negative control for the same respective periods (24 or 48 h)

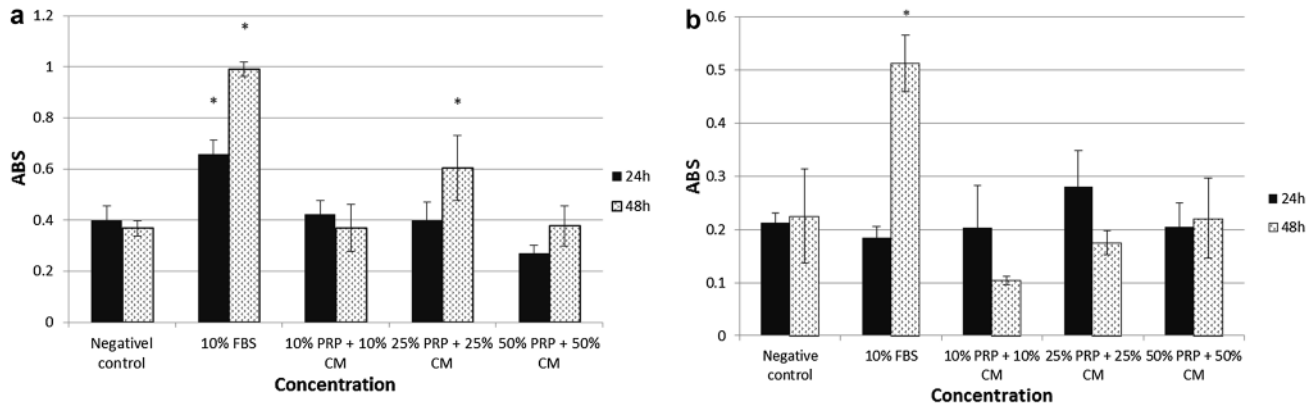

Fig. 6 Growth of **a** fibroblasts and **b** keratinocytes cultured with different PRP and CM concentrations, compared with negative control and FBS. *Statistically significant difference ($p < 0.05$) compared with negative control for the same respective periods (24 or 48 h)

cultured with 50 and 100 % CM, and the negative control after 24 h of culture. Fibroblasts cultured with 100 % CM (Fig. 5a), and keratinocytes cultured with 25, 50, and 100 % CM (Fig. 5b) showed statistically significant differences compared with the negative control after 2 days of culture.

As shown by the graphs in Fig. 6, fibroblast proliferation and keratinocyte proliferation were, to a certain extent, stimulated when only half the culture medium was supplemented with an equal proportion of PRP and CM.

Significant stimulation was observed after 48 h in fibroblasts cultured with 25 % PRP and 25 % CM (Fig. 6a). Higher stimulation of keratinocyte proliferation, although not significant, was observed after 24 h of cultivation (Fig. 6b).

Migration assay

A scratch assay was used to determine the influence of PRP and CM, derived from ADSC, on fibroblast migration. The stimulation promoted by PRP on ADSC migratory potential was also examined. Images of lesions made in vitro on the cell monolayer were acquired and the migration quantified after 24 and 48 h, as shown in Figs. 7 and 8.

Quantitative analysis of scratch closure, performed by analyzing the images on ImageJ, showed a statistically significant difference only for ADSC cultured with 10 % PRP, compared with the group without stimulation (negative control) after 24 and 48 h (Table 2).

The migratory stimulus was significantly different in fibroblasts cultured with 10 % PRP + 10 % CM (compared with the negative control) after 48 h (Table 3).

Discussion

In this study, we investigated the effect of PRP in ADSC, aiming to identify a repair process with growth factors that is more efficient than conventional treatments, including a powerful antinociceptive activity linked to lipid fraction of PRP [4] for treating chronic cutaneous ulcers [5, 7, 21].

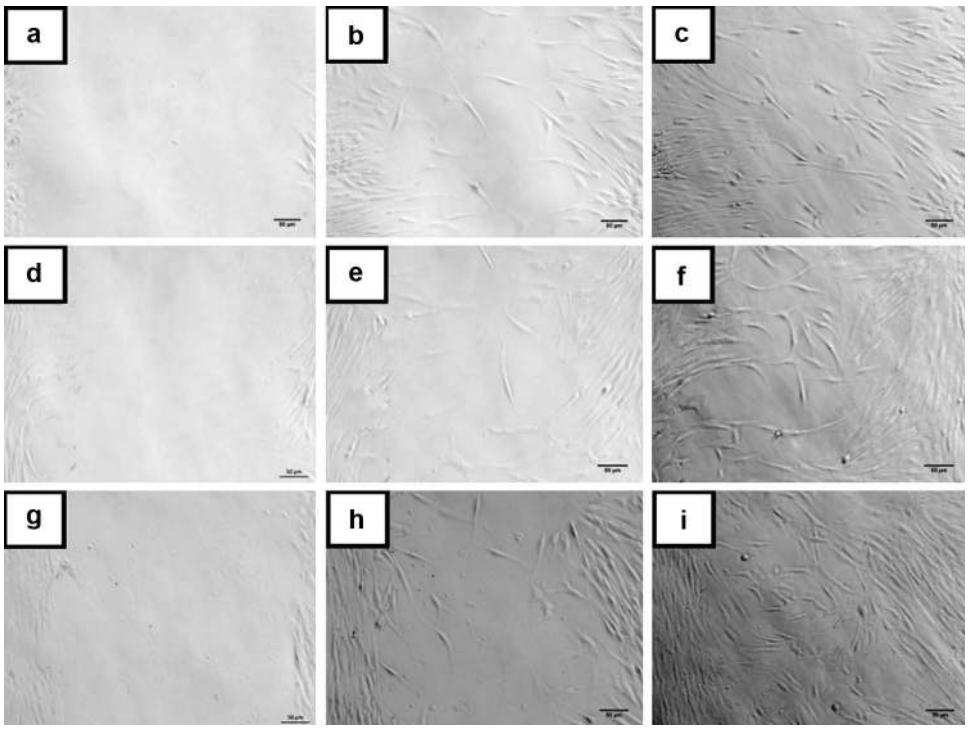

Fig. 7 Scratch assay on ADSC cultured without supplement/negative control (**a**–**c**), 10 % FBS (**d**–**f**), and 10 % PRP (**g**–i). Images captured at 0 h (**a**, **d**, **g**), 24 h (**b**, **e**, **h**), and 48 h (**c**, **f**, **i**), following a tracer on the ADSC monolayer

ADSCs were cultured with different PRP concentrations in the absence of FBS. The results showed that there was a proliferative and migratory stimulus in ADSC cultured with 10 % PRP after 48 h, compared with negative control. An inverse dose-dependent relationship was observed, i.e., higher PRP concentrations provided lower proliferative means, as shown in Fig. 3 (proliferation) and Fig. 8 (migration). These results are consistent with previous studies reporting significant proliferative stimulation of cultured MSCs with up to 10 % PRP [6, 10, 12, 15].

In in vitro cultures, higher PRP concentrations limit cell growth, as platelets release a complex protein composition with a negative regulatory activity that may reduce the effects of growth factors [9]. Furthermore, Mills et al. [17] proposed that when the proteolytic enzymes found in PRP—collagenase, elastase, cathepsin, and acid phosphatase—are highly concentrated, they contribute to cell growth inhibition.

To determine how the skin responds to growth factors, keratinocytes and fibroblasts were cultured with different concentrations of PRP, CM or both. Significant inhibition, similar to ADSC, was observed after 24 h of incubation with high PRP concentrations, as shown in the graphs of Figs. 4 and 5. Distinct reports in the literature refer to the proliferative stimulus produced by PRP. Kakudo et al. [12] cultivated dermal fibroblasts with different PRP concentrations and saw that after 24 h, 10 and 20 % PRP inhibited proliferation. Proliferative stimulation, however, was only observed on the 7th day in culture with 10 % PRP. The authors used buffy coat and a large amount of platelets ($132.26 \times 10^4/\mu L$), approximately three times higher than the amount used in this study ($476.66 \times 10^3/\mu L$). In accordance with data obtained by this study, Kakudo and colleagues found that no PRP proliferative stimulus occurs within 24 h for fibroblasts. However, in contrast with Kakudo et al. [12], the present study shows that low concentrations of PRP produce no inhibitory effect, even with minimal leukocyte and neutrophil fractions.

In a recent study, Shan et al. [21] reported that the best stimulus for cell growth is achieved with 2×10^6 platelets/μL. However, the authors performed leukodepletion of the PRP and achieved significant growth in human keratinocytes (HaCaT) within 24 h of cultivation with 10 % PRP. These results indicate that higher PRP platelet concentration and absence of leukocytes may contribute to the proliferation of fibroblasts and keratinocytes in a short period of time. Fibroblasts appear to respond better to PRP, while keratinocytes have shown a negative apparent specific growth rate when cultured in low concentrations of PRP, in a dependent manner [26]. Therefore, the absence of proliferative stimuli in keratinocytes exposed to PRP observed in the present study may be related to the presence of leukocytes and neutrophils, even if in minimum fractions, and to the smaller amount of platelets in the PRP.

CM provided a proliferative stimulus for fibroblasts and keratinocytes within 24 h. The higher the CM concentrations, the greater were the proliferative stimulus. The CM derived from ADSC contains a fraction that is rich in

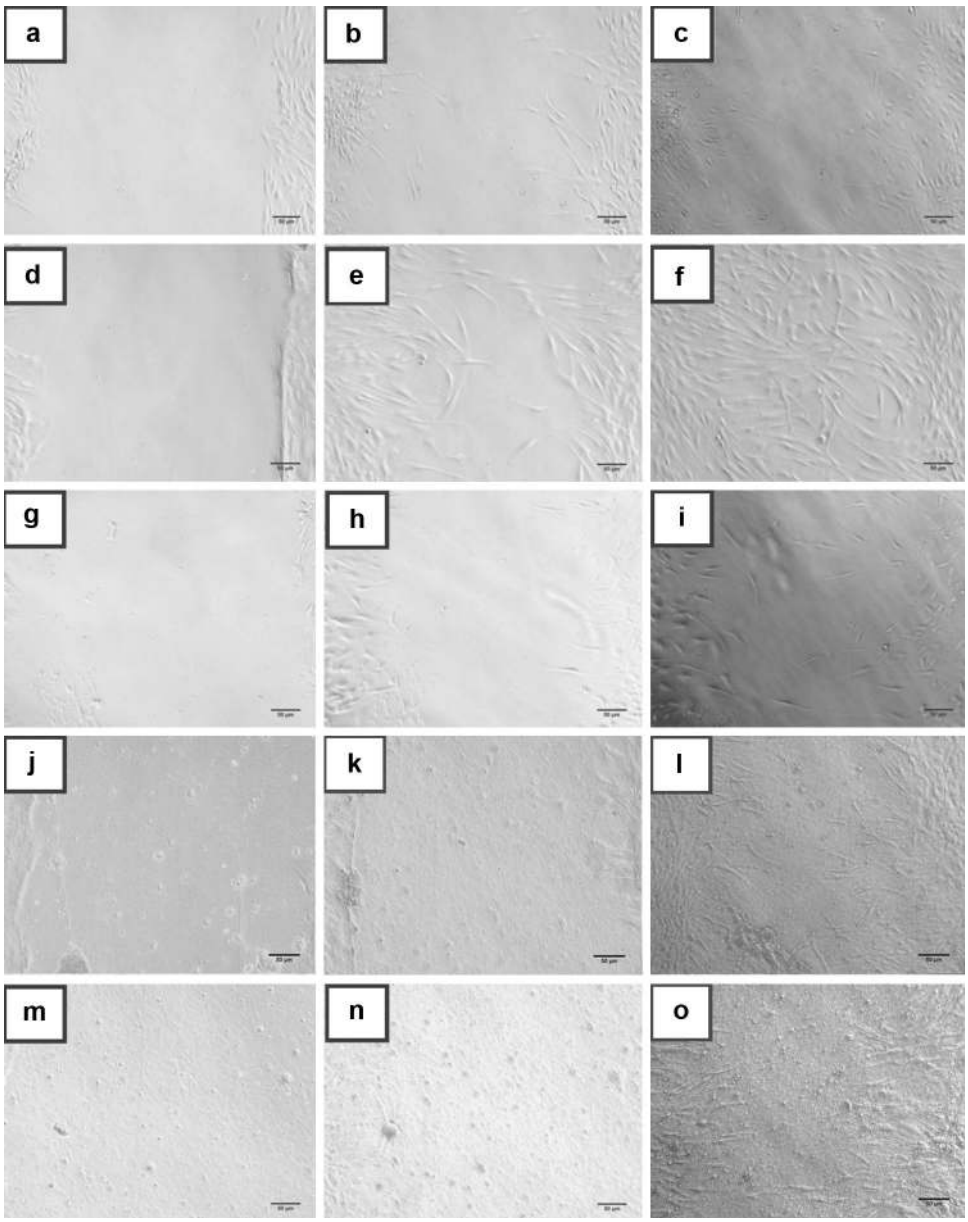

Fig. 8 Scratch assay on fibroblasts cultured without supplement/negative control (**a**–**c**), 10 % FBS (**d**–**f**), 10 % CM (**g**–**i**), 10 % PRP (**j**–**l**), and 10 % CM + 10 % PRP (**m**–**o**). Images captured immediately at 0 h (**a**, **d**, **g**, **j**, **m**), 24 h (**b**, **e**, **h**, **k**, **n**), and 48 h (**c**, **f**, **i**, **l**, **o**), following a tracer on the fibroblasts's monolayer

Table 2 Surface area (means percentage) occupied by ADSC after 24 and 48 h

Group	24 h Mean ± SD	48 h Mean ± SD
Control	10.48 ± 3.63^{a}*	11.89 ± 2.68^{a}*
10 % FBS	16.65 ± 3.48^{b}	18.56 ± 4.46^{b}
10 % PRP	17.07 ± 6.47^{b}	20.02 ± 2.30^{b}

* Letters indicate results of pairwise comparisons among means. Means with the same letter (within columns) do not differ significantly ($p > 0.05$)

Table 3 Surface area (means percentage) occupied by fibroblasts after 24 and 48 h

Group	24 h Mean ± SD	48 h Mean ± SD
Control	5.67 ± 1.76^{a}*	8.25 ± 3.04^{a}*
10 % FBS	13.69 ± 3.65^{b}	22.02 ± 4.11^{b}
10 % CM	7.80 ± 2.98^{a}	11.05 ± 5.83^{a}
10 % PRP	5.31 ± 2.05^{a}	13.02 ± 3.22^{a}
10 % PRP + 10 % CM	5.83 ± 1.74^{a}	17.78 ± 2.64^{b}

* Letters indicate results of pairwise comparisons among means. Means with the same letter (within columns) do not differ significantly ($p > 0.05$)

soluble factors with paracrine action on fibroblasts and keratinocytes, such as EGF, FGF, KGF, IGF-1, VEGF, and PDGF [3], important cytokines for repair of skin ulcers. Zhao et al. [30] showed that fibroblast proliferation depends on EGF and bFGF, which are present in CM and in PRP. This could explain the cell expansion observed in this study with 25 % PRP + 25 % CM (Figs.6, 8), which proposes a possible synergistic action.

Park et al. [18] found that 10 % PRP stimulates fibroblast migration within 24 h. However, similar to the proliferation assays, the authors used a PRP with a platelet concentration that is three-fold the one used in this study. The low CM concentration was also not sufficient to stimulate migration. Nevertheless, higher CM concentrations, such as 50 % CM [8] and 100 % CM [24], induce proliferation/migration. Our results showed that fibroblasts do not exhibit a significant migration behavior when cultured with 10 % PRP. On the other hand, fibroblast migration seems to rely on bFGF, VEGF, and PDGF [30]. This could explain why the migratory stimulus is observed only up on combination of PRP and CM, at low concentrations, indicating a synergistic or additive action.

In summary, the results of this study show that low PRP concentrations stimulate proliferation and migration of ADSC and fibroblasts in vitro. Furthermore, keratinocyte proliferation can be stimulated by ADSC paracrine action. These results propose an association between ADSC and PRP soluble mediators, which could potentially be used to promote healing and re-epithelialization in cutaneous ulcers.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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