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Platelet-rich plasma stimulates cytokine expression and alkaline phosphatase activity in osteoblast-derived osteosarcoma cells

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ABSTRACT

Objective: The aim of this study was to investigate the effects of PRP on SAOS-2 cells in terms of cytokine expression, cell activity and oxidative stress.

Design: Cell line SAOS-2 (1×10^5 cells/mL) were grown in culture medium α-MEM with 10% FBS for 24 h and stimulated (or not) with PRP at concentrations of 3, 10 and 20%, LPS (*E. coli*, 10 g/mL) and IL-1β (1 mg/mL) for 24 h. The supernatant was collected and analyzed for the expression of cytokines in a panel array, ALP using a commercial kit and NO_2^- with Griess reaction method. Also, the cells were analyzed using Western blot for RANKL and slot blotting for nitrotyrosine expression.

Result: There were no significant differences amongst the groups in terms of NO_2^-, protein nitrotyrosine content and RANKL expression. However, all stimuli increased ALP activity and in case of PRP, it was in a dose-dependent manner ($p < 0.001$). Also, all stimuli induced an increase in cytokines and chemokines expression, but only PRP promoted an increase of component C5, sICAM-1 and RANTES expression. Whilst IL-1 receptor antagonist (IL-1ra) expression was down-regulated by PRP, both LPS and IL-1β caused up-regulation of this cytokine.

Conclusions: PRP can stimulate osteoblast activity and cytokine/chemokine release, as well as indicate some of the mediators that can (and cannot) be involved in this activation.

1. Introduction

Bone formation is a prerequisite for tissue regeneration after the loss that occurs during periodontal disease and for implant osseointegration,[1] and requires the recruitment of osteoblast precursor cells, their differentiation into secreting cells, production of unmineralised osteoid and eventual calcification of the extracellular matrix.[2] It is a dynamic process that involves cell-to-cell and cell-to-extracellular matrix interactions, from two distinct lineages, the osteoblasts and the osteoclasts, coordinated by various polypeptide growth factors, such as platelet-derived growth factor (PDGF), insulin-like growth factors (IGFs), transforming growth factor

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(TGFs) and fibroblast growth factor (FGF) amongst other biological mediators.[1,3–5]

The activation of platelets at the site where infection or injury occurs, leads to the release of considerable quantities of potent endogenous bioactive substances including growth factors, such as vascular endothelial growth factor and endostatin, that can accelerate or delay the healing process respectively.[6–8] Therefore, their distinctive released substances, predominantly known for their involvement in both inflammatory/immunologic and healing process; clearly exceed an exclusive function in haemostasis and thrombosis.[8]

We have already demonstrated that in rats with thrombocytopenia the periodontal healing process after ligature-induced periodontitis is delayed. This process is associated, at least in part, with decreased serum concentrations of platelet-derived vascular endothelial growth factor (VEGF) and endostatin.[7] On the other hand, in another study[8] we have also shown that the systemic administration of aspirin or clopidogrel (both platelet activity inhibitors) attenuates the inflammation associated with periodontitis without affecting the bone repair process that follows the bone loss due to the periodontal disease.[8] Despite the recent advances in this field that reveal the prominent role that platelets can play in the inflammation and repair processes, a more comprehensive examination of the mechanisms involved is needed.[8]

The clinical use of platelet-derived factors was firstly shown by Lynch and colleagues, whom used platelet-rich plasma (PRP) in periodontal bone defects[9,10] and around implants,[11] and showed increased bone growth. PRP is an autologous concentrated, easily obtained in which the blood platelet concentration is above normal, with a higher concentration of the growth factors.[12] PRP has been used in several surgical specialties, involving both bone and soft tissues. Lately, there has been a growing interest in the use of PRP for the treatment of many intraoral clinical conditions. However, there has been no agreement about the advantages derived from the adjunct of platelet concentrates to periodontal surgical procedures as suggested by some reviews.[13]

Another important mediator of bone metabolism is nitric oxide (NO), an L-arginine-derived free radical that plays an important role in the host response against infection.[14] High levels of NO produced by the inducible NO synthase isoform (iNOS) have been observed in inflammatory bone resorption[15] and NO from the constitutive endothelial isoform (eNOS), the most abundant NOS isoform in bone, seems to be important for osteoblast function and involved in bone formation.[14] Lagumdzija et al.[16] showed that insulin growth factor I, which can be found in PRP, stimulates the proliferation of osteoblasts extracted from mouse calvaria. This stimulation was abolished in cells from eNOS knock-out animals, but recovered by a NO donor, and these results were shown to be similar in the human osteosarcoma cell line (SAOS-2) when using a NOS inhibitor.[16]

Previous studies have been investigating the mechanisms involved in PRP stimulation in bone *in vivo*,[4,9,10] and *in vitro*.[17–19] However, our study brings additional evidences that contribute to the elucidation of the underlying mechanisms of PRP on osteoblastic-like cells focusing on cytokine and chemokine release and nitric oxide production after PRP stimulation. In this context, the aim of this study was to investigate the effects of PRP in an immortalized osteoblastic-like cell lineage from osteosarcoma, SAOS-2, in terms of cytokine expression, alkaline phosphatase activity (ALP, a marker of cell activity), receptor activator of nuclear factor kappa-B ligand (RANKL) expression, NO production (measured as nitrite – NO_2^-) concentration and nitrotyrosine containing proteins (as an oxidative stress marker).

2. Materials and methods

2.1. Cell culture

SAOS-2 osteoblastic cells (The *Rio de Janeiro Cell Bank*, Rio de Janeiro, RJ, Brazil; CR019), derived from a primary osteogenic sarcoma, were cultured in Alpha Minimum Essential Medium with 100 μg/L streptomycin, 10 IE/L penicillin, and 10% foetal bovine serum (Invitrogen, Carlsbad, CA, USA) in a number of 1×10^5 cells/mL in a 48 well plate for 24 h, then stimulated for another 24 h with PRP with three different concentrations (3, 10 and 20%), LPS (*E. coli*, 10 μg/mL; Sigma Chem. Co., St. Louis, MO, USA) and IL-1β (1 μg/mL; R&D Systems Inc., Minneapolis, MN, USA).

2.2. Preparation of PRP, cell counting, viability, and activation

The present study was approved by the Ethics in Human Research Committee of the Araraquara School of Dentistry (UNESP, Araraquara, SP, Brazil; CAAE – 0053.0.199.019-08). It was conducted in full accordance with the ethical principles of the World Medical Association Declaration of Helsinki (2002). The volunteers were informed about the aims and methods of this study and provided a written consent to participate.

A simple technique was used to prepare PRP using common laboratory equipment.[20] The blood samples from 5 systemically health individuals were collected into 15-mL polystyrene tubes under sterile conditions and centrifuged at $500 \times g$ for 10 min at room temperature. The upper plasma fraction and the buffy coat layer were carefully collected and centrifuged at $700 \times g$ for 15 min. The pellet was used as the PRP. The samples were diluted in 1% ammonium oxalate# to haemolyze mature erythrocytes and a Neubauer haemocytometer were used for platelet count determination. The PRP samples were treated with 10% calcium chloride (final concentration of 1% of $CaCl_2$; Sigma Chem. Co.) and bovine thrombin (50 NIH/mL thrombin reference standard; low specific activity; Sigma Chem. Co.) mixture to activate the platelets. The platelets were allowed to aggregate within a few seconds of activation and centrifuged at $5000 \times g$ for 10 min. The supernatant was collected and immediately added to the SAOS-2 cultures. The experiment was performed one time with 4 samples in triplicate.

2.3. Alkaline phosphatase activity

ALP activity was determined by using a commercial kit (Labtest, Lagoa Santa, MG, Brasil). Briefly, ALP hydrolyses thymolphthalein monophosphate releasing thymolphthalein in alkaline medium, and the absorbance of the blue product is

read at 590 nm. SAOS-2 were previously washed with phosphate-buffered saline solution (PBS) (Invitrogen) and treated with 1.1 mL of sodium lauryl sulphate (1 mg/mL, SDS; Sigma Chem. Co.) for 30 min at room temperature. Fifty microliters from each sample were added to the kit solutions according to the manufacturer. Results were calculated as U/L and data were expressed as ALP activity normalized by the protein concentration (measured using the method of Bradford).[21,22]

2.4. Western blot analysis of RANKL expression

After measurement of protein contents in the samples (using the Bradford method [22]), the expression of RANKL receptor was analyzed in the cells as previously described[23] Immunoreactive bands were detected by chemiluminescence (*Thermo Scientific*, Waltham, MA, USA) and their intensities were estimated by densitometric analysis (ChemImager 5500 system, Alpha Innotech Corp., USA).

2.5. Slot blotting analysis of nitrotyrosine-containing proteins

The presence of proteins containing 3-nitrotyrosine (NT) residues, an index of oxidative stress and protein nitration, was analyzed in the cells using a protein slot blotting method, as described previously.[24] Immunoreactive bands were detected by chemiluminescence (*Thermo Scientific*) and their intensities were estimated by densitometric analysis (ChemImager 5500 system). Results were normalized by the band intensity values obtained after staining with Ponceau red.

2.6. Measurement of NO_2^- concentration

Nitrite concentration in the in the cell culture supernatants was quantified by the Griess reaction method.[25] Briefly, the supernatant (100 μL) reacted at room temperature with an equal volume of the Griess reagent (1% sulphanilamide plus 0.1% naphthylethylenedihydrochloride in 2.5% phosphoric acid; Sigma Chem. Co.) and the absorbance of the resulting chromophore was read at 450 nm. The samples were run in duplicate using 96-well microtiter plates, and nitrite concentrations were calculated from a sodium nitrite standard curve.

2.7. Proteome profiler human cytokine array

The cell culture supernatants were analyzed with the Proteome Profiler Human Cytokine Array Panel A Array Kit (R&D Systems Inc.). Nitrocellulose membranes, each containing 36 different anti-cytokine antibodies (anti-C5a, CD40 Ligand, G-CSF, GM-CSF, GRO, I-309, sICAM-1, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, IP-10, I-TAC, MCP-1, MIF, MIP-1α, MIP-1β, Serpin E1, RANTES, CXCL12, TNF-α, sTREM-1) in duplicate, were incubated with the supernatant samples and a cocktail of biotinylated detection antibodies. Any cytokine/detection antibody complex present in the sample is bound by its cognate immobilized capture antibody on the membrane. After washing (in order to remove unbound material), streptavidin–horseradish peroxidase and chemiluminescent detection reagents (*Thermo Scientific*) were sequentially added. The chemiluminiscence signal of the membranes were captured and the intensity of each spot was estimated by densitometric analysis (ChemImager 5500 system) after standardized preliminary image processing for noise reduction, background subtraction and signal enhancement.[26]

2.8. Statistical analysis

All data were analyzed by one-way analysis of variance and corrected for multiple measures by the Newman-Keuls correction. All values are reported as mean ± SEM. Statistical significance was set at 0.05.

3. Results

3.1. Alkaline phosphatase activity

As shown in Fig. 1, stimulation with PRP increased ALP activity in SAOS-2 cells after 24 h incubation in a dose-dependent manner (Control: 86.4 ± 0.7, 3% PRP: 94.6 ± 0.7, 10% PRP: 99.7 ± 1.6 and 20% PRP: 104.1 ± 1.1 U/mg of protein; $p < 0.001$), as it was also the case for both LPS (99.8 ± 1.1 U/mg of protein) and IL-1β (103.1 ± 1.0 U/mg of protein; $p < 0.001$). We did not observe differences between all the treatment groups.

3.2. Protein nitrotyrosine content, RANKL expression and NO_2^- concentration

There were no significant differences amongst the groups in terms of either NO_2^- concentration in the cell culture supernatants (Control: 1.31 ± 0.26, 3% PRP: 1.261 ± 0.22, 10% PRP: 1.37 ± 0.32, 20% PRP: 1.19 ± 0.32, LPS: 1.16 ± 0.33 and IL-1β: 1.16 ± 0.16 M), or protein nitrotyrosine content and RANKL expression in SAOS-2 extracts obtained 24 h after incubation with the different stimuli (Fig. 2).

3.3. Cytokine expression

During stimulation process, multiple cytokines and chemokines were secreted in SAOS-2 supernatants. As shown in

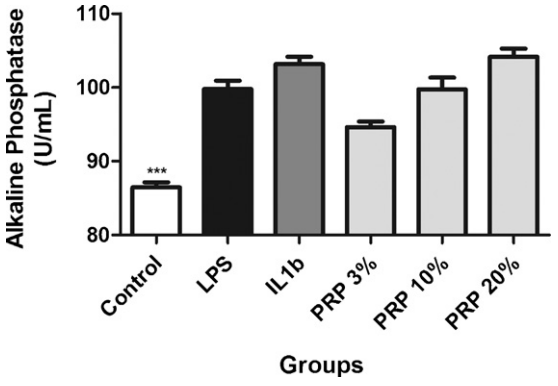

Fig. 1 – Alkaline phosphatase activity measured in homogenates of SAOS-2 cells 24 h after being stimulated with PRP, LPS and IL-1β ($n = 4$). ***$p < 0.001$ *vs.* all the remaining (stimulated) groups.

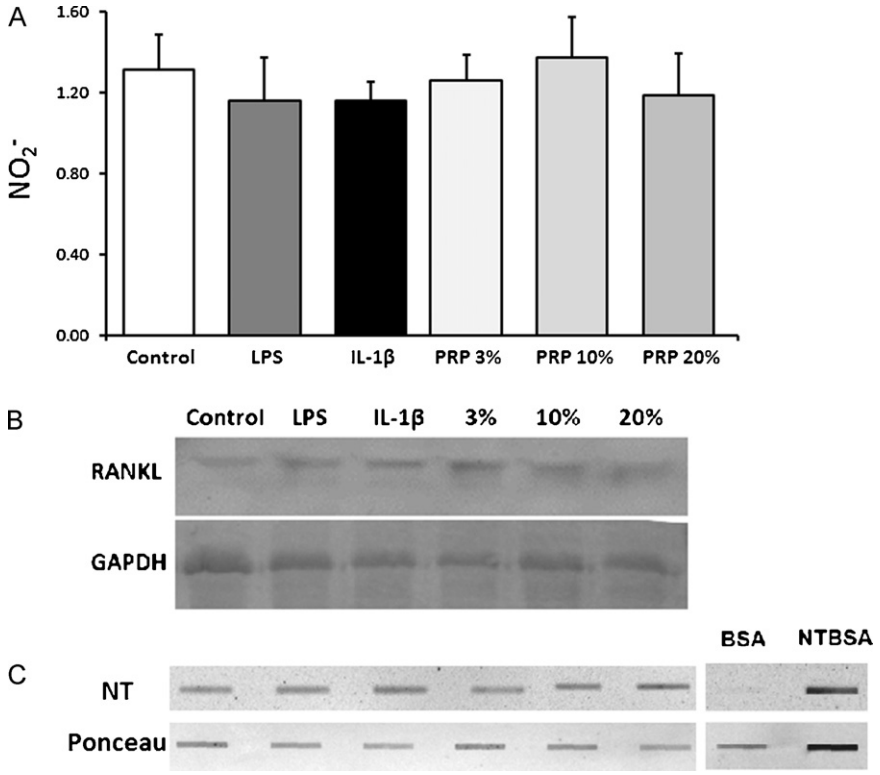

Fig. 2 – *Pannel A*: Nitrite concentration in supernatants collected 24 h after the stimulation of SAOS-2 cells with either PRP (3, 10 and 20%), LPS or IL-1 β. *Panel B*: RANKL and nitrotirosine (NT) expression in cell protein extract obtained 24 h after stimulation of SAOS-2 cells with either PRP (3, 10 and 20%), LPS or IL-1 β. Ponceau stained bands were used as loading control. BSA and NTBSA (nitrated bovine serum albumin) were used as negative and positive controls, respectively (n = 4).

Fig. 2, all the stimuli (20% PRP, LPS, IL-1β) up-regulated the expression of interleukin-6 (IL-6) and plasminogen activator inhibitor-1 (Serpin-1) when compared to the control. After PRP stimulation, the expression of *complement* component C5 (C5a), soluble Intercellular Adhesion Molecule-1 (sICAM-1) and Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) protein were up-regulated. Whilst IL-1 receptor antagonist (IL-1ra) expression was down-regulated by PRP, both LPS and IL-1β caused up-regulation of this cytokine. In addition, IL-1β up-regulated Granulocyte-macrophage colony-stimulating factor (GM-CSF), Chemokine (C-X-C motif) ligand 1 (CXCL1), IL-ra and IL-32α (Fig. 3).

4. Discussion

Inflammatory cytokines are key mediators of osteoclastogenesis and osteoclast action. They are present in the bone microenvironment in quite variable amounts, as a consequence of different disease conditions. However, there is not a general agreement about the advantages of using platelet concentrates in periodontal surgical procedures, as stated in the review from Del Fabbro,[13] and the role of platelets in controlling osteoblast differentiation, function, and apoptosis is not completely clear.[27]

Bacterial constituents, including Gram-negative-derived lipopolysaccharides (LPS), initiate inflammatory bone loss, as exhibited in periodontal diseases. LPS can stimulate the expression of IL-1β, TNF-α, IL-6, and RANKL by activating the innate immune response. In this study, we used LPS and IL-1b as positive control based on previous studies showing that IL-1b[28] and LPS[29] induce osteoblast-like cells stimulation, an important event in the etiopathogenesis of periodontal disease.

Several studies have been previously published aiming the elucidation of the actions of PRP on osteoblastic-like cells,[17–19] however the analyzed end-points and the experimental conditions (such as incubation time and PRP concentrations) are variable. For example, Kanno et al.[19] studied the effects of PRP at different concentrations (5 and 10%) and time points (first analysis were made after 24 h); Ogino et al.[17] stimulated the cells with PRP for 36 and 72 h but did not analyzed the effects on ALP; Bertoldi et al.[18] studied SAOS-2 cell culture during 19 days stimulated with 1% PRP (freshly added every 2 or 4 days) and evaluated ALP on day 21st.

In this study, instead, we focus on nitric oxide and cytokine/chemokine production by osteoblastic-like cells. Previous studies have already shown that PRP stimulates the proliferation of osteoblast-like cells in a dose-dependent manner, and that PDGF and TGF-β1, but not IGF-I, significantly contribute to this process,[17] resulting in enhanced bone regeneration and healing activation.[28]

Platelet-derived growth factors initiates connective tissue healing and activates bone regeneration, including mitogenesis, by increasing the number of healing cells, triggering

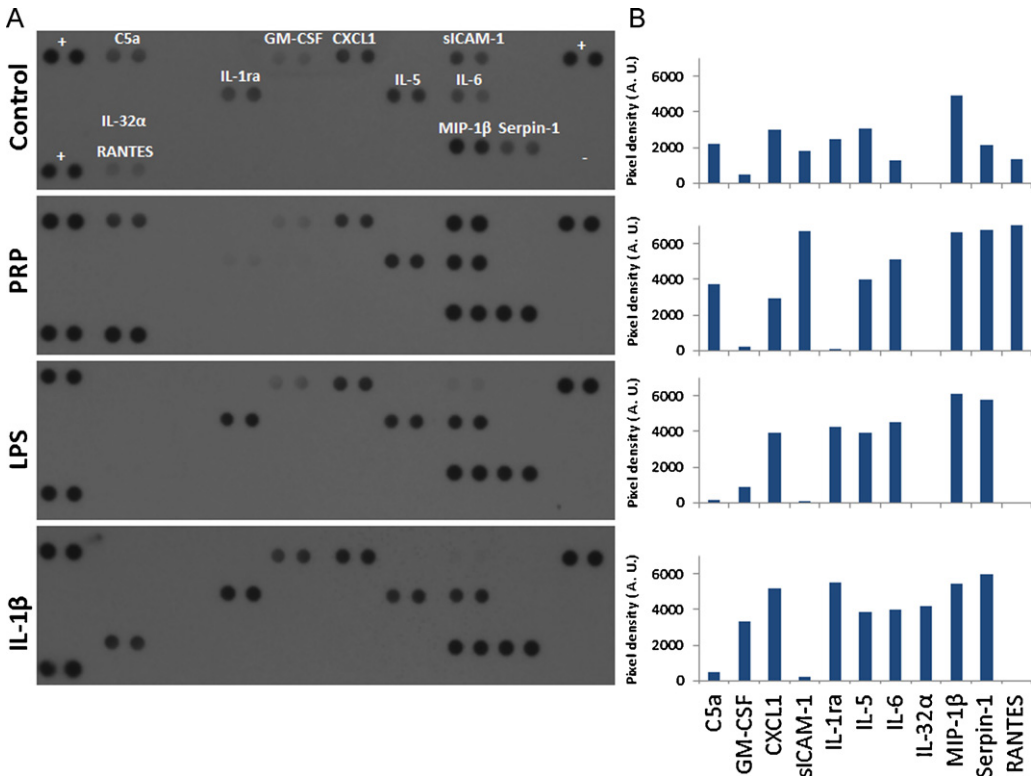

Fig. 3 – *Panel A*: Human cytokine array panel from cultured SAOS-2 cell supernatants obtained 24 h after stimulation with PRP, LPS or IL-1β. *Panel B*: Pixel density plot of the analytes detected in the corresponding cytokine array (in arbitrary units).

angiogenesis, and activating macrophages.[19] On the other hand, transforming growth factor β (TGF-β), a generic growth factor which can be produced by macrophages, fibroblasts and specially by platelets. TGF-β acts in connective tissue repair and regeneration; it is also a potent inhibitor of osteoclast formation and bone resorption, allowing bone formation,[30,31] triggering adjacent cells, such as pre-osteoblasts, to differentiate into mature osteoblasts favouring the initiation of bone remodelling, tissue healing, and bone mineralization.[19]

PRP is an easy available blood derivative that contains high concentrations of growth factors and cytokines which, when locally applied, increase tissue and bone repair acting on osteoprogenitor cell recruitment, proliferation, and differentiation.[32] The clinical use of PRP is an entirely safe procedure, causing no adverse events or postoperative complications.[33] In the present study, and in order to study the effects of PRP on osteoblasts, we made use of the SAOS-2 cell line given its well-documented characterization as a moderately to well-differentiated osteoblastic cell type.[34] Our results show that PRP induces ALP activity in a concentration-dependent manner, and to an extent comparable to those induced by either LPS or IL-1β stimulation (Fig. 1). Since the highest activity was observed with 20% PRP, we decided to use this concentration in the experiments entitled to analyze cytokine and chemokine release.

Other authors have also reported that PRP induces ALP expression. For example, Bertoldi et al.[18] showed that PRP induces SAOS-2 proliferation and differentiation, although repeated administration of PRP was needed in order to achieve better results. Kanno et al.[19] observed that PRP has favourable effects, enhancing the viability of the cells in a dose-dependent manner, ALP activity when the cells reached confluence and the levels of procollagen type I, osteopontin, osteoprotegerin, and core binding factor alpha 1 (cbfa1) mRNAs.[19]

In this study we show that, in a concentration-dependent manner (and similarly to Ogino et al.[17]), PRP obtained from five different donors increased the osteoblastic activity of SAOS-2 cells, as assessed by the increase in ALP activity. It is well known that PRP can differ amongst donors, mainly in terms of cell viability and platelet counts. In addition, the efficacy on bone regeneration might be very diverse, and individual differences in the amount of each growth factor should be considered.[19] For this reason, we made use of a 20% PRP pool in culture medium for stimulation of the SAOS-2 cells and further assessment of the release of pro- and anti-inflammatory cytokines and chemokines into the supernatant. PRP induced the expression of soluble intercellular adhesion molecule-1 (sICAM-1), complement component 5a (C5a) and RANTES, all chemokines important in bone metabolism. IL-6 expression was also increased and, according to previous studies,[35,36] can happen as result of the up-regulation of the above mentioned chemokines.

Chemokine-regulated chemotaxis is an important attribute of osteoblasts, by mediating their migration to sites of bone resorption.[37] Stromal cells and osteoblasts have a potential chemotactic property in response to growth factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGFs), transforming growth factor (TGFβ) and bone morphogenetic

proteins (BMPs)[38] amongst others stimuli.[37] During bone resorption, substances released from bone matrix influence osteoblast chemotaxis, with scant reference to the possible production of chemoattractants by osteoclasts *per se*.[37]

RANTES appears to be an important molecule for communication between osteoclasts and osteoblasts and this signalling is important to osteoblast biology.[37] RANTES is secreted by bone cells and participate in osteoclastogenesis, recruiting osteoblasts and protecting against apoptosis during the resorptive and formative phases of bone turnover, respectively. On the other hand, sICAM-1 exerts important osteotropic effects by mediating the adhesion of osteoblasts to osteoclast precursors, thereby facilitating osteoclast differentiation and bone resorption.[39] Lavigne at al.[35] showed that sICAM-1 expression in osteoblasts obtained from patients with osteoarthritis and osteoporosis was higher than that observed in normal human osteoblasts. In addition, IL-6 and PGE_2 levels were found to be significantly elevated with high ICAM-1 expression compared to low ICAM-1 expression.[35]

C5a, another potent chemoattractant, was also found upregulated in SAOS-2 cells in response to PRP, although downregulated by LPS or IL-1β. C5a can induce the release of cytokines (particularly IL-6) and proteolytic enzymes, and may thus represent an important modulator of osteoblast/osteoclast activities associated with bone resorption.[36]

More than two decades ago, Lynch et al.[10] show that PDGF, one of the most important growth factors found in PRP, promotes new bone formation around periodontal bone defects and stimulate a continuous layer of osteoblasts lining the newly formed bone around periodontal bone defects in Beagle dogs.[10] Since then several clinical and experimental studies have been performed to investigate role of PRP in bone regeneration. Also, mature osteoclasts can produce chemotactic factors, including PDGF, that attract osteoblasts and fibroblasts towards the injured or inflamed sites thereby promoting bone/tissue regeneration.[40–42]

Although we have previously shown that iNOS-derived NO is involved in periodontitis-induced alveolar bone loss by enhancing osteoclast differentiation and activity,[15] and that protein nitration can occur during periodontitis,[23] in the present study, no significant effects due to the different stimuli were observed in terms of either nitrotirosine-containing proteins in the SAOS-2 cells or nitrite (a nitric oxide end-product) concentrations in the cell culture supernatants (Fig. 2). These observations are supported by the study from Da Rocha et al.[43] whom using a primary human osteoblast-like cell obtained after hip arthroplasty, showed that the treatment with IL-1α or TNF-α (or their combination with IFN-γ) during 72 h did not result in altered NO production or increased nitrotyrosine-containing proteins.[43]

As a whole, we may suggest that the effects of NO on bone metabolism are highly dependent on the available amounts of this mediator. NO (especially from iNOS) is involved in bone loss by enhancing osteoclast differentiation and activity, and its potential sources periodontium are inflammatory cells, keratinocytes, fibroblasts, osteoclasts and blood vessels,[44] but not osteoblasts.

In addition, we did not observe any significant increase of RANKL expression in these cells in response to the stimuli, thus suggesting that osteoclast stimuli by the coupling of RANKL/RANK can be done by other cells that may be involved, such as T-cells[45] and periodontal ligament fibroblasts,[46] which are also sources of RANKL.

There is a growing interest on the use of platelet concentrates for treatment of many intraoral clinical conditions involving bone healing. Bone loss secondary to periodontal defects is amongst these situations; however, diverse outcomes showing both positive[47] and negative[48] results have been reported to date, probably reflecting the limited number and heterogeneity of the available data.[33] For example, in a systematic review, Del Fabbro et al.[13] report that PRP may have positive adjunctive effects when used in combination with graft materials, but not with guided tissue regeneration, for treatment of intrabony defects.[13] In this way, it becomes evident the need of additional research on the efficacy of each specific therapeutic approach envolving combination with PRP.[33]

In summary, from the results shown in this study, we can conclude that in cultured SAOS-2 cells PRP stimulates osteoblast activity and a specific cytokine/chemokine pattern release, indicating some of the mediators that can (and cannot) be involved in this activation. Whether these results can be, at least partially, extrapolated to the *in vivo* situation, it still remains to be determined.

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Competing interests

None declared.

Ethical approval

The present study was approved by the Ethics in Human Research Committee of the Araraquara School of Dentistry (UNESP, Araraquara, SP, Brazil; CAAE – 0053.0.199.019-08).

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