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“Impacto de drogas antiplaquetárias na doença e no reparo periodontal experimental em ratos.”

Tese apresentada à Faculdade de Odontologia de Araraquara, Universidade Estadual Paulista "Júlio de Mesquita Filho", como parte dos requisitos para a obtenção do título de Doutor em Odontologia, Área de Concentração - PERIODONTIA.

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DADOS CURRICULARES

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Com amor, dedico este trabalho aos meus pais ***Luiz Roberto*** e ***Ana Lúcia*** e as minhas irmãs ***Luciana*** e ***Izabel***.....

"Tudo o que um sonho precisa para ser realizado é alguém que acredite que ele possa ser realizado". (Roberto Shinyashiki)

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"Nas grandes batalhas da vida, o primeiro passo para a vitória é o desejo de vencer"

-Mahatma Gandhi-

FILOSOFIA DO SUCESSO

-Napoleon Hill-

Se você pensa que é um derrotado,
você será derrotado.
Se não pensar “quero a qualquer custo!”
Não conseguirá nada.
Mesmo que você queira vencer,
mas pensa que não vai conseguir,
a vitória não sorrirá para você.

Se você fizer as coisas pela metade,
você será fracassado.
Nós descobrimos neste mundo
que o sucesso começa pela intenção da gente
e tudo se determina pelo nosso espírito.

Se você pensa que é um malogrado,
você se torna como tal.
Se você almeja atingir uma posição mais elevada,
deve, antes de obter a vitória,
dotar-se da convicção de que
conseguirá infalivelmente.

A luta pela vida nem sempre é vantajosa
aos fortes nem aos espertos.
Mais cedo ou mais tarde, quem cativa a vitória
é aquele que crê plenamente....

EU CONSEGUIREI!

SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

ICAM-2: Receptor de superfície

VEGF: Fator de crescimento endotelial vascular

IL-6: Interleucina-6

TNF-alfa: Fator de necrose tumoral alfa

IFN-gama: Interferon gama

COX-1: Ciclooxigenase-1

P2Y12: Receptor de membrana plaquetário

IL-1beta: Interleucina 1beta

CXCL4: Fator de plaquetas-4

CCL 5: Rantes

GPIIb/IIIa: Receptor de membrana plaquetário

ADP: Adenosina di-fosfato

MPO: Atividade de mieloperoxidase

COBEA: Comitê de Ética de Experimentação Animal Local

CEJ-ABC: Distância da junção cemento-esmalte à crista óssea alveolar

CD40L: Glicoproteína de membrana

PRP: Plasma rico em plaquetas

COX-2: Ciclooxigenase-2

CXCL12: Fator derivado de células do estroma

PDGF: Fator de crescimento derivado de plaquetas

PMNs: Leucócitos polimorfonucleares

TRAP: Fosfatase alcalina tartarato resistente

MSC: Células mesenquimais indiferenciadas

CD 271: Marcador de células mesenquimais indiferenciadas

KI67: Marcador de proliferação celular

CD 45: Marcador de linfócitos

H&E: Hematoxilina e eosina

BV/TV: Volume de tecido ósseo/ volume tecidual

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RESUMO

As plaquetas são um reservatório natural de diversos mediadores biológicos e, uma vez ativadas, expressam fatores de crescimento pró e anti-angiogênicos, quimiocinas e monoaminas, modulando a ativação e manutenção dos processos inflamatório e de reparo. Os objetivos deste trabalho foram avaliar o impacto do tratamento com drogas antiplaquetárias, aspirina (Asp) e clopidogrel (Clop), sobre a doença periodontal e o processo de reparação dos tecidos periodontais após indução de periodontite experimental em ratos. Para a avaliação do processo inflamatório foram realizadas análise microscópica, análise da expressão das quimiocinas CXCL4 e CCL5 por ELISA e da atividade de mieloperoxidase (MPO). Para avaliação do reparo dos tecidos periodontais, foram realizadas análise microscópica, análise da expressão de CXCL12, CXCL4, CCL5 e PDGF por ELISA, contagem de osteoclastos e vasos sanguíneos através de reações de imunohistoquímica e caracterização das células mesenquimais indiferenciadas por imunohistoquímica para CD 271 e imunofluorescência de dupla marcação utilizando CD 271 associado ao CD45, KI67 e TUNEL. Células mesenquimais indiferenciadas derivadas da medula óssea de humanos foram utilizadas *in vitro* para avaliação da proliferação celular por imunocitofluorescência. Três dias após a instalação da ligadura, verificou-se que o tratamento com as drogas antiplaquetárias abreviou o processo inflamatório, observado através dos aspectos microscópicos, da redução da atividade da enzima mieloperoxidase ($p<0.001$), e queda da expressão de CXCL4 ($p<0.05$), entretanto o tratamento com Clop de forma mais acentuada que o da Asp. Após retirada da ligadura, o tratamento com Clop acelerou o processo de reparo através da redução significativa da expressão de CXCL12, CXCL4, PDGF, número de vasos sanguíneos e osteoclastos; por outro lado, o tratamento com Asp apresentou efeitos significativos apenas na redução da expressão de CXCL12 e CXCL4. Por sua vez, tratamento com Clop aumentou o número de osteoblastos, expressão de células mesenquimais indiferenciadas e os índices de proliferação e apoptose dessas células *in vivo*. *In vitro*, tratamento com Clop apresentou aumento na taxa de proliferação das células mesenquimais analisadas. Os resultados apontam

que tanto a Asp quanto o Clop, apresentam perfil antiinflamatório com repercursão positiva sobre a aceleração do processo de reparo, sendo o Clop mais eficaz em ambos os processos.

Palavras-chave: periodonto, plaquetas, inibidores da agregação de plaquetas.

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ABSTRACT

Platelets are a natural reservoir of several biological mediators and once activated, express growth factors, pro- and anti-angiogenic factors, chemokines and monoamines modulating the activation and maintenance of the inflammatory and repair process. The aim of this study was to evaluate the impact of antiplatelet drugs, aspirin (Asp) and clopidogrel (Clop) on periodontal disease and the repair of periodontal tissues after induction of experimental periodontitis in rats. For the evaluation of the inflammatory process, microscopic evaluation, analysis of the expression of the chemokines CXCL4 and CCL5 by ELISA and myeloperoxidase activity (MPO) were realized. To evaluate periodontal tissue repair we performed microscopic examination, analysis of the expression of CXCL12, CXCL4, CCL5 and PDGF by ELISA, and blood vessels and osteoclast count by immunohistochemistry. Also, characterization of undifferentiated mesenchymal stem cells by immunohistochemical staining for CD 271 (stem cell marker) and immunofluorescence double labeling using CD 271 associated with CD45, Ki67 and TUNEL were realized. Undifferentiated mesenchymal stem cells derived from human bone marrow were used for in vitro evaluation of cell proliferation by immunocitofluorescence. Three days after ligature placement, we found that treatment with antiplatelet drugs abbreviated the inflammatory process observed by the microscopic analysis, reduction of myeloperoxidase activity ($p < 0.001$) and decreased the expression of CXCL4 ($p < 0.05$), but treatment with Clop more pronounced than Asp. After removal of the ligature, Clop treatment accelerated the repair process by significantly reducing the expression of CXCL12, CXCL4, PDGF number of blood vessels and osteoclasts, on the other hand, treatment with Asp only showed significant effects in reducing the expression of CXCL12 and CXCL4. In turn, Clop treatment increased the number of osteoblasts, expression of undifferentiated mesenchymal cells and the proliferation and apoptosis index of these cells in vivo. In vitro treatment with Clop showed an increase in the proliferation rate of undifferentiated mesenchymal cells analyzed. The results showed that both Asp as Clop, exhibit anti-inflammatory profile with positive repercusion on accelerating the repair process, Clop being the most effective in both processes.

Keywords: periodontium, platelets, platelet aggregation inhibitors.

INTRODUÇÃO

1. Introdução

A doença periodontal é o resultado das reações imunoinflamatórias decorrentes da presença do biofilme dental cuja extensão alcançam níveis mais profundos do tecido conjuntivo e osso alveolar[9,15]. A partir da neutralização dos micro-organismos que compõem o biofilme dental, devolve-se ao organismo a capacidade de reparar total ou parcialmente as perdas das estruturas e função do periodonto[1,3]. Tanto o processo inflamatório quanto o processo de reparo são processos complexos, que envolvem diferentes tipos celulares os quais são coordenados por diferentes mediadores químicos e fatores de crescimento[2,6]. Dentre os componentes que fazem parte desses processos, as plaquetas são um reservatório natural de diversos mediadores biológicos e, uma vez ativadas, expressam fatores de crescimento pró e anti-angiogênicos, quimiocinas, monoaminas, e citocinas, modulando a ativação e manutenção dos processos inflamatórios e de reparo inclusive no caso da reparação da doença periodontal[12,14].

Tal afirmação é suportada por um estudo experimental demonstrando que a redução no número de plaquetas (trombocitopenia) induz uma queda na concentração de VEGF e endostatina com consequente comprometimento na angiogênese, interferindo negativamente na reparação do osso alveolar e tecido gengival[10]. Por outro lado, verificou-se em um modelo de periodontite experimental induzido por ligadura em ratos, que o tratamento com drogas antiplaquetárias mediam a inibição de diversas citocinas com atividade pró-inflamatória, como por exemplo IL-6, TNF-alfa e TXA-2, culminando com a redução do influxo de células inflamatórias[4]. Confirmando essas informações, Liu et al[8] (2011) mostraram que a administração local de aspirina, reduziu os níveis de IFN-gama e TNF-alfa no sítio de implantes e acelerou o processo de reparo com células mesenquimais indiferenciadas derivadas da medula óssea, aumentando também a sua taxa de sobrevivência em defeitos na calvária de camundongos, sugerindo uma relação direta entre a aspirina e o aumento na expressão e atividade das células mesenquimais indiferenciadas no processo de reparação/regeneração[8,13]. Levando em consideração a atuação de outras células que participam ativamente do processo inflamatório e de reparo tecidual, estes dados evidenciam a relevância das plaquetas nesses processos e tais funções têm sido dissociadas do conhecido envolvimento das plaquetas na trombose e hemostasia[7,12,14].

Diversas drogas são utilizadas como agentes antiplaquetários, sendo o ácido acetilsalicílico (aspirina) o mais comum, embora outras drogas como o clopidogrel, dipiridamol ou ticlopidina possam ser prescritos. A aspirina inativa irreversivelmente a

atividade da enzima ciclooxigenase tipo-1 (COX-1) e consequentemente a síntese de TXA-2 por difusão através da membrana das plaquetas enquanto o clopidogrel age através de uma ligação irreversível ao receptor de membrana P2Y12 das plaquetas. Esta ligação diminui a liberação dos grânulos denso e alfa-plaquetários pelas plaquetas, inibindo a ação da beta3 integrina e atenuando a síntese de IL-1beta após ativação plaquetária[5,11].

A partir de dados prévios que sinalizam que drogas antiplaquetárias interferem no processo inflamatório instalado a partir de um modelo experimental de doença periodontal, o objetivo do presente trabalho foi avaliar o impacto do tratamento com Asp e Clop nos períodos iniciais da indução da doença periodontal e reparo tecidual após a remoção do estímulo patogênico (ligadura).

PROPOSIÇÃO

2.1. Hipótese

Nossa principal hipótese tem o objetivo de responder a seguinte questão: Qual é o impacto das drogas inibidoras da ativação de plaquetas, principalmente Asp e Clop, sobre a doença periodontal e reparo do periodonto após indução de periodontite experimental por ligadura? Tais respostas podem identificar mediadores e aventar prováveis mecanismos que são importantes para o melhor entendimento da doença e do processo de reparo periodontal. Como relevância clínica, podemos aventar que a aspirina e o clopidogrel, drogas antiplaquetárias comumente prescritas para o tratamento de pacientes com doenças cardiovasculares, podem interferir tanto no processo inflamatório quanto no processo de reparo da doença periodontal.

2.2. Objetivos Específicos

- ✓ Avaliar *in vivo* o efeito da inibição da atividade de plaquetas sobre a expressão de CXCL4 e CCL5 e a atividade da enzima mieloperoxidase (MPO) que estão ligados ao recrutamento de leucócitos polimorfonucleares no perfil da resposta imune inflamatória na indução da doença periodontal experimental.

- ✓ Avaliar *in vivo* o efeito do tratamento sistêmico com Asp e Clop sobre a angiogênese e sobre o número de osteoclastos na reparação da doença periodontal experimental através da avaliação imunohistoquímica da vascularização tecidual com coloração específica do Fator de Von Willerbrand e fosfatase ácida tartarato resistente (TRAP), respectivamente; assim como a expressão por ELISA de CXCL12, CXCL4, CCL5 e PDGF que estão ligados ao recrutamento de leucócitos polimorfonucleares e indução da angiogênese e conseqüentemente à reparação tecidual após indução de periodontite experimental.

- ✓ Avaliar *in vivo* o efeito da Asp e Clop sobre a expressão de células mesenquimais indiferenciadas na reparação da doença periodontal experimental através da avaliação imunohistoquímica da expressão de CD271, assim como a caracterização fenotípica

desses tipos celulares e sua proliferação e apoptose através de imunofluorescência de dupla marcação de CD271 associado ao CD45, KI67 e TUNEL, respectivamente. Avaliar *in vitro* a proliferação de células mesenquimais indiferenciadas derivadas da medula óssea de humanos através de ensaios de imunocitofluorescência.

CAPÍTULOS

CAPÍTULO 1

Antiplatelet drugs reduce the immunoinflammatory response in a rat model of periodontal disease.

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Antiplatelet drugs reduce the immunoinflammatory response in a rat model of periodontal disease.

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Running title: Antiplatelet drugs and inflammation.

Keywords: periodontal disease; inflammation; systemic host effect; polymorphonuclear leukocyte.

ABSTRACT

Background and Objective: After activation, platelets express mediators that modulate inflammation. We hypothesized that drug-induced platelet inactivation may interfere in the inflammatory process in experimental periodontitis by suppressing the release of biological mediators from platelets to the injury.

Material and Methods: To evaluate the effects of antiplatelet drugs on experimental periodontitis 60 rats were randomly assigned in 6 groups (n=10) and ligatures were placed around lower first molars on three groups. The other three groups were not subjected to the induction of periodontal disease and were used as negative controls. During experimental period animals were given aspirin (Asp) (30 mg/kg) or clopidogrel (Clop) (75 mg/kg) intragastrically once daily for 3 days. At day 3 they were sacrificed and gingival tissue were used to evaluate myeloperoxidase (MPO) activity and the expression of CCL5 (Rantes) and CXCL4 (Platelet Factor 4). Hemi-mandibles were used for microscopic evaluation.

Results: Clop reduced the inflammatory infiltrate and increased the amount of collagen fibers. Histometric analysis showed that Clop impaired alveolar bone loss. Expression of CXCL4 was significantly increased and CCL5 decreased ($P<0.001$) in rats subjected to periodontitis. Systemic administration of Asp and Clop induced a significant decrease ($p<0.05$) in the expression of CXCL4; however it did not affect the expression of CCL5 compared to saline treated animals. Treatment with antiplatelet drugs resulted in significant reduction of MPO activity when compared to saline-treated animals with periodontal disease.

Conclusion: Systemic administration of Clop showed a more pronounced attenuation of the inflammation associated with periodontitis.

INTRODUCTION

Periodontitis is an infectious disease of periodontal tissues, initiated by a small group of anaerobic gram-negative bacteria that colonize the subgingival area[1]. It results in periodontal pocket formation and ulceration of the epithelial lining, which may lead to a transient bacteremia and loss of the teeth[2]. This intermittent bacteremia can result in an increase of circulating leukocytes and consequently in the concentration of leukocyte-derived mediators, in addition to enhanced activation and subsequent aggregation of platelets[3].

Systemic inflammation is characterized by an increase in the number of platelets and also their activation, and particularly periodontitis, has been associated with elevated number of circulating platelets[2]. Activated platelets release chemokines, monoamines, and pro-inflammatory mediators and express a variety of pro-inflammatory surface receptors[4]. In addition, upon activation, platelets also express both pro- and anti-angiogenic factors, such as vascular endothelial growth factor (VEGF) and endostatin, respectively[5], as well as a variety of adhesion molecules and receptors with known involvement in the immune response, such as P-selectin, ICAM-2, toll-like receptors and chemokine receptors[6]. CXCL4 (Platelet Factor 4) and CCL5 (Rantes) are platelet chemokines released upon proper activation. CXCL4 promotes blood coagulation by moderating the effects of heparin-like molecules, while CCL5 is chemotact for T cells and plays and an active role in recruiting leukocytes into inflammatory sites. They act in the onset of tissue repair and establish a pro-inflammatory environment that culminates in the recruitment and activation of a variety of immune cells[7].

In the context of periodontitis, we have previously demonstrated that drug-induced platelet inactivation results in attenuation of the inflammation by reducing the concentration of the inflammatory mediators Interleukin-6 (IL-6), Tumor Necrosis Factor-alpha (TNF-alpha) and Thromboxane A2 (TXA-2)[8]. Additionally, we have shown that thrombocytopenia leads to a delay in periodontal healing, which is associated, at least in part, with decreased serum concentrations of platelet- derived VEGF and endostatin[9].

In addition to their defensive role in inflammatory process, platelets appears to be involved in the pathogenesis of various inflammatory diseases such as cardiovascular disorders as atherosclerosis or atherothrombosis[6,10]. The modulation of platelet activity in vascular alterations is of great interest, considering that antiplatelet therapy is effective not only in the prevention of atherothrombosis, but also to inhibit atherosclerosis progression. Several drugs are used as antiplatelet agents, being acetylsalicylic acid (aspirin) the most commonly prescribed, although other agents, such as ticlopidine, clopidogrel, dipyridamole or GPIIb/IIIa

inhibitors (eptifibatide, the monoclonal antibody abciximab) are also prescribed. These drugs have in common the fact that none of them affect the number of platelets, but inhibit platelet function/aggregation[11]. Aspirin enters platelets by diffusion through the membrane and irreversibly inactivates cyclooxygenase-1 (COX-1) and, consequently, the synthesis of TXA_2. Clopidogrel acts by irreversible antagonism of platelet $P2Y_{12}$ receptors expressed in the platelet membrane thus inhibiting the effects of the endogenous ligand adenosine diphosphate (ADP).

Because platelets have a crucial role in the inflammatory process, we hypothesized that antiplatelet drugs may affect periodontal disease. The purpose of this study was to evaluate the effects of aspirin and clopidogrel; the two most frequently prescribed antiplatelet drugs[11], on ligature-induced experimental periodontitis in rats, by the production of CXCL4 and CCL5 and MPO (a marker for leukocyte migration), as well as histological analysis.

MATERIAL AND METHODS

Animals

Sixty male adult Holtzman rats (*Rattus norvergicus albinus*) weighing 250-300 g were housed under similar conditions in cages with access to food and water *ad libitum*. During the whole experimental protocol, the rats were kept in a quiet room with controlled temperature (22 ± 1^0C), humidity (65-75%) and a 12h light-dark cycle. All experimental protocols were approved by the local Ethics Committee for Animal Experimentation and performed in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

Chemicals

All drugs and reagents were purchased from Sigma-Aldrich (São Paulo, Brazil), unless otherwise stated. Ketamine and xylazine chloride (Francotar® and Virbaxil® respectively, both from Virbac do Brasil Ind. Com. Ltda, São Paulo, SP, Brazil) were used for dissociative anesthesia of the animals.

Experimental design

Rats were randomly distributed into 6 groups (10 animals/group) as follow: vehicle-treated: ‘NaCl’, Aspirin-treated: ‘Asp’, Clopidogrel-treated: ‘Clop’, Periodontitis+vehicle-treated: ‘DP+NaCl’, Periodontitis+Aspirin-treated: ‘DP+Asp’, Periodontitis+Clopidogrel-treated:

'DP+Clop'. The animals in the groups subjected to experimental periodontitis were anesthetized with 1 mg/kg body weight of ketamine and 0.4 mL/kg of xylazine for the induction of experimental periodontal disease by the insertion of a 3.0 cotton ligature in a submarginal position on the lower right and left first molars of each rat, as previously described[12]. The animals from the other three groups were not subjected to the induction of periodontal disease and were used as negative controls. During the 3-day experimental period, groups of animals were treated by gavage with intragastrical daily doses of aspirin (Asp; 30 mg/kg; Sigma-Aldrich), clopidogrel (Clop; 75 mg/kg; Sanofi-Aventis, São Paulo, Brazil) or the same volume of vehicle (NaCl 0.9%), as described above. These doses have been previously shown to cause effective inhibition of platelet aggregation and thrombus formation in rats[13-16].

Harvesting and processing of samples

At the end of the 3-day experimental period, animals were killed by anesthesia overdose and the mandibles were carefully removed and dissected. Gingival-mucosal tissues from the right mandibular first molars were removed and processed for the analysis of myeloperoxidase activity (see below) and CCL5 and CXCL4 contents by enzyme-linked immunosorbent assay (ELISA kits from Life Technologies, Carlsbad, CA USA), according to the manufacturer's instructions. The chemokine results were normalized to the total protein contents in each sample and expressed as pg/mg of protein.

Myeloperoxidase activity assay

The activity of myeloperoxidase (MPO), a hemoprotein located in azurophilic granules of neutrophils, was used as a biochemical marker for neutrophil infiltration into the studied tissues. MPO activity was measured according to the method originally described by Bradley et al (1982)[17] with some modifications[18], after heating the organ homogenates at 60°C during 2h in order to inactivate endogenous catalase[19].

Briefly, after homogenizing the tissue samples in the presence of hexadecyltrimethylammonium bromide (HTAB, Sigma Chem. Co., St. Louis, EUA) in order to disrupt the granules, the tubes were centrifuged at 10,000 g during 5 min. MPO activity was analyzed in the supernatants by its capacity to catalyze the oxidation of o-dianisidine dihydrochloride (Sigma Chemical Co., St. Louis, EUA) in the presence of hydrogen peroxide (Merck, Darmstadt, Germany). Absorbance increase rate was monitored at 460 nm (Espectra

max Plus 384, Molecular Devices Inc., Sunnyvale, EUA) and the obtained Vmax (maximum speed) parameter related to the enzyme activity using a molar extinction coefficient of 11,300 M^{-1} cm^{-1}.

Histomorphometric analysis

Subsequently, decalcification of the left hemi-mandible was carried out in 4.13% EDTA solution (pH 7.2) at room temperature for approximately 3 months. Serial paraffin sections 5 µm thick were obtained from buccal–lingual aspects of the whole left first molars and subsequently stained with hematoxylin and eosin (H&E) for histological/histometric evaluation. The severity of the inflammatory process in the connective tissue between the highest peak of the alveolar bone crest and the cemento-enamel junction was determined using a score of PMN/mononuclear cell infiltration as previously described[20]. Severity was ranked as 0 (no inflammatory cells), 1 (slight inflammation with a few inflammatory cells), 2 (moderate inflammation with a remarkable number of inflammatory cells scattered throughout the connective tissue above the bone crest) or 3 (severe inflammation with predominance of inflammatory cells). The amount of collagen fibers was determined by a semi-quantitative analysis using a score of number and density of collagen fibers as previously described[21]: score 1 = absent; score 2 = mild: individual collagen fiber arrangement resembling a normal connective tissue, permeated by negative spaces indicative of non-fibrous components of the extracellular matrix; score 3 = moderate: areas with individual collagen fibers alternated with areas of eosinophilic extracellular matrix without typical linear and undulate formations; and score 4 = intense: collagen fibers merged within an eosinophilic extracellular matrix without the typical linear and undulate formations, not allowing to distinguish the individual fibers. 5 randomly chosen sections of 5 animals per group were examined at 200X magnification. The analysis was conducted by a single examiner that was blind to the experimental groups using an optical microscope (Diastar Cambridge) set at 200 X magnification. In order to estimate the extent of alveolar bone loss caused by the progression of periodontitis, the distance between the cemento-enamel junction and the highest peak of the alveolar bone crest (CEJ–ABC) was measured in the buccal bone region in the other 5 animals per group as previously described[22].

Statistical analysis

All data are expressed as mean ± SEM. Differences among the groups were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons. P values less than 5% were considered statistically significant.

RESULTS

All animals were alive at the end of the experimental period, and the recorded body weights were 271.0±3.8, 262.0±3.3, 255.0±1.7, 248.0±7.1, 251.0±3.1 and 267.0±3.7 g for NaCl, Asp, Clop, DP+NaCl, DP+Asp and DP+Clop groups, respectively. Neither occult bleeding nor hematoma after gingival intervention was observed.

Microscopic analysis

Ligature placement for 3 days induced an inflammatory response consistent with periodontal disease in humans (Figure 1). Clop-treated animals with ligature-induced periodontitis showed a significant decrease (*$p<0.05$) decrease in the inflammatory infiltrate (Figure 2A) and increase (*$p<0.05$) in the amount of collagen fibers (Figure 2B).

After ligature placement, CEJ-ABC distance increased by 40% (*$p<0.0001$). Treatment with Clop during this process affected the loss of periodontal bone height by reducing CEJ-ABC distance in 40% (#$p<0.05$), indicating that bone resorption was significantly reduced by systemic administration of this drug (Figure 3).

ELISA analysis

After induction of periodontal disease, gingival content of CXCL4 increased by 40% ($p<0.001$). Systemic administration of Asp and Clop reduced by 20% ($p<0.05$) this expression compared to saline treated animals (Figure 4A).

The gingival level of CCL5 decreased by 75% ($p<0.001$) after ligature induced periodontal disease and it was not affected by treatment with antiplatelet drugs (Figure 4B).

Myeloperoxidase activity

After induction of periodontal disease, MPO activity increased significantly ($p<0.0001$). Systemic administration of Asp and Clop reduced by 50% ($p<0.001$) this activity when compared to saline treated animals (Figure 5).

DISCUSSION

Platelets are cellular corpuscles that once activated play an important role in the production of the inflammatory response and tissue repair in various conditions such as periodontal disease and periodontal tissue repair[23,24]. Furthermore, in humans, blood platelet count decreases after periodontal therapy[25], indicating that these blood cells are sensitive to alterations in the inflammatory status of the periodontium. In fact, blood platelets are key elements that link the processes of hemostasis, inflammation and tissue repair. Once stimulated, platelets express a variety of surface receptors and release growth factors and other biological mediators with pro and anti-angiogenic features[26]. As platelets are important cells in the development of the inflammatory process and the previous results obtained by our group showing that systemic administration of aspirin and clopidogrel attenuates the inflammation associated with periodontal disease without affecting the repair process once the pathogenic stimulus is removed, we hypothesized that the inhibition of platelet activation induced by drugs could influence the clearance of some inflammatory mediators, such as chemokines.

To the best of our knowledge, the effects of antiplatelet drugs on periodontal disease in rats were just evaluated by our group[8]. It has been documented elsewhere that the antiplatelet drug doses used in the present study were effective to reduce platelet aggregation[27-29], although no clinical effects were observe in the animals: the coat remained with normal appearance and both the skin and the oral mucosa showed no changes in color or texture, thus indicating the absence of hematological disorders. Bennett et al (1999 and 2001)[11,30] showed that treatment with antiplatelet drugs can cause undesirable side effects as thrombotic thrombocytopenic purpura, rash and reversible granulocitopenia with treatment- related with thienopyridines in humans.

We have used a well-established model of experimental periodontitis that presents histological and immunological characteristics similar to those of periodontal disease in humans[9,31,32]. After 3 days of ligature-induced gingival inflammation, we expected to see an acute inflammation compatible with inflammatory gingivitis without periodontal support involvement. According to our results, after 3 days of periodontal disease, alveolar bone loss, an increase in myeloperoxidase activity and inflammatory infiltrate and a reduction in the amount of collagen fibers was observed. However, Clop-treatment affected the loss of periodontal bone height by reducing CEJ-ABC distance, in addition to attenuate signs of inflammation, which were characterized by reduction of the inflammatory infiltrate and an

increase in the amount of collagen fibers, when compared to the remaining experimental groups. These microscopic results are in agreement with our previous study[8], where we also showed a decrease in the expression of the inflammatory mediators TNF-α, IL-6 and TXA_2 and reduction of the severity of the inflammatory process after antiplatelet therapy after 15 days of experimental periodontitis. The exact pathogenesis of clopidogrel-abreviating inflammation is well established in the literature. In animal models, antiplatelet therapy with clopidogrel or an inhibitor of TXA-2 receptor was associated with reduced expression of inflammatory markers such as P-selectin, CD40L, tissue factor, and improved endothelial cell function[33]. On the other hand, Garcia et al (2011)[34] showed a pro-inflammatory effect related to clopidogrel treatment in a rat-induced arthritis model. Clopidogrel is a thyenopiridine that acts by irreversibly binding to P2Y12 receptor. This binding diminishes the extent of alpha and dense granules release from platelets[34] and also inhibits beta3 integrin engagement attenuating the synthesis of IL-1beta induced upon platelet stimulation[35]. This cytokine is produced as an inactive precursor (pro-IL1beta) that is subsequently activated by caspase 1 before being secreted as a biologically active IL-1beta[36]. As beta3 integrin plays a critical role in osteoclast formation, adhesion and bone resorption[37], its inhibition by clopidogrel can explain our findings that was also observed by Su et al (2012)[38] that showed that mice treated with the clinical inhibitor of P2Y12, were protected from pathologic osteolysis. By irreversibly binding to cyclooxygenase-1 (COX-1), aspirin inhibits TXA-2 production, blocking TXA2-induced aggregation of platelets and vasoconstriction. It also modifies the COX-2 enzymatic activity, inducing lipoxin production (anti-inflammatory effect) and inhibiting prostanoid production (generally pro-inflammatory)[39.] In in vitro studies, aspirin inhibits NF-κB-dependent induction of adhesion molecules in endothelial cells and subsequent monocyte adhesion, suggesting this may be another mechanism through which aspirin acts as an anti-inflammatory agent[40].

In fact, in the inflammatory process established 3 days after ligature placement it is expected to occur a significant influx of polymorphonuclear and mononuclear cells, and it is possible that a platelet-derived CXCL4 and CCL5 signaling mechanism is involved in this recruitment. In fact, CXCL4 together with CCL5 participates in the recruitment of polymorphonuclear leukocytes and monocytes into the blood vessel wall[7]. In this study we showed that gingival contents of CXCL4 increased significantly after the induction of periodontal disease, and was significantly reduced by both aspirin and clopidogrel; CCL5 expression was dramatically reduced in rats with ligature-induced periodontal disease and unaffected by antiplatelet

treatment. This result was somewhat unexpected given the prominent role of CCL5 in the inflammatory process supported by the literature. Shah (2011)[41] demonstrated high plasma levels of systemic CCL 5 in patients with systemic lupus erythematosus and rheumatoid arthritis. Moreover, Offenbacher (2009)[42] and Thunell (2010)[43] showed high levels of CCL 5 in periodontal disease in humans. Also, Gamonal (2000)[44] demonstrated a significant reduction in CCL5 expression in gingival crevicular fluid, after periodontal treatment in humans. The differences in the main source of both chemokines can partially explain our results. While platelets are not the main source of CCL5, as it can also be produced by other cell types in the gingival tissue (e.g., epithelial cells, fibroblasts and macrophages), CXCL4 is only released from alpha-granules of activated platelets.

This background demonstrates the relevance of antiplatelet treatment on periodontal disease. The first point to be mentioned is that antiplatelet drugs are routinely prescribed in the prevention of cardiovascular diseases as stroke and atherosclerosis in elderly patients. Also, it is well established the role of platelets in inflammation and the mechanisms by which antiplatelet drugs affects periodontal microenviroment may provide clues to new therapeutic strategies.

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FIGURES

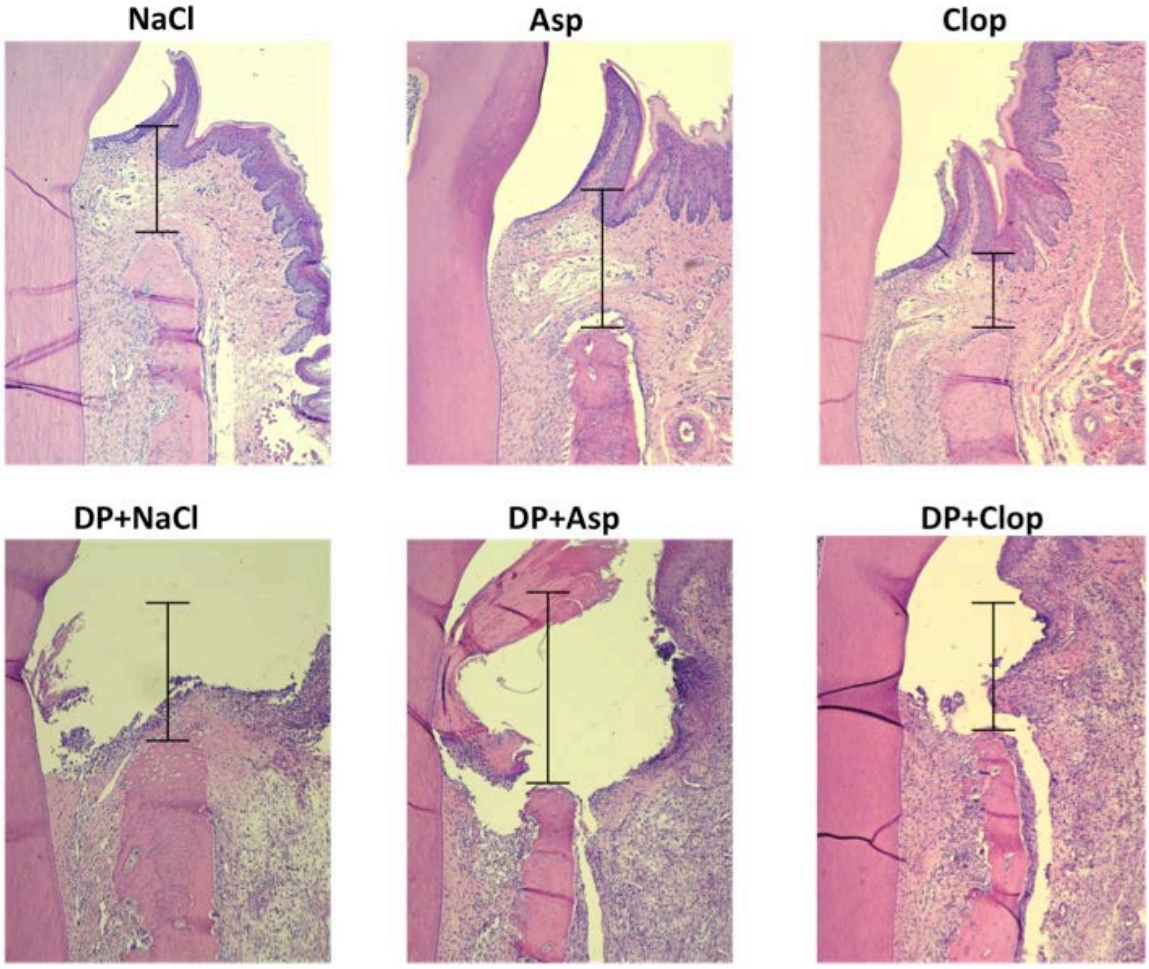

FIGURE 1. Histological analysis of the gingival margin region of left lower first molar of rats treated with NaCl 0.9%, Asp (30 mg/kg) or Clop (75 mg/kg) submitted or not to experimental periodontal disease (vehicle-treated: 'NaCl', Aspirin-treated: 'Asp', Clopidogrel-treated: 'Clop', Periodontitis+vehicle-treated: 'DP+NaCl', Periodontitis+Aspirin-treated: 'DP+Asp', Periodontitis+Clopidogrel-treated: 'DP+Clop') stained with hematoxylin and eosin (H&E). In animals with experimental periodontal disease, the presence of tissue destruction associated with vertical and horizontal bone resorption and intense inflammatory infiltrate can be observed, which was reduced by treatment with Clop for 3 days. Representative images (100X magnification) from each experimental group (n=10 each).

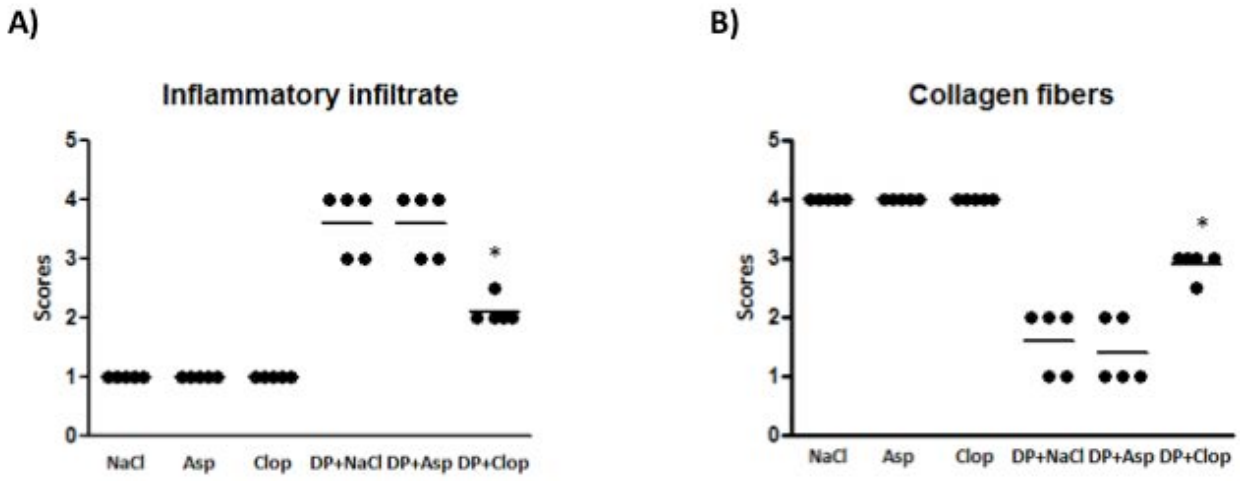

FIGURE 2. Effect of systemic treatment with Asp or Clop on the severity of inflammation secondary to ligature-induced periodontal disease in rats. Five H&E stained slides per animal, including the first molars and surrounding periodontal tissues, from 5 animals in each group (vehicle-treated: 'NaCl', Aspirin-treated: 'Asp', Clopidogrel-treated: 'Clop', Periodontitis+vehicle-treated: 'DP+NaCl', Periodontitis+Aspirin-treated: 'DP+Asp', Periodontitis+Clopidogrel-treated: 'DP+Clop') were analyzed for the intensity of inflammatory infiltrate (A) and the amount of collagen fibers (B). Gingival tissues from non-ligated teeth from control animals ('NaCl, Asp and Clop') were also analyzed for comparative purposes. Circles indicate the score of each animal and the horizontal lines the average of the scores for each experimental group. Values are expressed as mean ± SD. *P<0.05 vs DP+NaCl and DP+Asp.

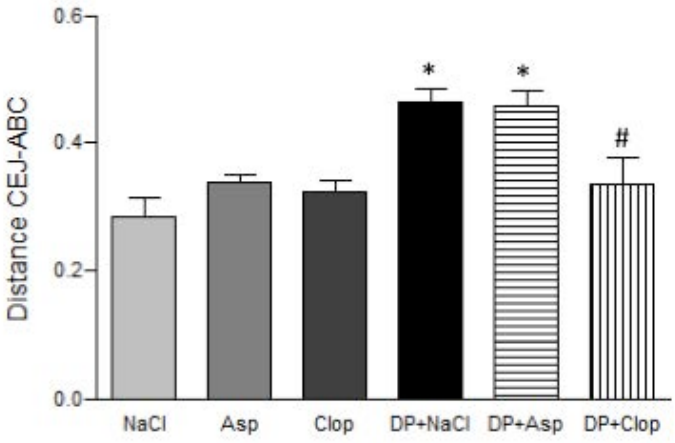

FIGURE 3. Effect of systemic treatment with Asp or Clop on alveolar bone loss secondary to ligature-induced periodontal disease in rats (measured as the distance CEJ-ABC). 3 H&E-stained slides per animal, including the first molars and surrounding periodontal tissues, from 5 animals in each group (vehicle-treated: 'NaCl', Aspirin-treated: 'Asp', Clopidogrel-treated: 'Clop', Periodontitis+vehicle-treated: 'DP+NaCl', Periodontitis+Aspirin-treated: 'DP+Asp', Periodontitis+Clopidogrel-treated: 'DP+Clop'). Values are expressed as mean ± SD. *P<0.0001 vs NaCl, Asp and Clop; #P<0.05 vs DP+NaCl and DP+Asp.

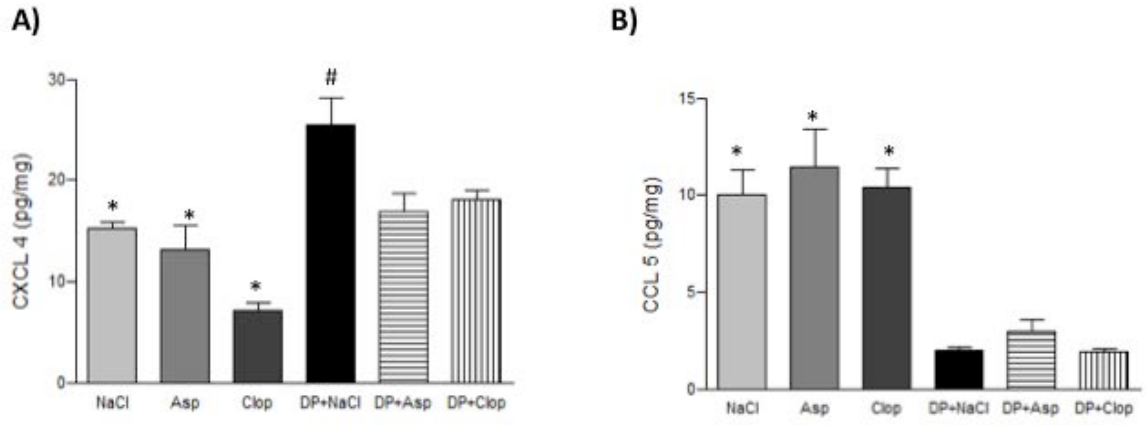

FIGURE 4. Effect of systemic treatment with Asp or Clop on the gingival contents of CXCL4 (panel A) and CCL5 (panel B) in rats with ligature induced periodontal disease. The samples were harvested from 10 animals in each group, and analyzed in duplicate. The chemokine results were normalized to the total protein contents in each sample and expressed as pg/mg of protein. Values are expressed as mean ± SD. $^{\#}$P<0.05 vs. DP+Asp and DP+Clop; *P<0.001 vs. DP+NaCl, DP+Asp and DP+Clop.

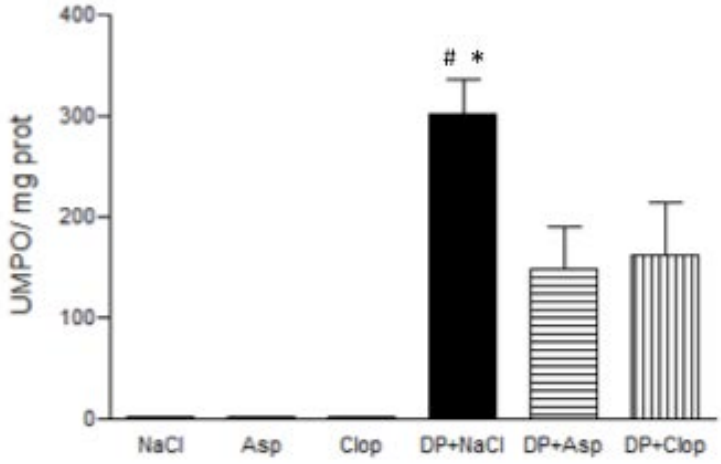

FIGURE 5. Effect of systemic treatment with Asp or Clop on the MPO activity present in gingival samples collected from rats with ligature induced periodontal disease (in units if MPO/mg of protein). Values expressed as mean ± SD. # P<0.0001 vs NaCl, Asp and Clop; *P<0.001 vs. DP+Asp and DP+Clop.

CAPÍTULO 2

Clopidogrel enhances periodontal repair through decreased inflammation.

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Clopidogrel enhances periodontal repair through decreased inflammation

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Running title: Clopidogrel and periodontal repair.

Key words: clopidogrel, periodontal repair, inflammation, innate immunity, chemokines.

ABSTRACT

Aim: Antiplatelet drugs may affect the transition from inflammation to repair following induction of periodontitis.

Material and Methods: To evaluate the effects of antiplatelet drugs on experimental periodontal repair 60 rats were randomly assigned to 6 groups (n=10) and ligatures were placed around lower first molars of three groups. The other three groups were used as negative controls. Ligatures were removed after 10 days of periodontitis induction and aspirin (Asp) (30 mg/kg) or clopidogrel (Clop) (75 mg/kg) was given intragastrically daily for 3 days. Periodontal tissue was assessed by the measurement of CXCL12, CXCL4, CCL5 and PDGF; histomorphometric analysis and quantification of osteoclast and blood vessels.

Results: CCL5 was decreased and CXCL12 increased when compared to negative control groups. Asp and Clop decreased CXCL12 expression but only Clop decreased CXCL4 and PDGF content. Clop increased blood vessel number, reduced PMN count, and decreased attachment and bone loss, also decreased osteoclast number in animals submitted or not to periodontal repair.

Conclusion: Clopidogrel had a stronger effect than aspirin, suggesting that it may have therapeutic value under situations where tissues undergo a transition from inflammation to repair.

CLINICAL RELEVANCE

Scientific rationale: Systemic administration of antiplatelet drugs might interfere with periodontal repair by suppressing the release of biological mediators.

Principal findings: Clopidogrel, but not aspirin, appears to have improved the repair process showed by an increase in blood vessels number, reduced leukocyte count, augmented bone percentage and significant reduction in the attachment loss and CEJ-ABC distance.

Practical implications: Aspirin and Clopidogrel, the two most frequently prescribed antiplatelet drugs in patients with cardiovascular diseases may affect the inflammatory and repair process in periodontal disease.

INTRODUCTION

Periodontitis is an inflammatory disease characterized by episodes of active destruction with intense inflammatory infiltration, loss of connective tissue attachment of the gingiva to the tooth surface and periodontal bone loss as well as periods of quiescence in which periodontal regeneration takes place, that is thought to partially restore damaged periodontal ligament, gingival connective tissue, periodontal bone, and cementum (Camargo et al, 2002; Van Dyke & Serhan, 2003; Zhao et al, 2012). The regeneration of any tissue is a complex biological process in itself, requiring intricately regulated interactions between cells, locally acting growth factors, chemokines, cytokines and the extracellular matrix components in which these entities interacts (Graves et al, 2011).

Platelet degranulation releases a plethora of biological mediators, such as chemokines, cytokines and growth factors that have a role in the inflammatory process and tissue regeneration. An alteration in the amount and function of platelets affects thrombotic and inflammatory events that contribute to cardiovascular diseases such as atherosclerosis (von Hundelshausen et al, 2007). Chemokines released by platelets contribute to modulating the inflammatory response at sites of injury (Weber, 2005; Gawaz, 2005).

In the context of periodontal regeneration, we have previously demonstrated that a pronounced reduction of circulating platelets (thrombocytopenia) leads to a delay in periodontal healing which is associated, at least in part, with decreased serum concentrations of platelet-derived VEGF and endostatin (Spolidorio et al, 2010). Furthermore, platelet inactivation by systemic administration of aspirin and clopidogrel attenuates the amount of destruction that occurs with periodontitis (Coimbra et al, 2011). Moreover, platelet rich plasma (PRP) has been shown to enhance regenerative treatment (Lekovic et al, 2012; Suaid et al, 2012; Dominiak et al, 2012).

Antiplatelet treatments include aspirin and thienopyridines (such as clopidogrel and dipyridamole), abciximab or eptifibatide (Schomig et al, 1997; Bennett, 2001). Aspirin irreversibly inactivates cyclooxygenase-1 (COX-1) activity and subsequent synthesis of thromboxane A_2 (TXA-2). It also modifies the COX-2 enzymatic activity, induces lipoxin production that facilitates the resolution of inflammation and inhibits prostanoid production that is generally pro-inflammatory (Warner & Mitchell, 2002). Clopidogrel acts by irreversible antagonism of platelet $P2Y_{12}$ receptors expressed in the platelet membrane by inhibiting binding of the endogenous ligand, adenosine diphosphate (ADP) (Bennett, 2001; Gao et al, 2009).

Because platelets contain an array of cytokines and growth factors they modulate inflammatory and repair processes. In this study, we evaluated the effects of aspirin and clopidogrel, the two most frequently prescribed antiplatelet drugs, on the transition from periodontal inflammation to periodontal repair by the measurement of growth factors and chemokines CXCL12, CXCL4, CCL5 and PDGF, histomorphometric analysis of PMN infiltration, attachment loss, bone loss and osteoclast numbers and quantification of blood vessels by imunnohistochemistry. Contrary to expectations, treatment with clopidogrel enhanced repair through a mechanism that involved reduced inflammation.

MATERIAL AND METHODS

Animals

Sixty male adult Holtzman rats (*Rattus norvergicus albinus*) weighing 250-300 g were housed under similar conditions in cages with access to food and water *ad libitum*. During the whole experimental protocol, the rats were kept in a quiet room with controlled temperature (22 ± 1^0C), humidity (65-75%) and a 12-h light-dark cycle. All experimental protocols were approved by the local Ethics Committee for Animal Experimentation and performed in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

Chemicals

All drugs and reagents were purchased from Sigma Aldrich (Brazil), unless otherwise stated. Ketamine and xylazine chloride (Francotar® and Virbaxil® respectively, both from Virbac do Brasil Ind. Com. Ltda, São Paulo, SP, Brazil) were used for dissociative anesthesia of the animals.

Evaluation of antiplatelet drug treatment on periodontal repair

The rats were randomly distributed into 6 groups (10 animals/group). Animals from three groups were anesthetized with 1 mg/kg body weight of ketamine and 0.4 mL/kg of xylazine for the induction of experimental periodontal disease by insertion of a 3.0 cotton ligature in a submarginal position on the lower right and left first molars of each rat, as previously described (Holzhausen et al, 2002). The animals from the other three groups were not subjected to the induction of periodontal disease and were used as negative controls. Rats in the remaining three groups with induction of periodontitis had ligatures removed 10 days after insertion to allow the spontaneous repair of periodontal tissues over the subsequent 3 days.

During these three days, animals were treated with intragastrical daily doses of aspirin (Asp; 30 mg/kg) or clopidogrel (Clop; 75 mg/kg). Control rats were treated with the same volume of vehicle (NaCl 0.9%). These doses have been previously shown to cause effective inhibition of platelet aggregation and thrombus formation in rats (Coimbra et al, 2011; Ma et al, 2001; Sasaki et al, 1996; Taka et al, 1999; Wallace et al, 1995). Animals without ligature-induced periodontitis were treated with Asp, Clop and NaCl 0.9% as described above.

Harvesting and processing of samples

At the end of the 13-day experimental period, animals were killed by anesthesia overdose and the mandibles were carefully removed and dissected. Gingivo-mucosal tissues from the right mandibular first molar were removed and processed for the analysis of PDGF, CXCL12, CCL5 and CXCL4 contents by enzyme-linked immunosorbent assay (ELISA) from Life Technologies (Carlsbad, CA, USA), according to the manufacturer's instructions.

Histomorphometric analysis

Subsequently, left hemi-mandibles were fixed in 4% paraformaldehyde at 4°C for 48h and decalcified in 4.13% EDTA solution (pH 7.2) at room temperature for approximately 3 months. Serial paraffin sections 5 μm thick were obtained from buccal–lingual aspects of the whole left first molars and subsequently stained with hematoxylin and eosin (H&E) for histological/histometric evaluation. Bone loss was measured as the distance between the cemento-enamel-junction (CEJ) and the highest peak of the alveolar bone crest (ABC). Attachment loss was measured by the distance between the CEJ and the most coronal extent of connective tissue attachment to cementum and the bone percentage by the difference between the total area and the bone area measured 1 millimeter above from the cemento-enamel junction. The number of polymorphonuclear (PMNs) leukocytes was counted in the connective tissue 0.5 mm above the CEJ. 5 randomly chosen sections of each animal were examined at 200X magnification. A blinded examiner who did not know the group to which an animal belonged analyzed all data.

Immunohistochemistry to identify osteoclasts and blood vessels

An anti-tartrate-resistant acid phosphatase primary antibody (Santa Cruz Biotechnology, 1:100) was used to identify multinucleated osteoclasts adjacent to bone. In separate sections an antibody specific for Factor VIII (Abcam, Cambridge, MA) diluted 1:100 was used to identify blood vessels. Primary antibody was localized by a biotinylated secondary antibody

followed by avidin-biotin complex staining (Burlingame, CA, USA). For blood vessels tyramide signal amplification was used to enhance the chromogenic signal (PerkinElmer, Waltham, MA, USA). Sections were examined at 600X magnification and counted in 5 randomly selected images. The number of TRAP+ cells was counted as the multinucleated cells adjacent to the bone 1 mm above the CEJ. Blood vessels was counted in 5 randomly fields in the connective tissue between the CEJ-ABC. There was no immunostaining with match control IgG (data not shown).

Statistical analysis

All data were expressed as mean ± SEM. Differences among the groups were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons. P values less than 5% were considered statistically significant.

RESULTS

All animals were alive at the end of the experimental period (13 days), and the recorded body weights were 283.7±1.8, 292.0±2.1, 275.0±1.5, 258.0±4.1, 261.0±2.3 and 277.0±2.6 g for NaCl, Asp, Clop, Rep+NaCl, Rep+Asp and Rep+Clop groups, respectively. Neither occult bleeding nor hematoma after gingival intervention was observed.

- ELISA analysis

After induction of periodontitis and repair there was no difference in CXCL4 levels compared to negative control groups. However, treatment with Clop significantly reduced the expression of CXCL4 by 60% to 70% with ($P<0.001$) or without ($P<0.001$) the induction of periodontitis. Asp induced a 50% decrease ($P<0.0001$) only after the induction of periodontitis and repair (Figure 1A).

The gingival level of CCL5 was significantly decreased by 80% ($P<0.0001$) and CXCL12 increased by 75% ($p<0.0001$) in rats subjected to the induction of periodontitis and repair compared to baseline. Treatment with antiplatelet drugs induced a 30% decrease ($P<0.001$) in CXCL12 expression compared with the saline-treated animals (Figure 1B and C).

PDGF-AB expression was stimulated approximately 10 fold by induction of periodontitis and repair ($P<0.001$) compared to negative control groups. Rats treated with Clop but not aspirin had a 75% reduction in PDGF-AB expression in the experimental group compared to saline treated (Figure 2).

- HISTOMORPHOMETRIC analysis

To evaluate the role of antiplatelet drugs on the repair process, ligatures were removed after 10 days in the animals of three experimental groups and they were submitted to treatments with Asp, Clop and vehicle as described above.

Administration of Clop during the period of repair appears to have reduced inflammation and improved the healing process. Clop-treated animals showed a reduced cellularity (polymorphonuclear leukocytes (PMN) infiltrate by 62% ($P < 0.001$), reduced attachment loss by 45% ($P < 0.001$) and reduced loss of periodontal bone height (CEJ-ABC distance) by 25% ($P < 0.001$); therefore an augmented bone percentage when compared to saline-treated animals (Figure 3 and 4 A, B, C and D).

Osteoclast numbers

To establish how antiplatelet therapy may affect bone resorption osteoclast numbers were measured. The number of osteoclasts per millimeter of bone length was increased 3 fold by induction of periodontitis and repair. Treatment of the experimental animals with Clop completely blocked this increase ($P<0.0001$) and also decreased levels of osteoclasts ($P<0.001$) in negative control group. In contrast Asp had no effect ($P>0.05$) (Figure 5 and 6).

Blood Vessels

Treatment with Clop during periodontal repair increased the number of blood vessels by 20% ($p<0.0001$) while Asp had no effect ($P>0.05$) compared to saline treated experimental animals (Figures 7 and 8).

DISCUSSION

In our experimental model, removal of the disease-causing stimulus (ligature) initiates a process that involves resolution of inflammation and a transition to repair. In the early transition phase there is an increased CXCL4, CXCL12, PDGF and higher levels of PMN infiltration and osteoclasts compared to baseline or animals that did not have periodontitis. Administration of clopidogrel significantly decreased levels of chemokines CXCL4 and CXCL12, PMN infiltration and osteoclast numbers suggesting that clopidogrel substantially reduced the level of inflammation during the transition phase. This decrease in inflammation is thought to be important in subsequent steps that involve repair. This is supported by an increase in the number of blood vessels observed with clopidogrel and a decrease in the loss of connective tissue attachment. Administration of aspirin had similar, but less marked effects on the production of platelet-derived chemokines and production of PDGF was barely attenuated. Also, aspirin did not attenuate loss of connective attachment and bone resorption. The latter is supported by the lack of effect on osteoclast numbers in the animals treated with aspirin. Interestingly, administration of aspirin was not as effective as clopidogrel in reducing inflammation as noted by a reduced effect on PMN infiltration or osteoclast numbers and was less effective in promoting the transition to repair as noted by relatively little effect on the number of blood vessels or connective tissue attachment levels.

The mechanisms by which clopidogrel improves the transition from inflammation to repair has not yet been fully elucidated. Our data suggests that modulation of the disease-associated inflammatory process in this early/transition phase is part of the mechanism. The differences between the results of clopidogrel- and aspirin-treated animals may reflect different biological mechanisms and potencies of these drugs. Clopidogrel is a thyenopiridine that acts by irreversibly binding to P2Y12 receptor. This binding diminishes the release of alpha and dense granules from platelets (Garcia et al, 2011) and also inhibits beta3 integrin engagement, attenuating the synthesis of IL-1beta induced upon platelet stimulation (Lindemann et al, 2001; Su et al, 2012). In the majority of studies Clopidogrel inhibits expression of inflammatory markers and reduces leukocyte activation (Ayral et al, 2007; Evangelista et al, 2005). Moreover, clopidogrel inhibits the progression of atherosclerotic lesions by reducing levels of inflammatory factors (Li et al, 2007). On the other hand, in a rat experimental arthritis model, treatment with clopidogrel had a pro-inflammatory effect measured by clinical manifestations of inflammation, elevated neutrophil blood count and the plasma levels

of pro-inflammatory cytokines (Garcia et al, 2011). Aspirin inhibits the activity of cyclooxygenases and promotes the expression of lipoxins and resolvins (Chiang & Serhan, 2006). In addition to inhibiting platelets aspirin works through a number of different pathways that can reduce inflammation (Dovizio et al, 2013; Berk et al, 2013). Aspirin has also been shown to reduce PMN infiltration related to inflammation (Gil-Villa et al, 2012).

Our results indicate that both clopidogrel and aspirin reduced production of chemokines CXCL4 and CCL5; although clopidogrel was more potent than aspirin in reducing PMN infiltration, attachment loss, bone loss, osteoclast numbers and increasing blood vessels. The greater differences between the antiplatelet drugs were related to the significant decrease of PDGF-AB and, most notably, to a bone and connective tissue sparing effect in clopidogrel-treated animals. This may be related to a potent effect of clopidogrel in modulating the cytokine network in this early repair/transition phase. Both clopidogrel and aspirin have been shown to reduce the production of TNF-α (Al-Baharani et al, 2007). Our research group has previously reported an anti-inflammatory effect associated with systemic administration of aspirin and clopidogrel in a rat ligature-induced periodontal disease model, which was characterized by a reduction in the influx of inflammatory cells and consistent decrease in the production of proinflammatory cytokines (IL-6, TNF-alpha and TXA-2) (Coimbra et al, 2011).

There was a marked reduction in osteoclast numbers in the tissues of clopidogrel-treated animals, which may be a direct effect of this drug on osteoclastogenesis or an indirect effect related with the modulation of the cytokine network and the pro- and anti-osteoclastic factors. P2RY2 receptors, which are inhibited by clopidogrel play a role in pathologic bone loss induced by arthritis, tumor growth in bone in osteoporosis (Su et al, 2012). In addition ADP, which is the natural ligand for P2RY2 receptors can stimulate osteoclast formation in vitro (Hoebertz et al, 2001). Gruber et al (2003) showed that treatment with aspirin had no effect in osteoclastogenesis, suggesting that the anti-inflammatory effects of aspirin are not related to this mechanism.

According to our results, clopidogrel but not aspirin enhanced angiogenesis by increasing in the number of blood vessels during periodontal repair. Interestingly, Clop has been shown to affect platelet degranulation, reducing secretion of PDGF, which plays an important role on tissue repair and angiogenesis (Graff et al, 2002). In fact, after Clop treatment we observed a significant reduction in PDGF expression. Regarding the angiogenic process we can partially explain the link between the increase in the number of blood vessels and PDGF expression as

platelets are a primary source of this growth factor. Moreover, Duelsner et al (2012) showed that aspirin, but not clopidogrel, inhibits arteriogenesis in vivo and in vitro.

In summary, we showed that after removal of the pathologic stimulus in the early/transition phase between disease-associated inflammation and repair both aspirin and clopidogrel decrease the production of chemokines associated with leukocyte chemotaxis in the periodontal microenvironment. Overall, the effects of clopidogrel were more potent than those of aspirin. Thus, clopidogrel had a stronger effect in reducing PMN infiltration, attachment and bone loss and osteoclast number and increasing bone percentage and blood vessels suggesting that it may have therapeutic value under situations where tissues undergo a transition from inflammation to repair.

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FIGURE

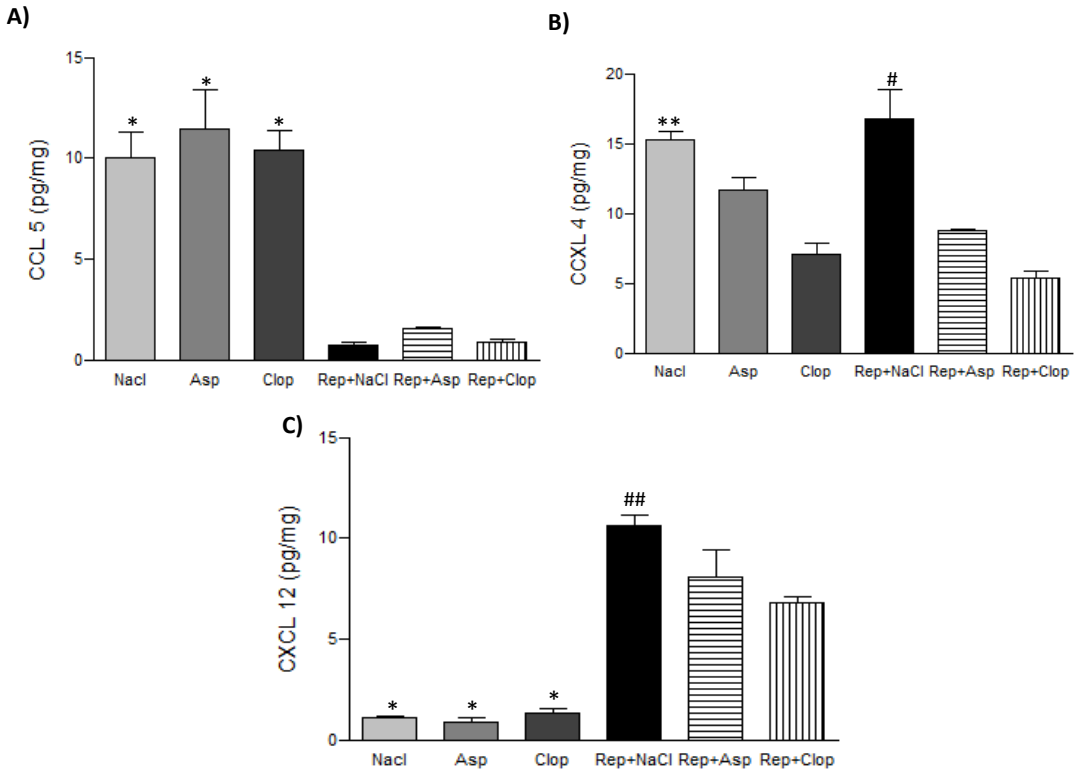

FIGURE 1- Effect of systemic treatment with Asp or Clop on the gingival contents of CCL5 (panel A), CXCL4 (panel B), CXCL12 (panel C) in rats with ligature induced periodontal disease when stimulus is removed. The samples were harvested from five animals from each group and analyzed in duplicate. The chemokine results were normalized to the total protein contents in each sample and expressed as pg/mg of protein. Values are expressed as mean±SEM. * P<0.0001 vs Rep+ NaCl, Rep+ Asp, Rep+ Clop; **P<0.001 vs Clop; #P<0.0001 vs Rep+ Asp, Rep+ Clop; ##P<0.001 vs Rep+ Asp and Rep+ Clop.

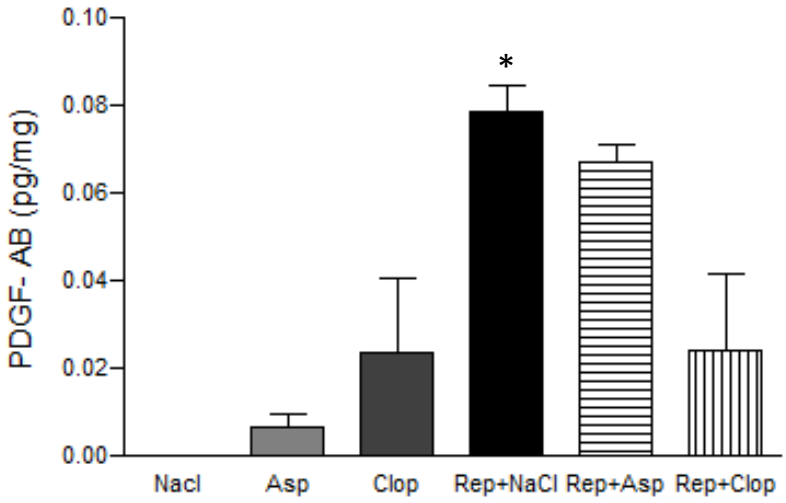

FIGURE 2- Effect of systemic treatment with Asp or Clop on the gingival contents of PDGF in rats with ligature induced periodontal disease when stimulus is removed. The samples were harvested from five animals from each group and analyzed in duplicate. The growth factor results were normalized to the total protein contents in each sample and expressed as pg/mg of protein. Values are expressed as mean±SEM. *P<0.001 vs Rep+ Clop.

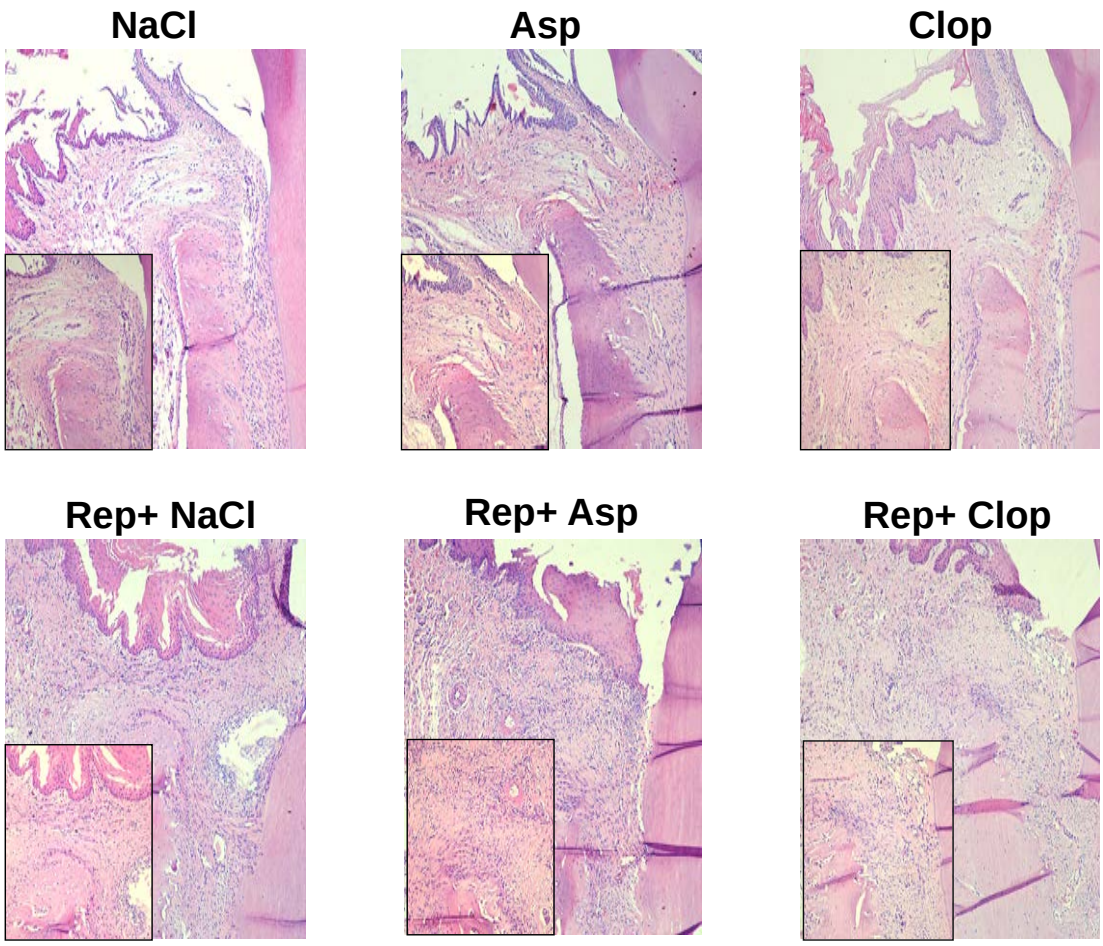

FIGURE 3- Histological analysis of the alveolar bone crest/gingival margin region of right lower first molar of rats treated with NaCl 0.9%, Asp (30 mg/kg) or Clop (75 mg/kg) submitted or not to experimental periodontal repair (vehicle-treated: 'NaCl', Aspirin-treated: 'Asp', Clopidogrel-treated: 'Clop', Repair+vehicle-treated: 'Rep+NaCl', Repair+Aspirin-treated: 'Rep+Asp', Repair+Clopidogrel-treated: 'Rep+Clop') stained with hematoxylin and eosin (H&E). Images are representative of the results observed in 5 animals in each group. 100X and 200X magnification.

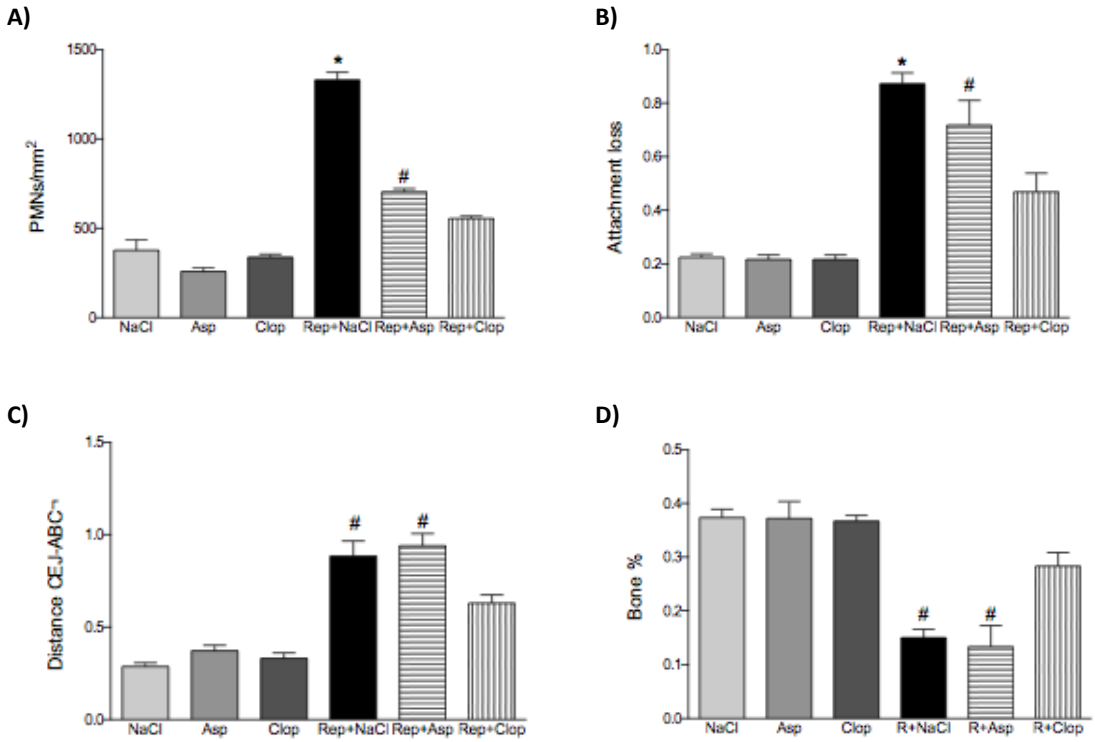

FIGURE 4- Effect of systemic treatment with Asp or Clop on the inflammatory infiltrate, measured as PMN count (panel A); attachment loss (panel B); alveolar bone loss, measured as the distance CEJ-ABC (panel C) and bone percentage (panel D) in animals submitted or not to experimental periodontal disease. Administration of Clop during periodontal repair improved the healing process. Clop treated animals showed a reduced inflammatory infiltrate; an augmented bone percentage and a significant reduction in the attachment loss and CEJ-ABC distance. The results are representative from 5 animals/group. Values are expressed as mean±SEM. *P<0.0001 vs Rep+Clop; #P<0.001 vs Rep+Clop.

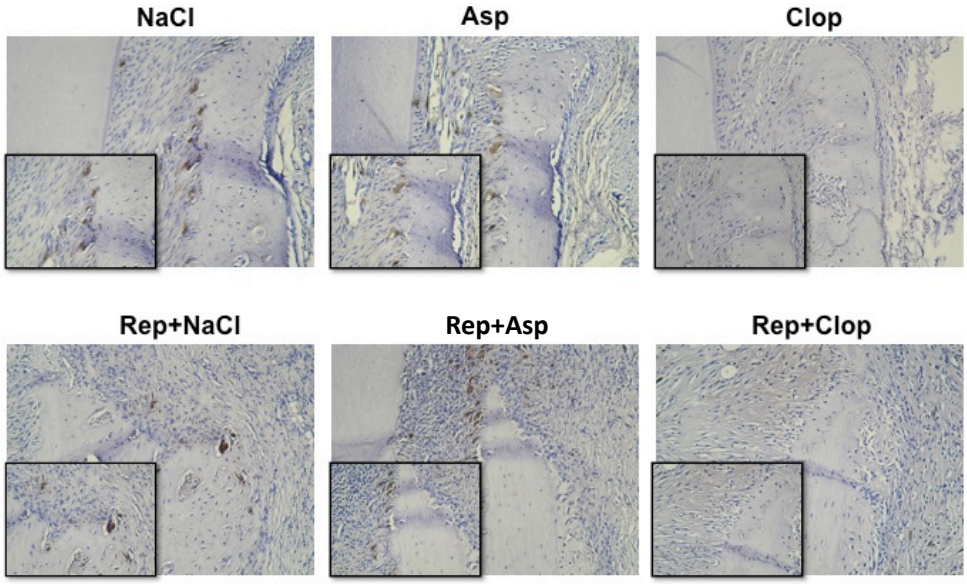

FIGURE 5- Immunohistochemistry sections of the alveolar bone crest of right lower first molar of rats treated with NaCl 0.9%, Asp (30 mg/kg) or Clop (75 mg/kg) submitted or not to experimental periodontal repair (vehicle-treated: 'NaCl', Aspirin-treated: 'Asp', Clopidogrel-treated: 'Clop', Repair+vehicle-treated: 'Rep+NaCl', Repair+Aspirin-treated: 'Rep+Asp', Repair+Clopidogrel-treated: 'Rep+Clop') stained with anti-tartrate-resistant acid phosphatase (TRAP) to measure osteoclast number. Images are representative of the results observed in 5 animals in each group. 100X and 200X magnification.

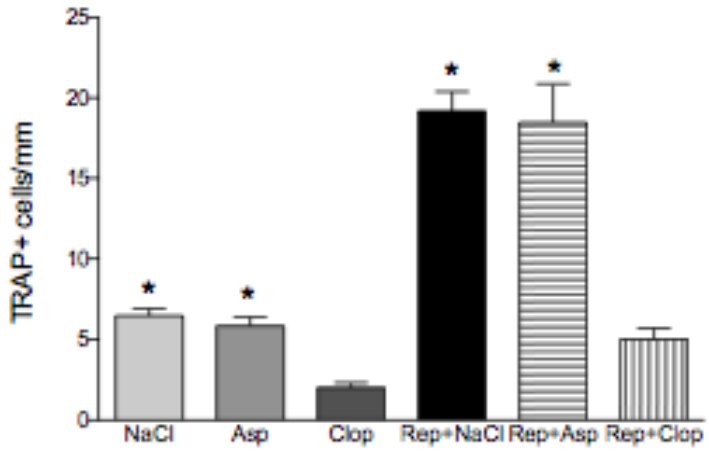

FIGURE 6- Effect of systemic treatment with Asp or Clop on the osteoclast number in rats with ligature induced periodontal disease when stimulus is removed. Clop-treated animals, with or without periodontal repair, showed a significant decrease in the osteoclast amount when compared to Asp and Saline treated animals. The results are representative from 5 animals/group. Values are expressed as mean±SEM. *P<0.0001 vs Clop, Rep+Clop.

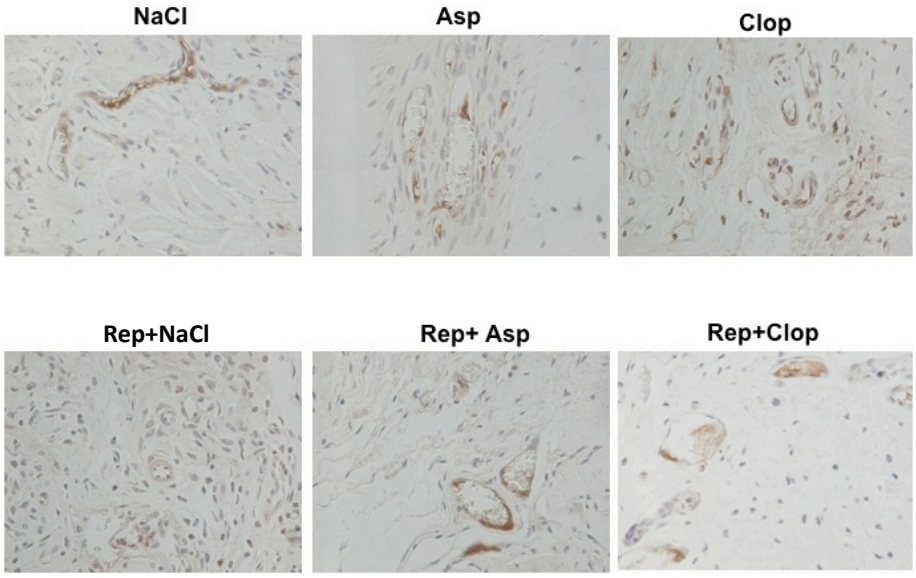

FIGURE 7- Immunohistochemistry sections of the alveolar bone crest/gingival margin region of right lower first molar of rats treated with NaCl 0.9%, Asp (30 mg/kg) or Clop (75 mg/kg) submitted or not to experimental periodontal repair (vehicle-treated: 'NaCl', Aspirin-treated: 'Asp', Clopidogrel-treated: 'Clop', Repair+vehicle-treated: 'Rep+NaCl', Repair+Aspirin-treated: 'Rep+Asp', Repair+Clopidogrel-treated: 'Rep+Clop') stained with an antibody against Factor VIII. Images are representative of the results observed in 5 animals in each group. 100X magnification.

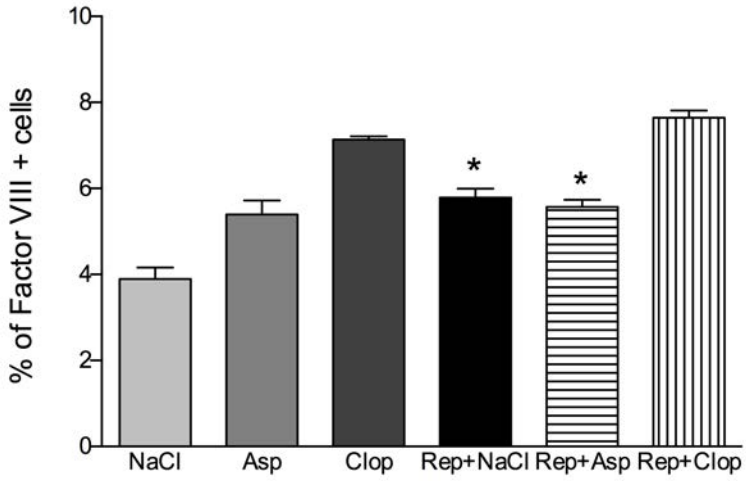

FIGURE 8- Effect of systemic treatment with Asp or Clop on the number of blood vessels in rats submitted or not to experimental periodontal repair. During the repair process, Clop-treated animals showed a significant increase in the number of blood vessels (Factor VIII+) when compared to Asp and saline treatment. The results are representative from 5 animals/group. Values are expressed as mean±SEM. *P<0.0001 vs Rep+Clop.

CAPÍTULO 3

Clopidogrel improves bone repair by a mesenchymal stem cell pathway.

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Artigo em processo de correção

Clopidogrel improves bone repair by a mesenchymal stem cell pathway.

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ABSTRACT

Background: Mesenchymal stem cells were regarded as the major source of reparative progenitor cells to replace damaged tissues. These cells can differentiate in megakaryocytes and functional platelets in vitro. We hypothesized that platelet inactivation induced by drugs might interfere with periodontal repair in experimental periodontitis by reducing the bioavailability of growth factors that are required for the differentiation of mesenchymal stem cells.

Methods: To evaluate the effects of antiplatelet drugs on experimental periodontal repair 60 rats were randomly assigned to 6 groups (n=10) and ligatures were placed around lower first molars of three groups. The other three groups were not subjected to the induction of periodontal disease and were used as negative controls. Ligatures were removed after 10 days of periodontitis induction and aspirin (Asp) (30 mg/kg) or clopidogrel (Clop) (75 mg/kg) was given intragastrically once daily for 3 days. Periodontal repair was assessed by histomorphometric analysis of osteoblast number; quantification of mesenchymal stem cells by immunohistochemistry, their behavior by double immunofluorescence staining associated with TUNEL, KI67 and CD45 and in vitro analysis of the human bone marrow stem cell proliferation rate.

Results: During periodontal repair, Clop treatment appears to have improved bone formation as shown by an increase in the osteoblast number and a decrease in CD 45 expression; also showed an increase in the amount of mesenchymal stem cells (CD 271+). Clop-treated animals submitted or not to experimental periodontal repair increased the amount of stem cells in apoptosis and proliferation. Also, different dosages of Clop increased the human bone marrow stem cell proliferation rate in vitro.

Conclusion: Systemic administration of Clop during 3 days affected periodontal repair by increasing the osteoblast number and mesenchymal stem cells in apoptosis and proliferation**.**

Keywords: Mesenchymal stem cell, periodontal repair, clopidogrel.

INTRODUCTION

Bone remodeling presupposes the recruitment of osteoclasts and osteoblastic cells to bone remodeling sites where both bone resorption and formation takes place. Although osteoclasts originates from hematopoietic precursors that reach bone surface through the circulation, the nature of osteoblastic cells recruited to bone formation sites is not completely understood. The description of osteoblastic cells is that they are fibroblast-like, derived from stem cells from the bone marrow stroma (MSCs)[1,2]. Originally, because of their multipotent capabilities, MSCs were regarded as the major source of reparative progenitor cells to replace damaged tissues[3]. According to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy a minimal criterion for defining multipotent mesenchymal stem cells was proposed. Their scheme is based in three criteria: adherence to plastic, a specific surface-antigen expression pattern and a multipotent differentiation potential[4,5].

MSCs are the focus of efforts worldwide not only to elucidate their nature and unique properties but also in the development of cell-based therapies for a diverse range of diseases[6]. Some studies have shown the MSC anticancer therapy[7,8] by the expression of modified forms of the TNF-related apoptosis-inducing ligand to complement radiation and chemotherapy in sarcoma cells. Moreover, clinical benefits were observed in children with osteogenesis imperfecta[9] and in cardiac cell therapy[10].

In the context of periodontal repair MSCs have been exploited in the field of tissue engineering[11]. Stem cells derived from gingival connective tissue showed significant periodontal regenerative potential in periodontal defects in pigs[12]. Also, Tcacencu et al (2012)[13] demonstrated that transplanted human stem cells could contribute to alveolar bone preservation after periodontal surgical trauma. Moreover, according to Ninomiya et al (2013)[14], MSCs from the periodontal ligament possess enhanced osteogenic potential and could be a potential source for cell-based regenerative therapy for alveolar bone.

Considering the major role of platelets in inflammation and in the repair process some studies have reported that host lymphocytes secrete IFN-gama and TNF-alpha to initiate apoptosis of transplanted MSCs and that aspirin can alleviate those effects to improve bone repair[15,16] .

Based on the considerations above, we decided to study in rats, the effects of either Aspirin or Clopidogrel on the mesenchymal stem cells focusing on the alveolar bone formation and how MSCs might interfere in periodontal repair. We assessed the number of osteoblasts by histological analysis, the expression of CD 271, a stem cell marker, by immunohistochemistry

and also their behavior by double immunofluorescence staining using CD 271 associated with TUNEL, KI67 and CD 45. In addition, we analyzed in vitro the human bone marrow stem cell proliferation rate.

MATERIAL AND METHODS

- Animals

Sixty male adult Holtzman rats (*Rattus norvergicus albinus*) weighing 250-300 g were housed under similar conditions in cages with access to food and water *ad libitum*. During the whole experimental protocol, the rats were kept in a quiet room with controlled temperature (22 ± 1^0C), humidity (65-75%) and a 12-h light-dark cycle. All experimental protocols were approved by the local Ethics Committee for Animal Experimentation and performed in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

- Chemicals

All drugs and reagents were purchased from Sigma Aldrich (Brazil), unless otherwise stated. Ketamine and xylazine chloride (Francotar® and Virbaxil® respectively, both from Virbac do Brasil Ind. Com. Ltda, São Paulo, SP, Brazil) were used for dissociative anesthesia of the animals.

- Evaluation of antiplatelet drug treatment on periodontal repair

The rats were randomly distributed into 6 groups (n=10 animals/group). Animals from three groups were anesthetized with 1 mg/kg body weight of ketamine and 0.4 mL/kg of xylazine for the induction of experimental periodontal disease by insertion of a 3.0 cotton ligature in a submarginal position on the lower right and left first molars of each rat, as previously described[17]. The animals from the other three groups were not subjected to the induction of periodontal disease and were used as negative controls. Rats in the three groups with induction of periodontitis had the ligature removed from both sides (10 days after insertion of the ligatures) to allow the spontaneous repair of periodontal tissues over the subsequent 3 days (the animals in these three groups were killed 13 days after insertion of the ligatures).

During these three days, animals were treated with intragastrical daily doses of Aspirin (Asp; 30 mg/kg) or Clopidogrel (Clop; 75 mg/kg). Control rats were treated with the same volume of vehicle (NaCl 0.9%). These doses have been previously shown to cause effective inhibition of platelet aggregation and thrombus formation in rats[18-22]. Animals with no ligature-induced periodontitis were treated with Asp, Clop and NaCl 0.9% as described above.

- Harvesting and processing of samples

At the end of the 13-day experimental period, animals were killed by anesthesia overdose and the mandibles were carefully removed and dissected. Subsequently, hemi-mandibles were fixed in 4% paraformaldehyde at 4°C for 48h and decalcified in 4.13% EDTA solution (pH 7.2) at room temperature for approximately 3 months. Serial paraffin sections 5 μm thick were obtained from buccal–lingual aspects of the whole left first molars and subsequently stained with hematoxylin and eosin (H&E) for histological evaluation. Osteoblast number was counted as cuboidal bone lining cells in areas of bone remodeling. 5 randomly chosen sections of each animal were examined at 200X magnification. A blinded examiner who did not know the group to which an animal belonged analyzed all data.

- Immunohistochemistry

The percentage of cells expressing CD 271 (stem cell marker) in the connective tissue was measured in 5 fields/place using the software SVCell. Sections were stained by immunohistochemistry using paraffin sections with an antibody specific for CD 271 (Abcam, 1:10.000). Primary antibody was detected by avidin-biotin horseradish peroxidase complex using a biotinylated secondary antibody. To enhance the signal to noise ratio, citrate (pH 6) antigen retrieval was used along with tyramide signal amplification that enhances the chromogenic signal (PerkinElmer, Waltham, MA, USA). Sections were examined at 600X magnification. There was no immunostaining with match control IgG (data not shown).

-Immunofluorescence

CD 271, KI67, TUNEL and CD 45 positive cells were detected and co-localized by fluorescence microscopy. Primary antibody to CD 271 (Abcam, 1:10.000), KI67 (Vector Laboratories, 1:20), TUNEL (Promega KIT, ready to use) and CD 45 (Abcam, 1:100) was

detected with a biotinylated secondary antibody followed by streptavidin-Texas Red (Vector Laboratories, Burlingame, CA, USA), avidin- flourescein with DAPI nuclear staining in the mounting medium (Vector Laboratories). We scanned the connective tissue for the presence of CD 271 and co-localization with KI67, TUNEL and CD 45 by comparing CD 271 staining with Texas Red, KI67, TUNEL and CD 45 staining with avidin-flourescein and nuclear stain with DAPI and a corresponding merged image. Matched control antibody was used as a negative control. Sections were examined at 600X magnification and measured in 5 fields/place using the software NISElements.

- In vitro proliferation assay

Human bone marrow derived mesenchymal stem cells were cultured in alpha-MEM media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. For proliferation assay, $0.8x10^4$ cells/well were seeded in 8 well chamber slides in triplicate. Cells were treated with serum, Clopidogrel (10 and 50 uM) and Aspirin (0.1 and 0.5 uM). In the baseline and after 3, 6 and 9 days of treatment the wells were removed and the slide mounted with DAPI mounting medium (Vector Laboratories) to count the number of cells. Five fields per well were examined at 600X magnification.

- Statistical analysis

All data were expressed as mean ± SEM. Differences among the groups were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons. P values less than 5% were considered statistically significant.

RESULTS

All animals were alive at the end of the experimental period (13 days), and the recorded body weights were 283.7±1.8, 292.0±2.1, 275.0±1.5, 258.0±4.1, 261.0±2.3 and 277.0±2.6 g for NaCl, Asp, Clop, R+NaCl, R+Asp and R+Clop groups, respectively. Neither occult bleeding nor hematoma after gingival intervention was observed.

-Histomorphometric analysis

Clop-treated animals showed a significant increase by 46% ($P<0.0001$) in the osteoblast count/length when compared to the other groups (Figure 1).

-Immunohistochemistry

Treatment with Clop during periodontal repair significantly increased by 40% ($p<0.0001$) CD 271 (stem cell marker) expression when compared to Asp and Saline treated animals (Figure 2).

-Immunofluorescence

Clop administration in animals submitted or not to experimental periodontal repair significantly ($p<0.0001$) increased by 60% and 23% the percentage of apoptotic cells (TUNEL+) and cells in proliferation (KI67+), respectively; during periodontal repair, Clop-treated animals significantly decreased ($p<0.001$) the inflammatory process by supressing the CD 45 expression (Figures 3, 5 and 7 panel A).

Moreover, to establish how stem cells may affect periodontal repair, double immunofluorescence with CD 271 and TUNEL or KI67 was performed. Clop-treated animals submitted or not to experimental periodontal repair had a significant ($p<0.0001$) increase in the percentage of stem cells in apoptosis (TUNEL +) and in proliferation (KI67+) when compared to Saline and Asp treatment (Figures 3, 4, 5 and 6). In order to characterize phenotipically stem cells, double immunofluorescence with CD 271 and CD 45 was performed (Figures 7 and 8). A small amount of double positive cells were observed among the experimental groups without significant difference.

-In vitro proliferation assay

For proliferation assay, human bone marrow stem cells were counted at baseline, 3, 6 and 9 days after treatment with serum, Clop (10 and 50 uM) and Asp (0.1 and 0.5 uM). On day 3, no significant difference was observed among the experimental groups. On day 6 and 9 treatment with Clop in different dosages increased significantly ($p<0.001$) the proliferation rate, when compared to the other experimental groups (Figure 9).

DISCUSSION

In the present study we evaluated the effects of aspirin and clopidogrel on alveolar bone formation and in the mesenchymal stem cells behavior during periodontal repair. Among the negative control groups treatment with clopidogrel increased the percentage of mesenchymal stem cells in apoptosis and in proliferation. In the repair phase, after treatment with antiplatelet drugs, clopidogrel increased osteoblast number and supressed CD 45 expression. Also increased CD 271 expression and the percentage of mesenchymal stem cells in apoptosis and in proliferation. On the other hand, aspirin treatment had no effects on osteoblast count and in the mesenchymal stem cells expression and behaviour in the periodontal microenvironment. This result was unexpected given our original hypothesis where we expected that platelet inactivation induced by aspirin would accelerate mesenchymal stem cell function through TNF-alpha and IFN-gamma inhibition.

According to some studies[4,11] phenotypically a mesenchymal stem cell is characterized by more than 95% of the population expressing the CD105, CD73, CD90 and CD 271 surface antigens and less than 2% of the population expressing the leukocyte marker CD45, the primitive hematopoietic progenitor and endothelial cell marker CD34 and the monocyte and macrophage markers CD14 and CD11. This is in agreement with our study where we evaluated by double staining CD 271 and CD 45 and we observed a small amount of double positive cells among the experimental groups.

The exact pathogenesis of clopidogrel affecting bone formation by a mesenchymal stem cell down-regulation has not yet been established. It is well known that mesenchymal stem cells can differentiate into several cell types, including osteoblasts, but the mechanisms by which clopidogrel affects osteoblast differentiation and function still remains unknown. In the present study we found an increase in osteoblast number per bone surface in animals treated with clopidogrel but not aspirin during periodontal repair. According to Syberg et al (2012)[23] clopidogrel treatment during 14 days slowed osteoblast growth and reduced cell viability, while also decreased bone formation. On the other hand, Su et al (2012)[24] did not detect

differences in osteoblast number per bone surface, but an absolute increase in osteoblast number, given the higher BV/TV observed. Moreover, aspirin delayed bone healing by a decreasing in osteoblast amount[25] and also had an inhibitory effect in a dose dependent manner in osteoblast growth in vitro[26].

According to our results, treatment with clopidogrel, but not aspirin enhanced apoptosis and proliferation in animals submitted or not to periodontal disease induction. These results were somewhat unexpected given the strong effect of clopidogrel in the negative control group and also the increase in the rate proliferation/apoptosis observed. In the literature it is established that P2Y12 activation is able to prevent platelets from apoptosis by reducing phosphatidylserine exposure and decreasing caspase-3 activation in human and mouse platelets in vitro and in vivo[27]. Also, specific P2Y12 receptor agonist as clopidogrel is able to increase cell proliferation in diabetic mice wound healing[28]. We also observed the same proliferation/apoptosis increased in the mesenchymal stem cells analysis. However, the pathways of how clopidogrel but not aspirin affects mesenchymal stem cells still remain unknown. To the best of our knowledge this is the first study relating platelet inactivation and mesenchymal stem cell proliferation/apoptosis in an experimental periodontal disease model. According to Wang et al (2013)[29] mesenchymal stem cells have been found to interact closely with immune cells, such as lymphocytes, which secrets pro-inflammatory cytokines as IFN-gama and TNF-alpha that synergistically can impair self-renewal and differentiation of MSCs via NFkB and one strategy to improve MSCs based-tissue engineering involves the reduction of IFN-gama and TNF-alpha by systemic administration of aspirin. Our research group has previously reported an anti-inflammatory effect associated with antiplatelet treatment, more pronounced after systemic administration of clopidogrel rather than aspirin in a rat ligature-induced periodontal disease model, which was characterized by a reduction in the influx of inflammatory cells and consistent decrease in the production of pro- inflammatory cytokines (IL-6, TNF-alpha and TXA-2)[22]. The increased anti-inflammatory effects after clopidogrel treatment was also observed in other studies[24, 30-32] and can partially explain the increase in the amount of mesenchymal stem cells (CD 271+) observed.

In summary we showed that after the removal of the pathologic stimulus in the early/transition phase between disease-associated inflammation and repair, the effects of clopidogrel were more potent than those of aspirin by increasing osteoblast amount, suppressing CD 45 expression, increasing the percentage of cells in apoptosis and proliferation and also the amount of mesenchymal stem cells showing also an increase in proliferation/apoptosis of those cells. This data suggests that further understanding of the

mechanisms underlying the interplay between lymphocytes, MSCs and systemic administration with clopidogrel may be helpful in the development of promising approaches to improve cell-based regenerative medicine and immune therapies.

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FIGURES

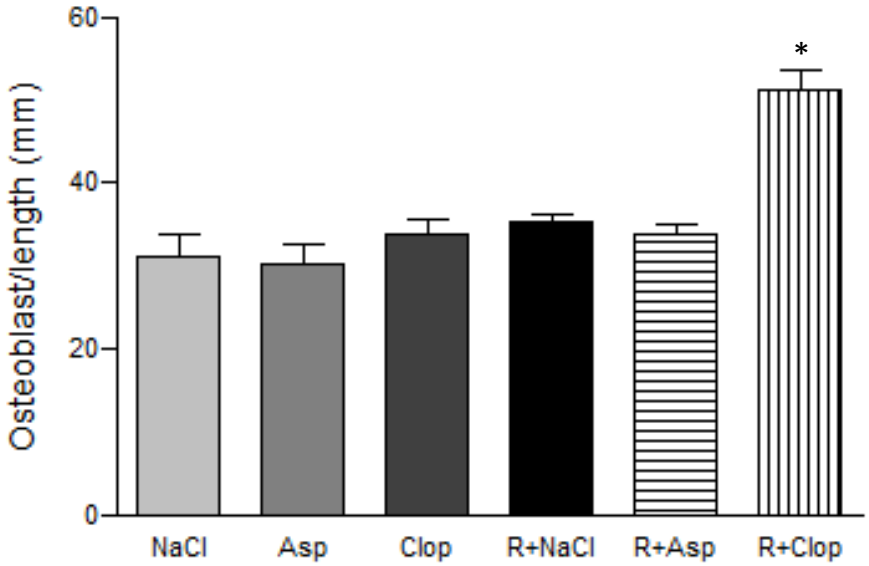

FIGURE 1- Effect of systemic treatment with Asp or Clop on the number of osteoblast per lenght in areas of bone remodeling in animals submitted or not to experimental periodontal disease. Administration of Clop during periodontal repair showed a significant increase in the number of osteoblasts. The results are representative from 5 animals/group. Values are expressed as mean± SEM. *P<0.0001 compared to the other groups.

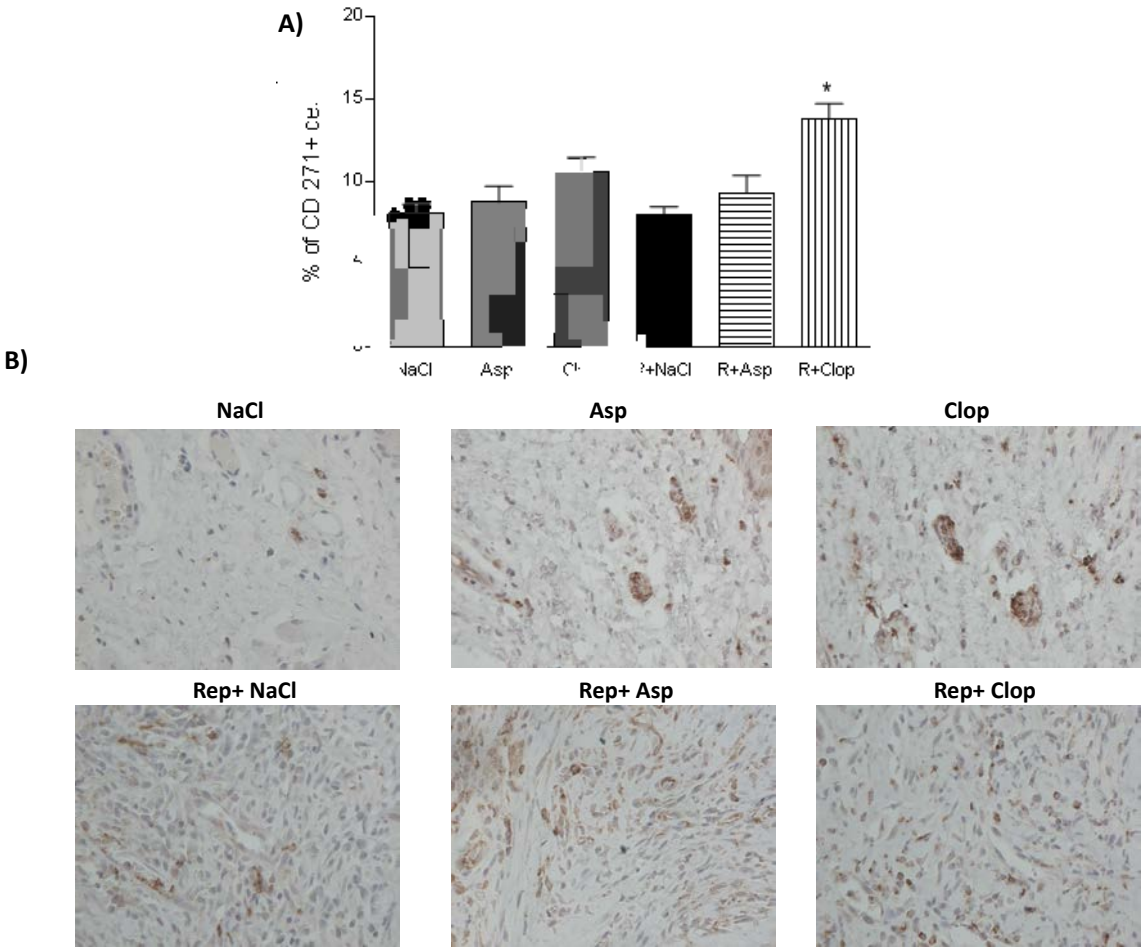

FIGURE 2- Effect of systemic administration of Asp and Clop on the percentage of mesenchymal stem cells (CD 271+) (panel A) and the immunohistochemistry sections (panel B) from the connective tissue of rats treated with NaCl 0.9%, Asp (30 mg/kg) or Clop (75 mg/kg) submitted or not to experimental periodontal repair (vehicle-treated: 'NaCl', Aspirin-treated: 'Asp', Clopidogrel-treated: 'Clop', Repair+vehicle-treated: 'Rep+NaCl', Repair+Aspirin-treated: 'Rep+Asp', Repair+Clopidogrel-treated: 'Rep+Clop'). Administration of Clop during periodontal repair showed a significant increase in CD 271+ cells compared to the other groups. Images are representative of the results observed in 5 animals in each group. Values are expressed as mean± SEM. *P<0.0001 compared to the other groups.

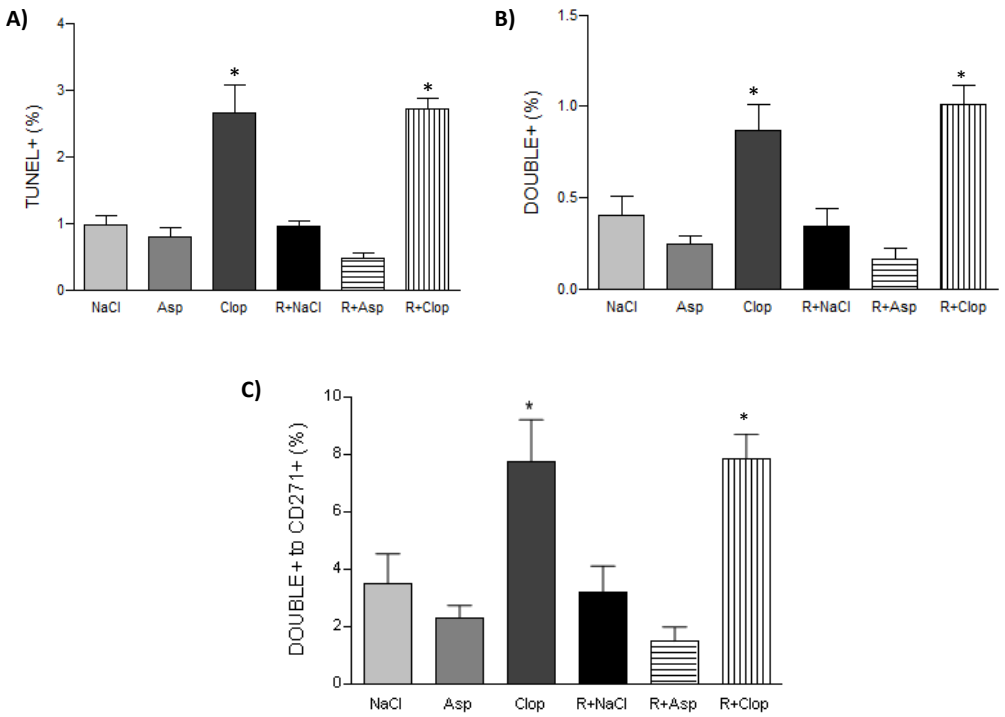

FIGURE 3- Effect of systemic administration of Asp and Clop on the percentage of apoptotic cells (panel A), mesenchymal stem cells in apoptosis (panel B) and the percentage of CD 271 and TUNEL positive cells normalized by CD 271+ cells (panel C). Treatment with Clop showed a significant increase in the percentage of apoptotic cells, CD 271+ cells in apoptosis and CD 271 and TUNEL positive cells normalized by CD 271 positive cells. The results are representative from 5 animals/group. Values are expressed as mean± SEM. *P<0.0001 compared to NaCl, Asp, R+NaCl, R+Asp.

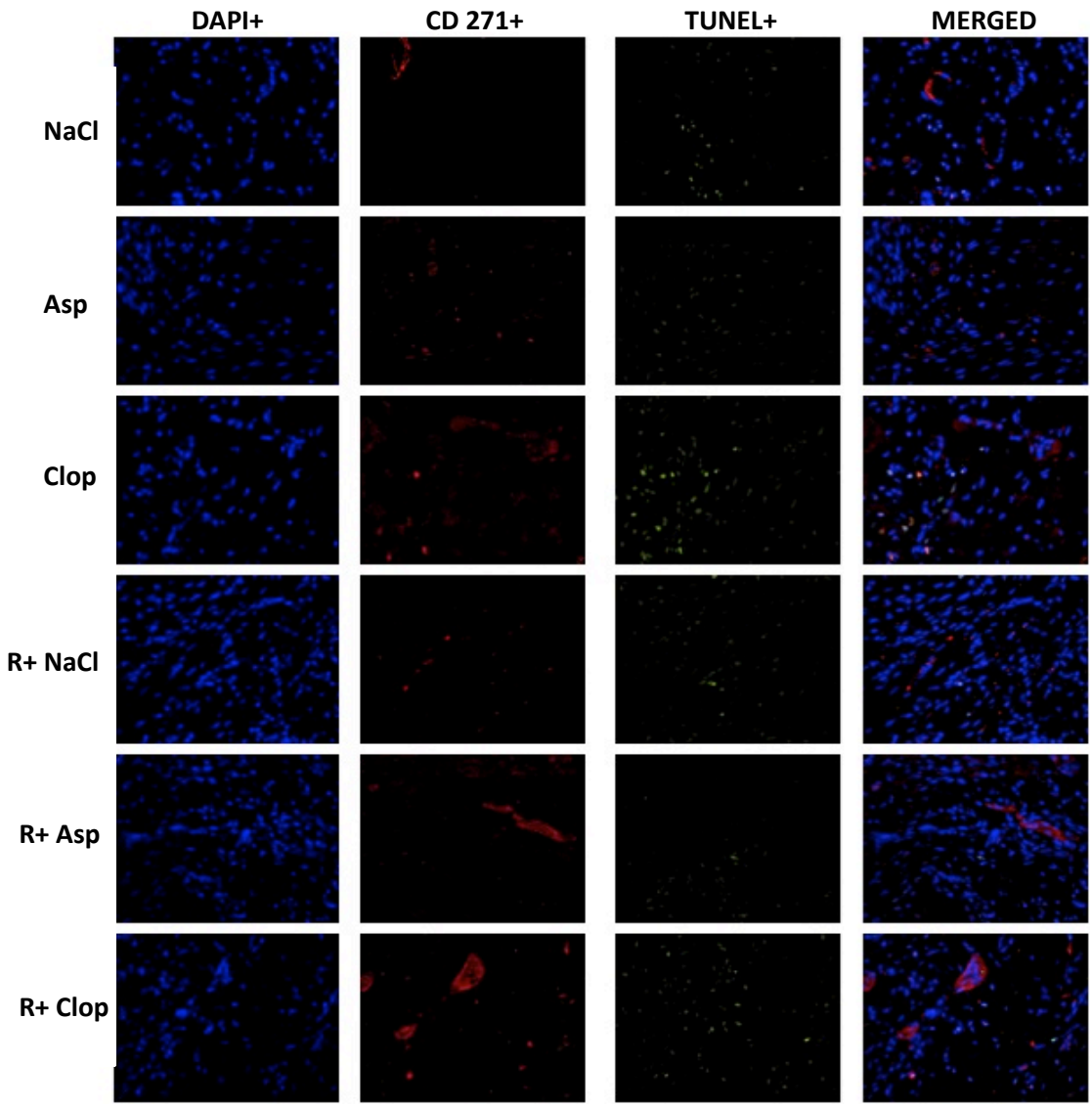

FIGURE 4- Representative fluorescence images of the connective tissue between the top of the alveolar bone crest and the cemento-enamel junction stained for CD 271 (stem cell marker) and TUNEL (apoptosis marker) in rats treated with NaCl 0.9%, Asp (30 mg/kg) or Clop (75 mg/kg) submitted or not to experimental periodontal repair (vehicle-treated: 'NaCl', Aspirin- treated: 'Asp', Clopidogrel-treated: 'Clop', Repair+vehicle-treated: 'R+NaCl', Repair+Aspirin-treated: 'R+Asp', Repair+Clopidogrel-treated: 'R+Clop'). Images are representative of the results observed in 5 animals in each group. 100X magnification.

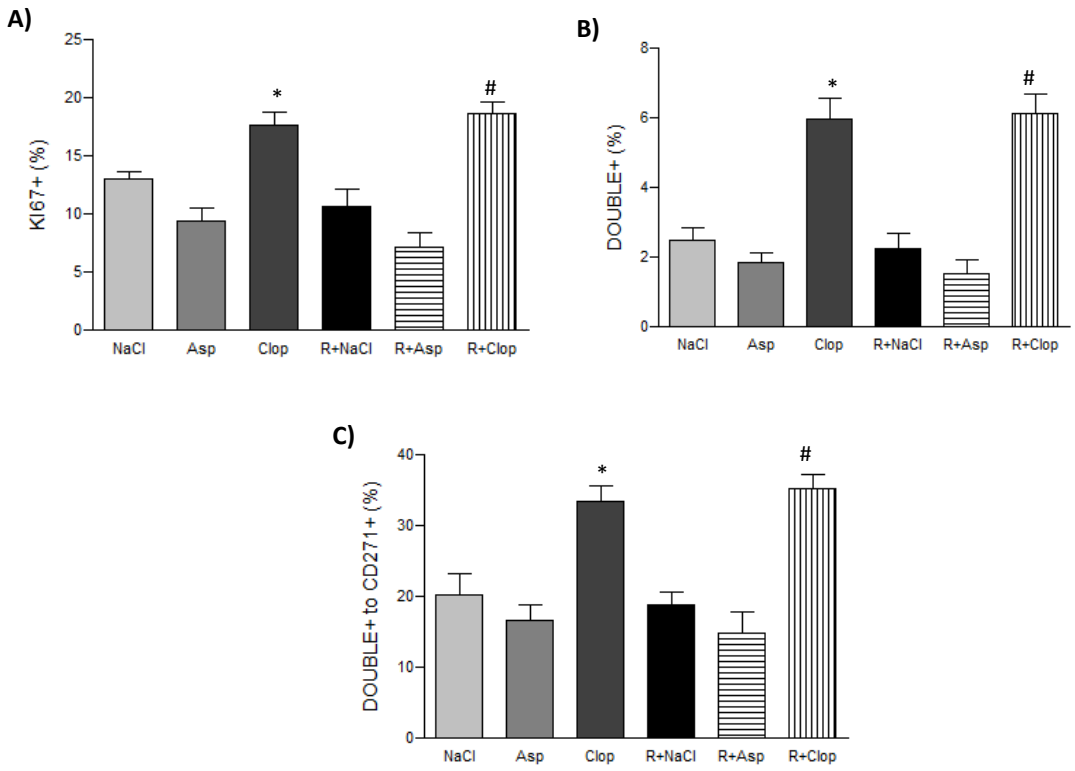

FIGURE 5- Effect of systemic administration of Asp and Clop on the percentage of cells in proliferation (panel A), mesenchymal stem cells in proliferation (panel B) and the percentage of CD 271 and KI67 positive cells normalized by CD 271+ cells (panel C). Treatment with Clop showed a significant increase in the percentage of cells in proliferation, CD 271+ cells in proliferation and CD 271 and KI67 positive cells normalized by CD 271 positive cells. The results are representative from 5 animals/group. Values are expressed as mean± SEM. *P<0.0001 compared to NaCl and Asp; #P<0.0001 compared to R+NaCl and R+Asp.

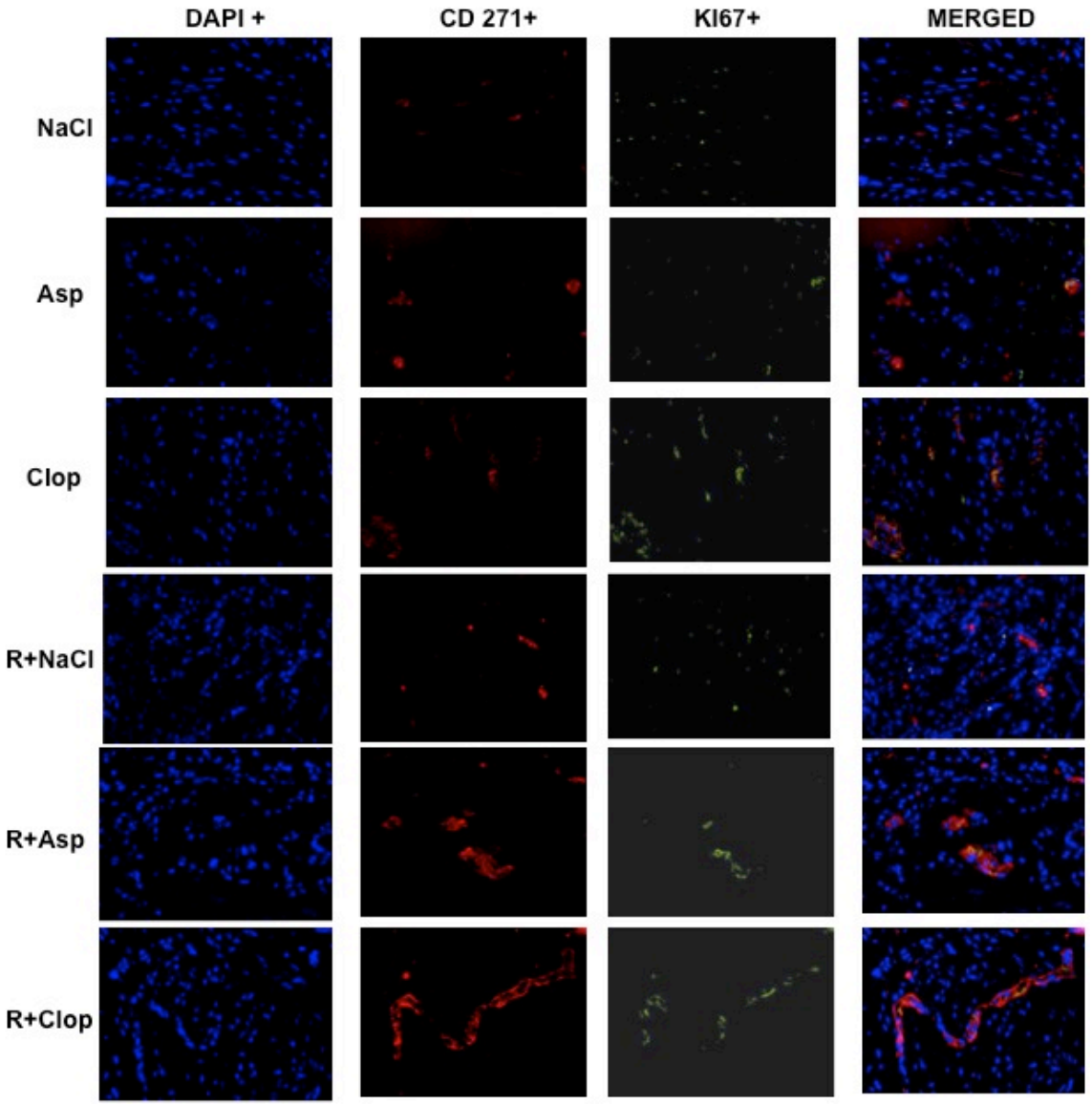

FIGURE 6- Representative fluorescence images of the connective tissue between the top of the alveolar bone crest and the cemento-enamel junction stained for CD 271 (stem cell marker) and KI67 (proliferation marker) in rats treated with NaCl 0.9%, Asp (30 mg/kg) or Clop (75 mg/kg) submitted or not to experimental periodontal repair (vehicle-treated: 'NaCl', Aspirin- treated: 'Asp', Clopidogrel-treated: 'Clop', Repair+vehicle-treated: 'R+NaCl', Repair+Aspirin-treated: 'R+Asp', Repair+Clopidogrel-treated: 'R+Clop'). Images are representative of the results observed in 5 animals in each group. 100X magnification.

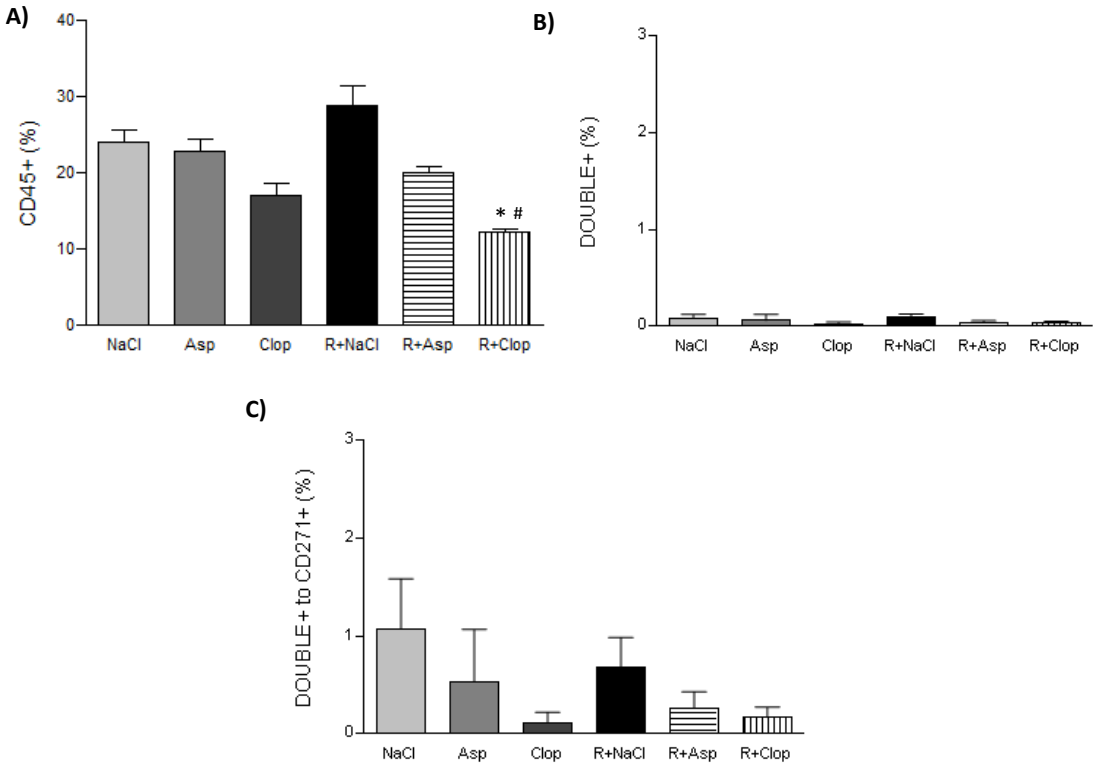

FIGURE 7- Effect of systemic administration of Asp and Clop on the percentage of CD 45+ cells (panel A), CD 271 and CD 45 positive cells (panel B) and the percentage of CD 271 and CD 45 positive cells normalized by CD 271+ cells (panel C). Treatment with Clop during periodontal repair significantly decreased the percentage of CD 45+ cells. It was not observed significant differences in the CD 271 and CD 45 positive cells and the percentage of CD 271 and CD 45 positive cells normalized by CD 271+ cells among the experimental groups. The results are representative from 5 animals/group. Values are expressed as mean± SEM. *P<0.001 compared to R+ NaCl; #P<0.05 compared to R+Asp.

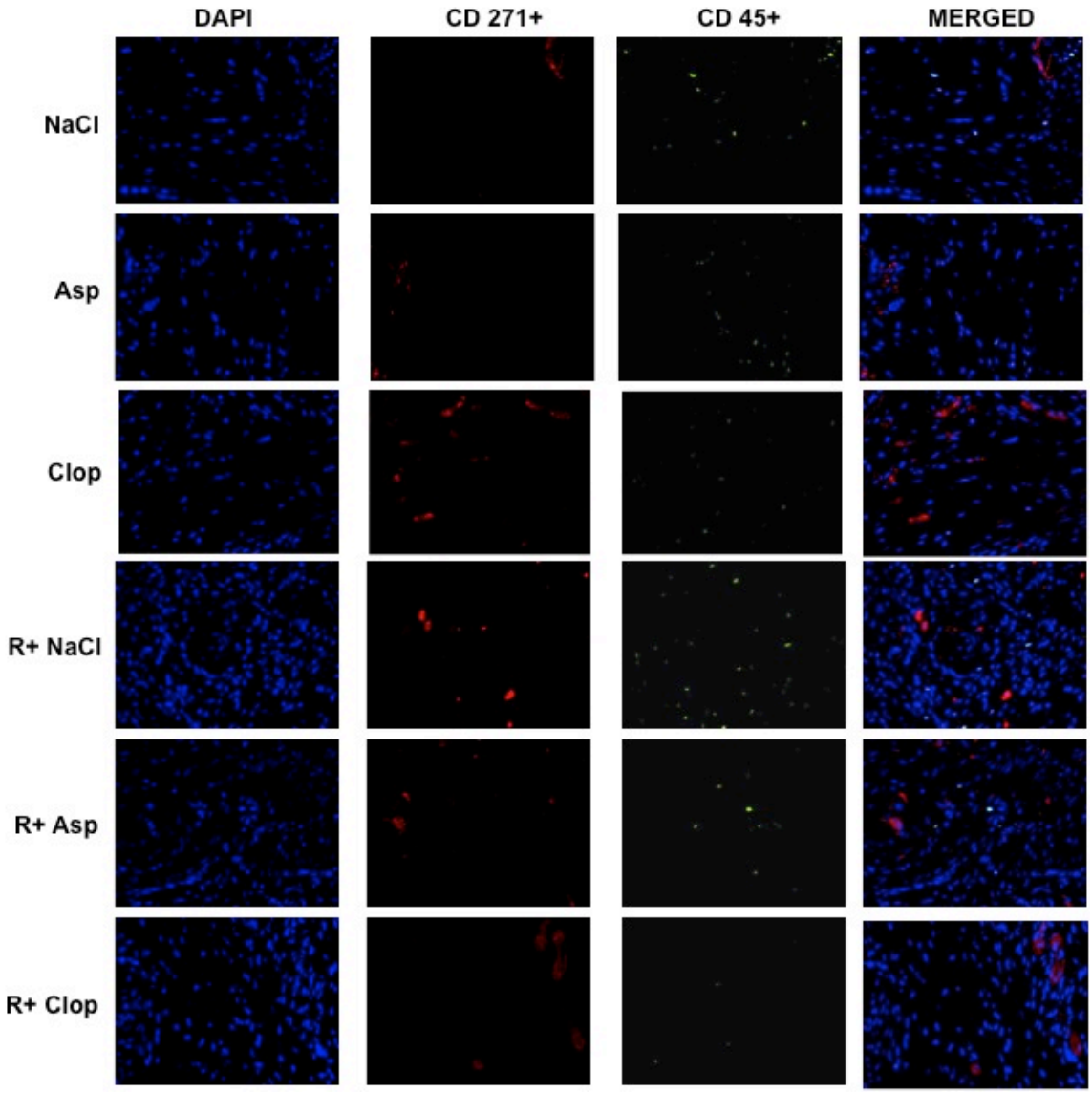

FIGURE 8- Representative fluorescence images of the connective tissue between the top of the alveolar bone crest and the cemento-enamel junction stained for CD 271 (stem cell marker) and CD 45 (lymphocyte marker) in rats treated with NaCl 0.9%, Asp (30 mg/kg) or Clop (75 mg/kg) submitted or not to experimental periodontal repair (vehicle-treated: 'NaCl', Aspirin- treated: 'Asp', Clopidogrel-treated: 'Clop', Repair+vehicle-treated: 'R+NaCl', Repair+Aspirin-treated: 'R+Asp', Repair+Clopidogrel-treated: 'R+Clop'). Images are representative of the results observed in 5 animals in each group. 100X magnification.

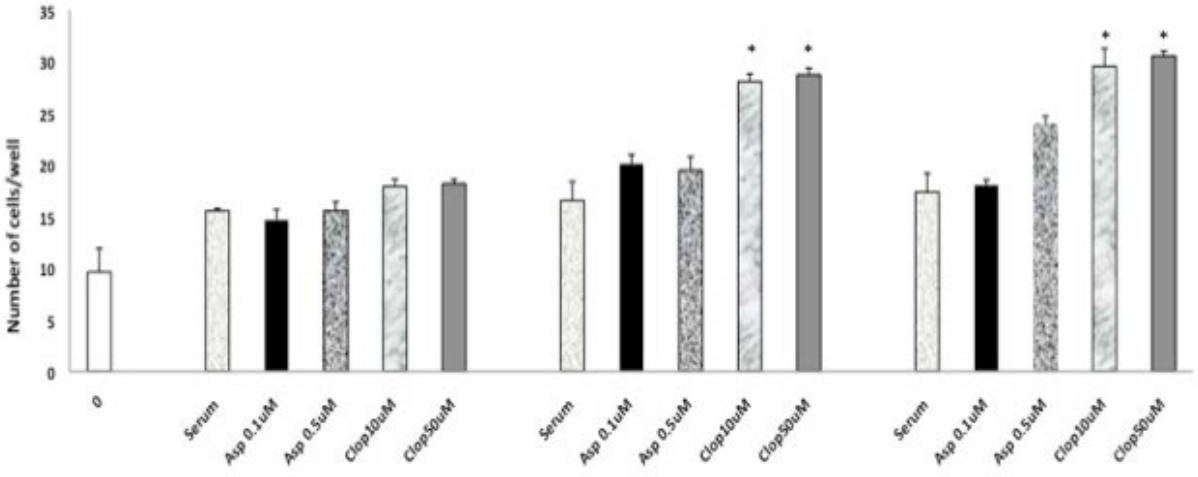

FIGURE 9- Effects of Asp and Clop in different dosages in the proliferation rate analysis of human bone marrow mesenchymal stem cells. Treatment with Clop after 6 and 9 days of stimulation showed a significant increase when compared to the other groups. Five fields per well were examined in triplicate at 600x. Values are expressed as mean± SEM. *P<0.001 compared to the other groups.

CONCLUSÕES

4. CONCLUSÕES

Com base nos resultados deste trabalho, conclui-se que:

1. O Clop apresenta uma promissora atividade antiinflamatória induzindo, dessa forma, aceleração da fase reparadora no modelo de doença periodontal experimental induzida por ligadura.

2. O tratamento com Clop, mas não com Asp, proporcionalmente estimulou a proliferação das células mesenquimais indiferenciadas quando comparado ao número de células em apoptose.

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APÊNDICE

APÊNDICE 1

PLATELET INACTIVATION DELAYS WOUND HEALING IN CONNECTIVE TISSUE IN RATS.

Running title: Platelet inactivation delays wound healing

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ABSTRACT

Wound healing involves several cell types including leukocytes, endothelial cells and platelets and is regulated by a signaling network involving growth factors, cytokines and chemokines, many originated from platelets. Since platelets contain growth factors that can modulate the healing process, we assessed the role of platelet activation in a murine model of wound healing. Polyvinyl alcohol sponges were implanted subcutaneously in rats. Animals were treated daily for 7, 14 and 21 days with aspirin (Asp), at equieffective antiplatelet dose. Tissue reaction was evaluated by microscopic examination (H&E), expression of type I collagen, growth factors VEGF, PDGF and endostatin (ELISA) and fibroblast proliferation. After 7 and 14 days, Asp (30 mg/kg) significantly decreased ($p<0.05$) collagen deposition at the wound site. In the same experimental periods Asp suppressed VEGF and PDGF ($p<0.05$), but did not affect endostatin levels. These Asp-mediated effects were transient, as there was no difference on VEGF and PDGF levels and collagen deposition after 21 days. Also, Asp did not affect fibroblast proliferation. These results demonstrated that Asp-induced platelet inactivation can significantly delay wound healing in subcutaneous connective tissue. Such effects may be mediated through the decrease of VEGF and PDGF levels released from platelets.

Keywords: angiogenesis, cell culture, VEGF, wound repair, histopathology

INTRODUCTION

Several studies focus in current evidence that Aspirin (Asp) may be used as a chemopreventive agent in various types of malignant tumours (Raza et al, 2011; Thiagarajan and Jankowski, 2012; Mills et al, 2012; Thun et al, 2012). Despite major advances in cancer and anti-inflammatory research, Asp has been widely used as a therapeutic agent in patients with atherosclerosis and various cardiovascular issues, including patients receiving coronary artery stents attributed to their antiaggregatory effects on platelets (Rao and Fareed, 2012; Kessler et al, 2012; Dai and Ge, 2012; Spectre et al, 2011). In addition to treatment of many complications of coronary syndromes, bleeding and delayed healing of ulcers are well recognized clinical problems associated with the use of Asp and other nonsteroidal anti-inflammatory drugs (NSAIDs). These serious adverse effects have been attributed in part to their antiaggregatory effects on platelets (Ma et al, 2011). Platelets contain a wide range of biological mediators capable of promoting tissue growth and new blood vessel formation (angiogenesis), including vascular endothelial growth factor (VEGF) (Maloney et al, 1998), growth factor of endothelial cells derived from platelets (EGF) (Myazono and Heldin, 1989), and platelets derived growth factor (PDGF) (Linder et al, 1979) as well as anti-angiogenic factors such as endostatin (Ma et al, 2001) which are released during the healing process. Administration of platelet rich plasma (PRP) has been shown to improve results in a variety of regenerative therapies (Camargo et al, 2002; Powell et al, 2009). It is also suggested that platelets act as inflammatory cells in innate immune responses, as sentinels against microbial invasion, orchestrating leukocyte recruitment and migration through the tissue and influencing the repair process in chronic diseases. Platelet activation and the formation of leukocyte-platelet complex are directly influenced by cytokines and occur rapidly by low levels of ADP (Pitchford, 2007). Platelets also contain numerous enzymes such as metalloproteinases (MMPs) that affect the composition and integrity of the cell membrane and extracellular matrix production (Ciferri et al, 2000; Falcinelli et al, 2005).

As platelets contain considerable quantities of potent endogenous bioactive substances and considering their important role in inflammation and healing processes, we hypothesized that Asp at dose known to inhibit platelet aggregation and thrombus formation, might interfere with the healing process in the subcutaneous connective tissue by suppressing the release of growth factors. Therefore, in this study, we evaluated the effects of Asp, the most frequently antiplatelet drug prescribed, on subcutaneous tissue reaction by histological

analysis as well as by the measurement of growth factors VEGF, PDGF, endostatin and collagen type I by enzyme linked immunosorbent assay – ELISA in a classic model of wound healing in rats.

MATERIAL AND METHODS

Animals

The experimental protocol was approved by the Animal Experiment Ethics Committee of the School of Dentistry at Araraquara, São Paulo State University (UNESP). Thirty male Holtzman rats (*Rattus norvegicus albinus*), weighing 250-300 g, were used in the experiments. During the length of the experimental protocol, the rats were kept in a quiet room with controlled temperature (21±1°C), humidity (65-70%) and a 12-h light-dark cycle. Animals were fed standard rat chow and received tap water *ad libitum*. The animals were randomly distributed into six experimental groups (n = 5 animals/group), defined according to the experimental period. Three groups were treated with Asp and 3 groups were used as vehicle controls.

Chemicals

Aspirin and other reagents were purchased from Sigma Aldrich (Brazil), unless otherwise stated.

Wound Induction

The model of wound healing was previously described (Barbul et al, 1985) and widely used by others. Briefly, the rats were anesthetized by the administration of a mixture of ketamine (80 mg/kg; Francotar®, Virbac do Brasil Ltda, São Paulo, SP, Brazil) and xylazine (20 mg/kg; Virbaxil®, Virbac do Brasil Ltda). After tricotomy in the dorsal region (5 cm diameter, approximately), an 8 mm dorsal skin incision was made under sterile conditions. Subcutaneous pockets were created on both sides of the incision and 3 cylindrical sponges (Clinicel ™PVA Sponge, Eudora Kansas U.S.A.), 0,7 mm diameter and 0,3 mm thick, previously autoclaved, were implanted. The wounds were closed with running 3-0 silk sutures (Ethicon 3-0). The rats were randomly assigned to one of the experimental periods study and sacrificed after 7, 14 and 21 days of the sponge implantation.

Treatment with aspirin

After the sponge implantation the animals were treated daily with saline (NaCl 0,9%) and aspirin (30 mg/kg) intragastrically, by gavage during the experimental periods (7, 14 and 21 days). This concentration had been shown to be able to inhibit platelet aggregation and thrombus formation in rats (Ashida and Abiko, 1979; Sasaki et al, 1996; Wallace et al, 1995; Wallace et al, 1999; Taka et al, 1999; Sugidachi et al, 2000; Coimbra et al, 2011).

Microscopic evaluation

At each of indicated experimental periods, the animals were sacrificed, and one sponge and the surrounding tissues were removed from each rat for histological evaluation. These tissue samples were soaked in 10% neutral buffered formalin for 48 hours. After routine processing and paraffin embedding, serial sections of 5 μm were obtained and subsequently stained with Hematoxylin and Eosin (H&E) for qualitative analysis. The analysis was conducted by a single examiner, who was blinded to the experimental groups, using an optical microscope (Diastar; Cambridge Instruments, Buffalo, NY) set at x40 and x100 magnification.

Measurement of Collagen Type I, VEGF, PDGF and Endostatin

Two sponges of each rat were carefully dissected from the surrounding tissues and the wound healing exsudate was collected in an eppendorf tube containing 1 ml of heparinized saline solution by applying mechanical pressure with tissue forceps. We used commercially available ELISA kit to measure the concentration of VEGF, PDGF, endostatin and collagen type I in the wound healing exsudate (Gu et al, 2006). In brief, the samples were kept in ice-cold PBS buffer (50 mg/ml), and the total proteins were extracted using M-PER Extraction Reagents (PIERCE, Rockford, Illinois) according to the manufacture's protocol. The total protein concentration of tissue supernatant was determined using Bio-Rad Protein Assay (Bio Rad Laboratories, Hercules, California). The protein levels of VEGF, PDGF, endostatin and collagen type I in the samples were normalized and expressed as pg per mg of total protein.

Cell Culture

A continuous lineage of periodontal ligament fibroblasts from mouse (a gift from Dr. Martha Somerman, Univ of Washington, Seattle, WA, USA) was used in this study. The cells were grown on DMEM (Gibco) supplemented with 7.5% FBS, 100 U/mL penicillin, 100

µg/mL estreptomicin and maintained in an atmosphere of 98% humidity, 5% CO_2 and 37°C.

Cell Proliferation

Cell proliferation was determined by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) assay (Ma et al, 2012). The cells were plated on 24-well plates (2 x 10^3/well) in triplicate. After 4 days of culture in complete growth medium, 1 ml of serum from rats that had been treated daily (for 7, 14 and 21 days) with vehicle or Asp (30mg/kg) was added to the medium and the cells incubated for an additional 24 h. Thereafter, the culture medium was aspirated and MTT was added to each well (0.25 mg/mL). After 4 h of incubation at 37°C, medium was removed and the cells were lysated with DMSO. 50ul of the lysate was transferred to a 96-well plate and cell proliferation was determined as being proportional to the absorbance measured at 570 nm wavelength on a microplate reader (model 3550-UV microplate reader; Bio-Rad, Hercules, CA).

Statistical analysis

All the results are expressed as mean ± SEM. Differences between the groups were analyzed by one-way analysis of variance followed by the Student-Neuman-Keuls test for multiple comparisons, and values of $p<0.05$ were considered significant.

RESULTS

All animals were alive at the end of all experimental periods. The recorded body weights were 360±25.20, 352.52±34.45, 362.52±24.81, 376.66±24.70, 379±20.94 and 376.66±30.86 g for groups treated with saline (7, 14 and 21 days) and aspirin (7, 14 and 21 days), respectively. Neither occult bleeding nor hematomas were observed.

Microscopic analysis

In vehicle-treated animals, implantation of the PVA sponge resulted in classical maturation of granulation tissue. After 7 days, presence of leukocytes, fibroblasts, new blood vessel formation (angiogenesis) and thin bundles of collagen fibers were observed. At the 14-day period, there were a greater number of fibroblasts, associated with increased fibroplasia, characterizing advanced maturation of granulation tissue, which continued until the period of

21 days.

In Asp-treated rats, there was a marked reduction on fibroblast numbers and fibroplasia at both 7 and 14 days, indicating an impairment of maturation of the granulation tissue. However, at the 21-day period, the microscopic characteristics of the implanted sponges were similar in both groups (Figure 1).

Measurement of Collagen type I, VEGF, PDGF and Endostatin

In the exsudate from vehicle-treated animals, the type I collagen levels increased progressively from 0.85±0.12 (7 days) to 1.73±0.21 (14 days) and 1.97±0.08 (21 days) ug/ml. The Asp-treated group showed a significant decrease in the type I collagen levels in the 7 and 14 days periods (60% and 37%, $p < 0.05$ respectively); however, at the 21-day period there was no significant difference between groups ($p > 0.05$).

The measured levels of VEGF, PDGF and Endostatin in the samples obtained from the control animals were 2100±1.2; 500±0.8; 18±0.6 ng/ml, 2200±1.4; 500±0.8; 12±0.4 ng/ml and 100±0.5; 250±0.8; 12±0.2 ng/ml during the 7, 14 and 21 day periods respectively. In the Asp treated groups, the values of VEGF and PDGF decreased significantly (18% and 38%, $p<0.05$) in the 7 and 14 days periods. On the other hand Endostatin concentrations in PVA sponges implanted were not significantly different from those found in control group (18±0.6 ng/mL).

Proliferation of fibroblasts

Exposure of fibroblasts to serum from vehicle-treated rats resulted in a concentration-dependent increase in proliferation. Serum from Asp-treated rats increased proliferation to the same extent as serum from vehicle- treated rats.

DISCUSSION

Research efforts have increasingly focused on the use of Asp as a chemopreventive agent (Mills et al, 2012; Thun et al, 2012). Asp is also widely used as a therapeutic target in patients with atherosclerosis and other cardiovascular diseases (Kessler et al, 2012; Dai and Ge, 2012). It can be attributed to its antiaggregatory and anti-inflammatory effects on platelets (Spectre et al, 2011). It is well established that the dosage of aspirin used in this study effectively reduced platelet aggregation (Ma et al, 2001; Wallace et al, 1999; Sugidachi et al,

2000; Coimbra et al, 2011) without affecting the number of circulating platelets of the experimental animals. Throughout the periods of treatment the coat of the animals remained normal in appearance and there were no color changes in skin and abnormal bleeding that might indicate hematological disorders.

Systemic administration of aspirin within the same dose range used in a thrombosis model (Judge et al, 2008) during the subcutaneous tissue reaction, i.e. in the presence of the sponges, significantly reduced the amount of collagen fibers in the granulation tissue surrounded the sponges and consistently decreased the production of collagen type I in the three periods analyzed and the growth factors VEGF and PDGF in the periods of 7 and 14 days.

We observed a significant decrease on the expression of VEGF on days 7 and 14 in the animals treated with aspirin. This can be explained by the non-steroidal anti-inflammatory properties associated with the antiplatelet function related to the aspirin. According to Maloney et al (1995), platelets secrete proteins during aggregation that are important for coagulation, inflammation and repair. The fact that platelet aggregation occurs predominantly at sites of endothelial injury and VEGF is an endothelial growth factor as a mediator of permeability, together these data suggest that VEGF is an important mediator of tissue repair. Spolidorio et al (2010) found that there was a decrease in the amount of circulating VEGF and that it impaired gingival repair with 15 days after removal of ligatures in thrombocytopenic rats, suggesting that platelets play an important role in the process of periodontal repair after induction of periodontal disease and that reduction of angiogenic growth factors such as VEGF is associated with delayed healing in this periodontal disease model. Also, Ma et al (2001) reported a delay in the repair process of experimentally gastric ulcers in rats treated with antiplatelet drugs. This delay was associated with decreased release of VEGF and increased serum levels of endostatin (Ma et al, 2001; Sibilia et al, 2007).

However, this Asp-induced delay on healing was transient, as no significant differences were found after 21 days of repair. This lack of difference may be associated with a natural decrease on the levels of VEGF with the progress of healing, which was previously reported by Cooke et al (2006) in a periodontal wound repair model in humans. These authors showed that the highest levels of VEGF in sites of periodontal healing are observed after 12-15 days, with a marked decrease in the following days.

We observed a similar effect of Asp on PDGF levels in the initial experimental periods of 7 and 14 days. PDGF is responsible for the migration and proliferation of fibroblasts, smooth muscle cells and monocytes, as well as being related to the repair of epithelial cells.

According to Raja et al (2009), PDGF is important for the stimulation of chemotaxis, cell proliferation and matrix synthesis, increasing the influx of fibroblasts to sites of wounds and increasing the production of extracellular matrix. Platelet inactivation by aspirin could impair epithelial repair, and this fact could corroborate with the findings observed in this study. Similarly to what we observed for VEGF, this effect was transient, since there was no significant difference on PDGF levels between vehicle- and Asp-treated animals at 21 days. This is also in agreement with the report of Cooke et al (2006), using a different model, in a periodontal wound repair, they observed an increase in PDGF expression within 6-9 days of healing and no significant difference among the different surgical treatments analyzed after 19-24 days.

Lanas et al (1994) showed that treatment with aspirin inhibits the mitogenic action of platelets on fibroblast culture by inhibiting the release of putative growth factors. Such effect might explain the adverse effects of aspirin on ulcer healing. Our results are in disagreement and showed that treatment with aspirin inhibited the expression of VEGF and PDGF, but did not affect fibroblast proliferation.

CONCLUSION

In summary, the present study shows that systemic administration of aspirin delayed the wound healing associated with subcutaneous tissue reaction in rats. Despite recent advances revealing the prominent role that platelets can have in inflammation and repair processes, more studies are necessary to investigate the role of antiplatelet drugs on the inflammatory and repair processes and the biological mechanisms involved.

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FIGURES

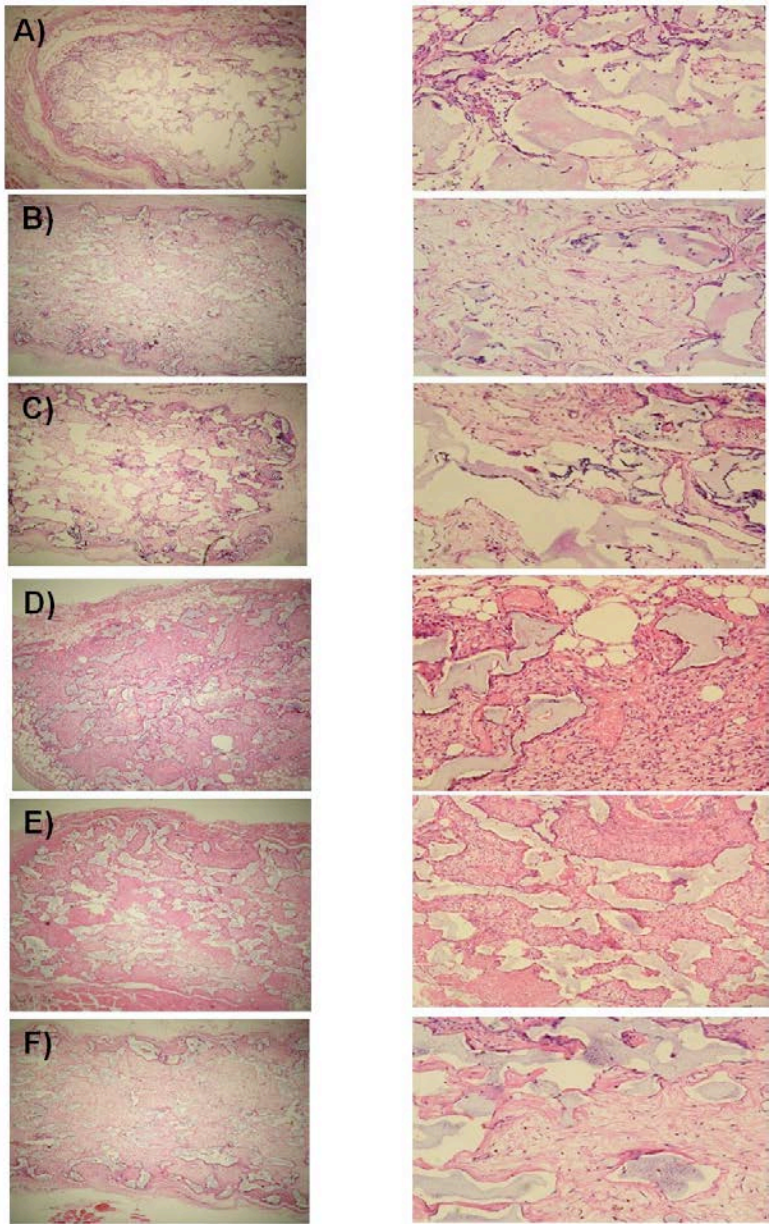

FIGURE 1- Histological analysis of the sponges surrounded by granulation tissue, stained with H&E of the control animals after 7, 14 and 21 days (A, B and C, respectively) and treated with Aspirin (30 mg/kg) after 7, 14 and 21 days of experimental period (D, E and F, respectively). In the initial periods granulation tissue with intense cellularity surrounded by dilated vascular figures and collagens fibers can be observed in the group treated with aspirin. After 21 days no differences were observed in the granulation tissue. Magnification 40X and 100X.

FIGURE 2- Expression of type I collagen in ug/ml in the animals treated with Aspirin (30 mg/kg) in the experimental periods of 7, 14 and 21 days (A, B and C, respectively). Values are expressed as mean ± SEM. *p<0.05 vs control.

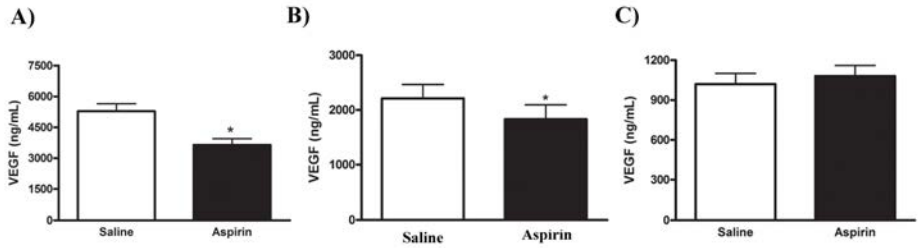

FIGURE 3- Expression of VEGF in ng/ml in the animals treated with Aspirin (30 mg/kg) during 7, 14 and 21 days (A, B and C, respectively). The expression was significantly reduced (*p<0.05) after 7 and 14 days of treatment. On day 21 there was no difference among the experimental groups. Values are expressed as mean ±SEM.

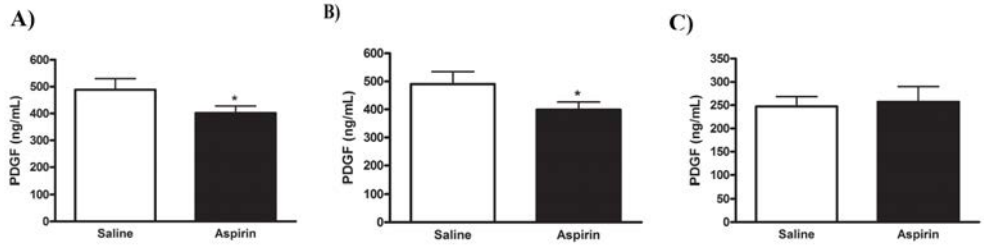

FIGURE 4- Expression of PDGF in ng/ml in the animals treated with Aspirin (30 mg/kg) during 7, 14 and 21 days (A, B and C, respectively). The expression was significantly reduced (*p<0.05) after 7 and 14 days of treatment. On day 21 there was no difference among the experimental groups. Values are expressed as mean ±SEM.

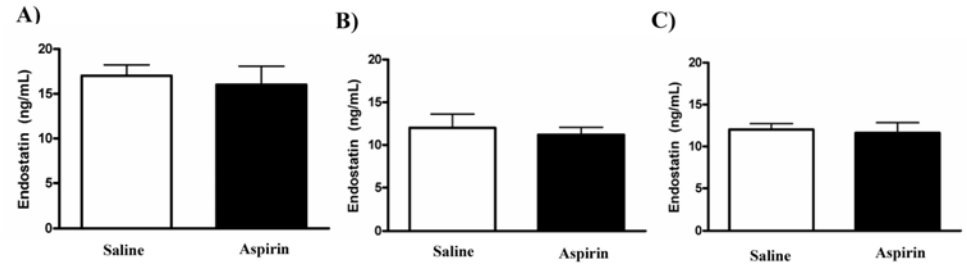

FIGURE 5- Expression of endostatin in ng/ml in the animals treated with Aspirin (30 mg/kg) during 7, 14 and 21 days (A, B and C, respectively). There was no difference among the experimental groups in the time points analyzed. Values are expressed as mean ±SEM.

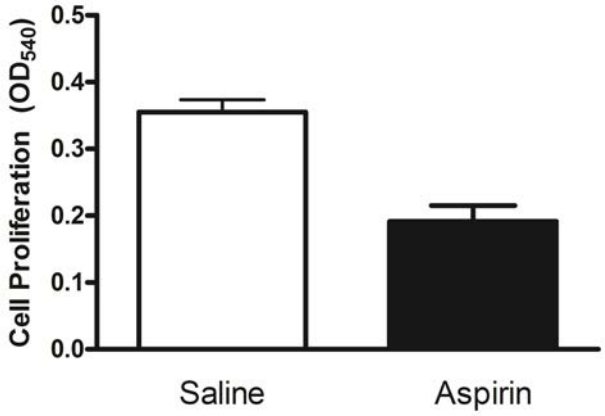

FIGURE 6- Fibroblast cell proliferation expression after exposure to the serum from vehicle and aspirin treated rats. Serum from Asp-treated rats increased proliferation to the same extent as serum from vehicle- treated rats Values are expressed as mean ±SEM.

ANEXOS

ANEXO 1

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Araraquara

FACULDADE DE ODONTOLOGIA

Proc. CEEA nº 18/2010

Araraquara, 12 de agosto de 2010

Senhores Pesquisadores:

O Comitê de Ética em Experimentação Animal-CEEA desta Faculdade reunido em 10/08/2010, após a avaliação do projeto de sua responsabilidade intitulado "Avaliação das citocinas liberadas por plaquetas durante o desenvolvimento da doença periodontal e reparo dos tecidos periodontais de ratos tratados ou não com drogas antiplaquetárias" (Proc. CEEA nº 18/2010) AUTORIZA a realização da pesquisa, ficando a apresentação do RELATÓRIO FINAL para SETEMBRO/2013. O CEEA sugere correção da temperatura constante no protocolo e no projeto, uma vez que a temperatura do Biotério é de 23 ± 2ºC.

Atenciosamente.

Profª Drª ELENY ZANELLA BALDUCCI
Coordenadora do CEEA

Ao
Prof. Dr. LUIS CARLOS SPOLIDÓRIO
DD. Pesquisador Responsável
a/c Leila Santana Coimbra
Departamento de Fisiologia e Patologia

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ANEXO 2

Journal of
PERIODONTAL RESEARCH

Antiplatelet drugs reduce the immunoinflammatory response in a rat model of periodontal disease: a pilot study.

Journal:	*Journal of Periodontal Research*
Manuscript ID:	JRE-04-13-2218
Manuscript Type:	Original Article
Date Submitted by the Author:	25-Apr-2013
Complete List of Authors:	Coimbra, Leila; Dental School of Araraquara, State University of São Paulo (UNESP), Physiology and Pathology Steffens, Joao Paulo; São Paulo State University, School of Dentistry at Araraquara, Physiology and Pathology Muscara, Marcelo; University of Sao Paulo, Pharmacology; Rossa Jr, Carlos; Fac Odontologia de Araraquara-UNESP, Diagnosis and Surgery Spolidorio, Luis; São Paulo State University, School of Dentistry at Araraquara, Physiology and Pathology
Keywords:	Periodontal disease, Inflammation, Systemic host effect, Polymorphonuclear leukocytes

Journal of Clinical Periodontology
Official Journal of The European Federation of Periodontology
Founded by the British, Dutch, French, German, Scandinavian, and Swiss Societies of Periodontology

Clopidogrel enhances periodontal repair through decreased inflammation

Journal:	*Journal of Clinical Periodontology*
Manuscript ID:	CPE-05-13-4510
Manuscript Type:	Animal Experiment
Date Submitted by the Author:	16-May-2013
Complete List of Authors:	Coimbra, Leila; Dental School of Araraquara, State University of Sao Paulo, Physiology and Pathology Steffens, Joao Paulo; São Paulo State University - School of Dentistry at Araraquara, Phisiology and Pathology Rossa, Carlos; UNESP - Univ Estadual Paulista, Department of Diagnosis and Surgery Graves, Dana; University of Pennsylvania, Periodontics Spolidório, Luis; Dental School of Araraquara, State University of Sao Paulo, Physiology and Pathology
Topic:	Aetiology
Keywords:	Periodontal disease, Immunohistochemistry
Main Methodology:	Animal Model

Araraquara, 26 de julho de 2013

LEILA SANTANA COIMBRA