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ORIGINAL RESEARCH

Standardized *Arrabidaea chica* Extract Shows Cytoprotective Effects in Zoledronic Acid-Treated Fibroblasts and Osteoblasts

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Introduction: Osteonecrosis of the jaw is a condition associated with intraoral ulceration and bone necrosis induced by antiresorptive medications, such as zoledronic acid, a bisphosphonate. Previous data on *Arrabidaea chica* (H&B.) Verlot wound healing activity prompted the study reported herein on *A. chica* standardized hydro alcoholic extract in vitro cytoprotective activity data on epithelial and osteoblastic cells exposed to zoledronic acid (ZA).

Methods: Primary human gingival fibroblasts and murine pre-osteoblasts were treated with ZA 10 µM together with 5 or 10 $\mu g.mL^{-1}$ *A. chica* extract for 24h and 48 h. At both times, cells were submitted to viability assay and caspase 3/7 activation evaluation. Statistical analysis used one-way ANOVA and p=0.05.

Results: In cell viability assay, a drastic damage effect of ZA appeared after 48 h in both epithelial (55.8%) and pre-osteoblastic cells (39.7%). When treated with ZA in combination with *A. chica* extract, cells showed higher viability values: 74.1%–82.3% for fibroblasts and 66% for pre-osteoblasts. Furthermore, the combined treatment presented lower caspase 3/7 activation in fibroblasts and pre-osteoblasts.

Conclusion: At low concentrations, *A. chica* extract showed promising cytoprotective effects against ZA-induced damage actions; however, further in vitro and in vivo studies are required to establish the mechanism of action.

Keywords: *Arrabidaea chica*, zoledronic acid, epithelial cells, osteoblasts, fibroblasts

Introduction

Bisphosphonates, as the potent third-generation intravenous zoledronic acid (ZA),[1] are antiresorptive medications widely used to treat bone disorders and complications of bone metastases in cancer patients.[2] Despite bisphosphonates beneficial therapeutic effects, the occurrence of Osteonecrosis of the jaw induced by bisphosphonates (BRONJ) is a devastating side effect, clinically characterized by a persistent exposed bone (more than 8 weeks) in the maxillofacial region of patients previously treated with bisphosphonates, without history of radiation therapy of the jaws.[2]

Studies revealed that oral mucosa tissues can be directly affected by bisphosphonates through different mechanisms including apoptosis,[3] inhibition of migration, proliferation, growth and influence on cells viability,[1,3–7] inhibition of angiogenesis[5,8] and decrease in collagen and extracellular matrix production.[9] Furthermore, oral bisphosphonate presents a potency comparable to intravenous bisphosphates that compromise cell activities, reinforcing the theory that bisphosphonates detrimentally affect not only osteoclasts but also several other somatic cell types.[10]

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Once BRONJ occurs, there is not an established treatment for the oral lesion, and surgical excisions of the necrotic bone, systemic or topical application of antibiotics and the improvement of oral hygiene are palliative applied therapies.[11–13]

In order to improve the healing process, many studies have focused therapeutic and cytoprotective benefits of secondary metabolites from plant origin.[14–16] In that context, *Arrabidaea chica* (Bignoniaceae), a native South American vine used in folk medicine for epithelial diseases,[17] has been reported for important healing properties attributed to the anthocyanin content found in the standardized extract. Among these activities are inducement of in vitro dermal fibroblasts growth and synthesis of collagen[18,20] apart from improvement of wound healing in vivo.[18]

Considering the side effects that patients under BRONJ treatment encounter prompted data reported herein on the benefits that *A. chica* standardized extract can play on bisphosphonates-modified healing process. Therefore, this study investigated the cytoprotective effect of *A. chica* extract on cells affected by ZA, using cell viability and apoptosis tests.

Material and Methods

Plant Material and Extraction Procedures

Arrabidaea chica Verlot. (Bignoniaceae) leaves were obtained at Chemical, Biological and Agricultural Pluridisciplinary Research Center of University of Campinas, Brazil, experimental field [Voucher deposit 1348 at CPQBA-Herbarium – Germoplasm bank]. The use of plant genetic material of the present study was approved by the Genetic Patrimony Management Board (CGEN/MMA) by CNPq through Access and Shipment Component of Genetic Heritage of Genetic Heritage for scientific research purposes (no. 010150/2012-9).

A. chica hydroalcoholic standardized extract was obtained according to the method described by Jorge et al,[18] with modifications. One kilogram of dried ground leaves was extracted using 5 L of 70% hydroethanol solution acidified with 0.3% citric acid with three one hour and a half period extraction, at room temperature with mechanical stirring. Thereafter, workup involved extract filtration, organic solvent evaporation using vacuum and residual water removed by spray drying using a Mini Spray Drier B-290, loop B-295; Büchi®, Switzerland, with inlet temperature 100°C ± 2°C and outlet temperature 60°C ± 2°C, with aspiration of 250 $mL.h^{-1}$ of non-filtrated N_2 atmosphere, injection pressure of 414 $L.h^{-1}$ and feed flow of 5 $mL.min^{-1}$ at room temperature, yielding the final product.

Analytical Analysis of the Extract

Analytical analysis was according to Wen et al,[21] with adaptions performed with a Shimadzu series High Pressure Liquid Chromatography (HPLC) system.

Cell Culture

Human primary gingival fibroblasts (FG; the use of these cells was approved by the Ethics Committee in Research of School of Dentistry of Piracicaba - State University of Campinas, register number 008/2012) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco®) supplemented with 10% fetal bovine serum (FBS; Atlanta biologicals®), 100 $U.mL^{-1}$ penicillin, 100 $\mu g.mL^{-1}$ streptomycin and 250 $ng.mL^{-1}$ Fungizone® (Amphotericin B; Gibco®). Also, murine osteoblast MC3T3-E1 (ATCC® CLR-2593™) were maintained in minimum essential medium alpha (Alpha-MEM; Gibco®) supplemented with 10% FBS, 100 U. mL^{-1} penicillin and 100 $\mu g.mL^{-1}$ streptomycin. The cells were cultured in a humidified atmosphere of 5% CO_2 in air.

Zoledronic Acid Treatment and Experimental Groups

The cells were exposed to 10 µM zoledronic acid (ZA, Novartis®, Basileia, Switzerland) in combination with *A. chica* extract at 5 $\mu g.mL^{-1}$ (ZAAC5) or 10 $\mu g.mL^{-1}$ (ZAAC10) concentrations. Control groups were cells without treatment (Control), 10 µM ZA (ZA) and *A. chica* extract at 5 $\mu g.mL^{-1}$ (AC5) or 10 $\mu g.mL^{-1}$ (AC10) concentration. All experiments were done in triplicate and repeated at three different occasions.

Cell Viability Tests

Cells (FG at $4x10^3$ cells/well and MC3T3-E1 at $1x10^4$ cells/well) were cultured in 96-well plates, in triplicate, for all the experimental groups. To avoid serum protective effects, FBS concentration was set to 5%. After 24 h or 48 h of treatment, viable cells were determined using crystal violet staining (CV) according to manufacturer's recommendation. Briefly, cells were washed with PBS (200 µL/well, pH 7.4), stained with crystal violet solution (0.05%, 50 µL/well), incubated at room temperature for 10 min, washed twice in tap water and drained upside down on paper towels. After 24 h, sodium

dodecyl sulfate (SDS) solution (1%, 200 µL/well) was added to solubilize the stain and absorbance was read using a microplate reader (SpectraMax® Plus 384 Absorbance Microplate Reader; SoftMax® ProData Acquisition & Analysis Software, Molecular Devices' industry) at 562 nm.

Apoptosis Test

Cells (FG at $4x10^3$ cells/well and MC3T3-E1 at $1x10^4$ cells/well) were cultured in 96-well plates. After 24 h, cells were treated according to the experimental groups and further incubated for 24 h or 48 h. The caspase 3/7 activity in cell extracts was determined using Caspase-Glo 3/7 Assay® (Promega® Corporation, Madison, USA) according to the manufacturer's instructions. Briefly, caspase 3/7 reagent was added into the wells (100 µL/well), the plates were protected from light, shook during 30 s (300 rpm) and then incubated at room temperature for 1 h. The luminescence was measured using Promega®-GloMax®-Multi detection system.

Statistical Analysis

The results were analyzed by one-way ANOVA for each cell type, with the post hoc test Tukey, and considering 0.05 as the significance level, using the IBM SPSS Statistic software 23.

Results

Analytical Analysis of the Extract

The final *A. chica* standardized extract presented pH 4.88 ± 0.02. The chromatographic fractionation afforded Carajurin (Table S1; Figure S1) that was used as analytical marker. Analytical HPLC quantitative analysis provided 6.51 ± 0.07 mg g^{-1} Carajurin yield content (Figure S2).

Cell Viability

For fibroblasts, a significant decrease in cell viability was observed at 48 h with ZA treatment. The combination ZA with *A. chica* 5 µg mL^{-1} or 10 µg mL^{-1} concentration maintained cell viability above 70% (Table 1).

Osteoblasts viability was significantly decreased by ZA at both times, whilst ZA with *A. chica* combination achieved higher viability values than ZA alone. At 48 h, osteoblasts treated with ZA showed viability of 39.7% ± 5.7% ($p < 0.0001$) whereas the combination of ZA with *A. chica* 5 µg mL^{-1} maintained cells viability above 65% ($p < 0.0001$) (Table 1).

Apoptosis Test

After 24 h, ZA significantly increased caspase 3/7 activation ($p<0.0001$) in fibroblasts in comparison to Control, whilst the combination of ZA with *A. chica* 5 µg mL^{-1} showed caspase 3/7 activation values statistically similar to the Control and to ZA in combination with *A. chica* 10 µg mL^{-1} ($p>0.05$). ZA in combination with *A. chica* 10 µg mL^{-1} showed a significant reduction in caspase 3/7 activation when compared to ZA ($p<0.0001$). After 48 h, ZA in combination with *A. chica* 5 µg mL^{-1} showed caspase 3/7 activation similar to Control, whereas ZA increased the luminescence values ($p<0.001$) (Figure 1A).

The analyses of osteoblasts's active caspase 3/7 revealed, at both evaluated times, that ZA presented the highest caspase 3/7 activation ($p<0.0001$), whereas the combination of ZA with *A. chica* 5 µg mL^{-1} extract was statistically similar to the Control group ($p>0.05$) (Figure 1B).

Discussion

The promising therapeutic actions of *Arrabidaea chica* Verlot leaves' extract, mainly in wound healing and

Table 1 Cell Viability of Fibroblasts and Osteoblasts After Isolated or Combined Zoledronic Acid and *Arrabidaea Chica* Extract Treatments

		Control	AC5	AC10	ZA	ZAAC5	ZAAC10
Fibroblasts*	24 h	102.8±7.1^{a}	85.3±4^{a}	90.7±5^{a}	98.3±8.9^{a}	75.4±5.9^{b}	92.9±12.4^{a}
	48 h	100.1±11.8^{c}	96.3±4.3^{c}	100.2±1.6^{c}	55.8±7.5^{d}	82.3±7.6^{c,e}	74.1±10.3^{e}
Osteoblasts*	24 h	108.4±5.1^{a}	100.24±7^{a}	97.2±10.9^{a}	63.3±3.5^{b}	82.4±8.1^{c}	68.7±2^{b,c}
	48 h	104.8±3.7^{d}	97.8±1.1^{d}	70.5±0.7^{e}	39.7±5.7^{f}	66±4.5^{g}	55.2±7.1^{g}

Notes: Results expressed as mean ± standard deviations of at least 3 independent experiments in triplicate (%); cell viability assessed by Crystal violet staining after 24 h or 48 h treatment; cell viability expressed in percentage considering untreated cells as 100%. *Comparisons are valid only for the same type of cells and time of treatment. The same superscript letters indicate statistical insignificance, and different letters indicate statistical significance; one-way ANOVA Tukey's Test ($\alpha=0.05$); $p<0.0001$. Cell lines: Fibroblasts = human gingival fibroblast cell line (FG); Osteoblasts = murine pre-osteoblast cell line (MC3T3-E1). Treatments: Control (untreated cells), AC: *Arrabidaea chica* hydroalcoholic standardized extract at 5 (AC5) or 10 (AC10) µg.mL^{-1}, ZA: Zoledronic acid 10 µM, ZAAC5: zoledronic acid 10 µM in combination with *A. chica* hydroalcoholic standardized extract 5 µg.mL^{-1}, ZAAC10: zoledronic acid 10 µM in combination with *A. chica* hydroalcoholic standardized extract 10 µg.mL^{-1}.

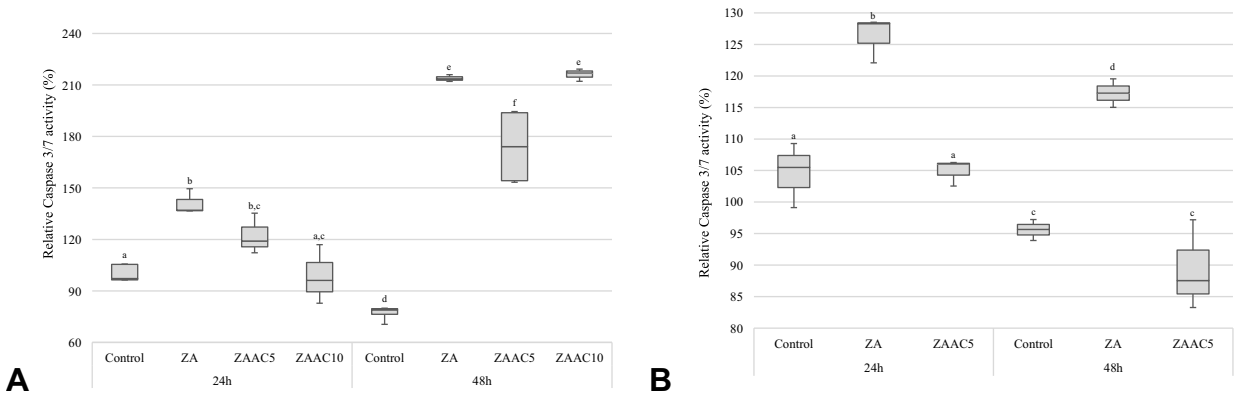

Figure 1 Caspase 3/7 activation on human fibroblasts (**A**) and murine pre-osteoblasts (**B**). Comparisons are valid only for the same type of cells and time of treatment. The same superscript letters indicate statistical insignificance, and different letters indicate statistical significance; one-way ANOVA Tukey's Test (α=0.05); p<0.0001. (**A**) Human gingival fibroblast cell line (FG). (**B**) Murine pre-osteoblast cell line (MC3T3-E1). Percentage of active caspase 3/7 related to control after 24 h or 48 h treatment. Zoledronic acid 10 μM (ZA); zoledronic acid 10 μM in combination with 5 μg.mL^{-1} *Arrabidaea chica* (ZAAC5); zoledronic acid 10 μM in combination with 10 μg.mL^{-1} *Arrabidaea chica* (ZAAC10). Caspase 3/7 Glo® Assay Promega®. Averages of at least two independent experiments in triplicate.

antioxidant properties,[18] justify the growing interest in studies involving this species. The pharmacological activity demonstrated a straight relationship with Carajurin, the main anthocyanidin identified in *A. chica* extract. Therefore, the pigment was used in this study for sample extract monitoring. All in vitro BRONJ experiments were conducted using standardized samples with 6.51 ± 0.07 mg Carajurin content per 1 g of *A. chica* extract.

BRONJ pathogenicity is yet to be explained, however, this work was based on studies that described a hypothetic mechanism of a toxic effect to the oral mucosa tissues (periosteum, connective tissue and epithelium), caused by bisphosphonate deposited on maxillary's bones; therefore, tissues would be unable to properly heal after trauma, favoring secondary infection to bone.[4,22,23] Considering *A. chica* wound healing properties and the necessity of new approaches for BRONJ treatment, prompted this study that evaluated the capacity of *A. chica* extract to revert zoledronic acid-induced damage on fibroblast and osteoblast (MC3T3-E1).

Data demonstrated that fibroblasts and osteoblasts viability varied according to treatment and experimental period (either 24 h or 48 h). During isolated treatments, both *A. chica* extract and ZA, at the selected concentrations, did not affect significantly human fibroblasts viability, independently on time exposure. For ZA, these results corroborated the evidences reported for ZA-treated (10 μM) gingival fibroblasts (HGF) viability evaluated by resazurin (Zafar et al)[24] and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Açil et al)[25] assays. In the present study, interestingly, regardless of preserving fibroblast viability after 24 h ZA exposure, caspase 3/7 was active at the same evaluated time.

The analysis of conjugated effector caspase 3/7 activation supports the identification for cell death mechanism related to apoptosis.[26] Despite bisphosphonates effects on osteoclasts cause ultimately cell apoptosis,[27] bisphosphonates' activity in epithelial and osteoblastic cells is still to be elucidated. Studies have described the occurrence of apoptosis and caspase activation, mainly caspase 3,[3,4,28] or necrosis, DNA and cellular cycle alterations[7,29–31] as mechanisms for bisphosphonates toxicity to epithelial cells. Herein, apoptosis initiated after 24 h ZA treatment could explain the reduction on fibroblast viability (55.8%) observed at 48 h. Moreover, *A. chica* extract in combination with ZA achieved a significant increase in fibroblast viability at 48 h in comparison to ZA group. Therefore, when ZA was associated with *A. chica*, a significant decrease in caspase 3/7 activation was observed after 24h and 48 h, in comparison to ZA alone. The extract was capable of partially avoiding fibroblast activating caspase 3/7 at 24 h, that evoked in the increased cell viability at 48 h, regardless of ZA treatment.

Since the promising cytoprotective outcomes on fibroblast cells were evidenced for *A. chica* at low concentrations, a similar evaluation was conducted using murine osteoblasts cell line, MC3T3-E1. Even though the promotion of osteoclasts's apoptosis represents the main mechanism of action of bisphosphonates,[2] osteoblasts have been shown to play an important role in bone homeostasis as well.[32] These cells regulate bone metabolism by directly interacting with osteoclasts through direct cell-cell contact, cytokines and extracellular matrix interaction. Osteoblasts also affect osteoclast production, differentiation or

apoptosis through several pathways.[33] Therefore, one can assume that any interference on osteoblast activity may directly affect osteoclasts and bone homeostasis.

Previous studies verified ZA actions against osteoblasts with 24 h and 72 h, showing harmful effects of bisphosphonate occurring in a time-dependent manner.[25,34,35] In this study, using a murine osteoblast cell line (MC3T3-E1), the damaging effects caused by ZA started at 24 h and increased at 48 h. Furthermore, there was significant caspase 3/7 activation at both 24 h and 48 h after treating cells with ZA, which corroborates the already described effect on caspase 3/7 activation in human osteoblasts after ZA (up to 50 μM) treatment (72 h) (Jung et al).[10]

When administrated alone, *A. chica* extract did not affect osteoblast viability except at 10 $\mu g.mL^{-1}$ concentration after 48 h exposure. Some studies have reported that polyphenolic and others antioxidant compounds might react with culture medium components generating hydrogen peroxide in situ.[36,37] This situation is dependent on diverse experimental conditions such as the selected culture medium and sample concentration range.[38,39] Probably, under our experimental conditions, MC3T3-E1 cells were slightly more sensitive to AC-induced H_2O_2 production than fibroblasts (FG), as described elsewhere.[40] This could explain the slight reduction in MC3T3-E1 cell viability after 48 h exposure to *A. chica* extract at 10 $\mu g.mL^{-1}$ concentration. Moreover, just at 5 $\mu g.mL^{-1}$, *A. chica* extract significantly reverted ZA-induced reduction on osteoblast viability after 24 h and 48 h treatment. Considering these results, we evaluated caspase 3/7 activation only for *A. chica* extract at 5 $\mu g.mL^{-1}$ concentration. Therefore, the cytoprotective effects of *A. chica* extract at 5 $\mu g.mL^{-1}$ concentration in ZA-treated osteoblasts could be partially explained by reduction on caspase 3/7 activity.

Despite the lack of consensus regarding BRONJ pathophysiology, several studies were reported in attempt to establish an effective treatment for the condition. The management for BRONJ consists of therapies' associations and palliative measures such as local hygiene with antimicrobial agents, excision of bone sequestrum, surgical resections and antibiotic regimen.[2,41,42] Alternative treatments were studied in vitro, such as geranylgeraniol application,[43,44] low-intensity lasers application,[45,46] platelet rich plasma (PRP) and platelet-derived growth factor (PDGF).[6,45] Draenert et al[47] described cytoprotective effects for epithelial cells treated with 50 μM ZA in combination with 600 μM dexrazoxane, a cardio-cytoprotectant agent with antioxidant property. In vivo studies conducted with rats described preventive BRONJ pos tooth extraction strategies', as using teriparatide, a synthetic analogue of parathyroid hormone,[48,49] platelet-rich plasma[50] and laser therapy.[51] Moreover, *A. chica* extract cytoprotective effect at low concentration could be a new, low cost and promising herbal product to treat BRONJ wounds; showing many advantages, among them, an easier accessibility of population to therapy.

Conclusion

A. chica at low concentration showed important cytoprotective effects for fibroblast and osteoblastic cells exposed to harmful effects of ZA. However, further in vitro and in vivo studies are required to elucidate the mechanism of action.

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Disclosure

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