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In Vitro Osteogenesis Modulation By Natural Products In Murine Osteoblast Precursors (MC3T3)

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Abstract*— Biological mechanisms, such as osteogenesis, osteoinduction and osteoconduction, are involved to the process of bone neoformation, but such processes are slow and undergo cellular competition. In this sense, the action of some substances and growth factors activation and release may play a promising role in accelerating bone neoformation. In this scenario, Dentistry field can be benefited from the use of natural products with lower toxicity, greater pharmacological activity and that present themselves as biocompatible, in addition to more affordable costs to the population. The aim of this study was to evaluate the possible modulation of osteoblast proliferation by natural products using murine osteoblast precursors (MC3T3). For this purpose, in vitro cell viability experiments were performed on MC3T3 by bromide colorimetric method (MTT) with: taxifoline; gallic acid; resveratrol and beta-escine at concentrations from 1000 to 7.81 µg / mL. Taxifoline (31.25 µg / mL to 7.81 µg / mL) was not cytotoxic, but at the higher concentrations it reduced cell viability (39.2% - 67.8%). Gallic acid at concentrations of 15.62 µg / mL and 7.81 µg / mL was not cytotoxic, with cell viability of 90.56% ± 5.53 and 104.16 ± 13.17, respectively. Resveratrol reduced cell viability (38.08% ± 0.94 - 65.29% ± 4.04) at all the tested concentrations and the effect does not appear to be dose dependent. Beta-escine provided cell proliferation at concentrations of 31.25 µg / mL and 15.62 µg / mL (104.0% ± 8.3 and 117.5% ± 12.0, respectively). The joint analysis of the data shows that both beta-escine and gallic acid were able to modulate MC3T3 proliferation in vitro. Thus, we suggest conducting new studies to confirm and broaden the findings presented here.

Keywords— Osteogenesis; Osteoblasts; Natural products.

I. INTRODUCTION

Several biological mechanisms (osteogenesis; osteoinduction; and osteoconduction) are involved in the process of bone neo-formation. Osteogenesis is the transport of living bone cells (osteoblasts), whereas osteoinduction is the ability of some materials to induce differentiation of pluripotent mesenchymal cells into osteoprogenitor cells. Osteoconduction, in turn, is characterized by the ability to conduct or direct bone neo-formation over and into the structure of another material, which means that adjacent cells actively participate. Such processes are slow and undergo cellular competition. In this context, the action of some substances and the activation and release of growth factors may play a promising role in accelerating bone neo-formation [1-3].

In Dentistry, the use of medicinal plants to treat oral or systemic diseases with oral manifestations is still little explored [4-7]. However, this scenario is expected to change as the use of phytomedicines as well as researching on this field has been stimulated by the implementation of the National Policy of Medicinal Plants, allowing the practice of phytotherapy in the Brazilian Unified Health System (SUS). Aside from being a therapeutic alternative for the Brazilian population treatment, this governmental action represents the incorporation of an ancient practice that combines popular and scientific knowledge [8-10] and progress is stimulated towards scientific confirmation of the efficacy and safety of medicinal plants by expanding treatment options [10-12]. In this scenario, Dentistry can be favored by the use of natural products with lower toxicity, greater pharmacological activity and that present themselves as biocompatible, in addition to more affordable costs to the population.

In this sense, the present study aimed evaluate the possible modulation of osteoblast proliferation by

natural products in in vitro experimental model using murine osteoblast precursors (MC3T3).

II. MATERIAL AND METHODS

Cell viability assays were performed using murine osteoblast precursor strains (MC3T3) and following the protocol previously described by Mosmann (1983) with some modifications. MC3T3 cells were cultured in Minimum Essential Medium Eagle - Alpha Modification (Alpha-MEM) culture medium (Nutricell-Nutrientes Celulares Ltda, Brazil) supplemented with 10% fetal bovine serum (FBS) (Invitrogen ™, USA), 1% antibiotic (10,000 U / mL penicillin and 10 mg / mL streptomycin) and 10 mM 4-(2-Hydroxy-Ethyl) -1-Piperazin Sulfonic Ethanol (HEPES) acid buffer. At the 80% confluence stage, the cells were trypsinized and plated into a 96-well microplates at a density of $5x10^3$ cells / well, and incubated at 37 ± 2 ºC with 5% CO_2 for 24 hours. Then, the culture medium was replaced by the treatments taxifoline, gallic acid, resveratrol, beta-escine, concentrations ranging from 1000 to 7.81 µg / mL. The plates were then reincubated at 37 ± 2ºC with 5% CO2 for 48 hours. Positive control was performed by adding only culture médium, whereas 1% dimethyl sulfoxide (DMSO) was used as solvent controls. After the incubation period, the culture medium was replaced for 10% 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT; 5 mg / mL; 100 µl / well) in Alpha-MEM (Vitrocell, Germany). The plates were immediately reincubated in an oven at 37 ± 2 ºC with 5% CO2 for 3 hours. The formazane crystals produced were dissolved with DMSO and the absorbance was read on a spectrophotometer (Multiskan GO, ThermoFisher Scientific, USA) at the fixed 540 nm wavelength. Results were expressed as percentage (%) of cell viability (Equation 1) comparing the control of the medium (untreated cells) with the cells receiving the different treatments. The 50% inhibitory concentration (IC_{50}), the concentration that reduces cell viability by 50%, was calculated by plotting cell mortality as a function of concentrations using Microsoft Office Excel.

$$\text{Cell viability \%} = [(As / Ac) \times 100] \quad (1)$$

Where A_s and A_c are the absorbance for the sample and control, respectively. The percentage of cell viability for the treated cells was expressed as mean ± standard deviation (n = 5). Statistical analyzes (ANOVA followed by Bonferroni post-test) were performed via Graphpad Prism 5.0 software (GraphPad, USA). Differences were considered significant when $p < 0.05$.

III. RESULTS AND DISCUSSION

The description of biocompounds able to induce and / or lead to osteoblast differentiation, which would facilitate bone regeneration, may benefit several Dentistry areas. In this work, some natural products (taxifoline, gallic acid, resveratrol and beta-escine) were evaluated for its osteogenesis induction capacity. To the best of our knowledge, it is the first time those above mentioned substances were evaluated on MC3T3 cell culture. In 2015, Tonder and colleagues reported the cell culture as the gold standard for determining cell viability and proliferation.

Platelet-rich plasma (PRP) is one of the most used substances to accelerate the healing process [3], being considered an adjuvant therapy in the bone healing process [15, 16]. However, the effects of PRP on the acceleration of bone regeneration are still under discussion [17-20], since it presents the limitation of having to be used fresh due to the reduction of its osteogenic properties when freezing [21]. Therefore, it is relevant to search for other substances with the same benefits, but with no such disadvantages presented by the PRP.

In this context, we saw the opportunity to assess natural compounds with biological potential that may be interesting. Taxifoline (3,5,7,3,4-pentahydroxyflavanone or dihydroquercetin) is abundantly found in citrus fruits and onions, being a component of dietary supplements or functional foods that are antioxidant-rich [22, 23]. Previously, it was described the antioxidant action and anti-inflammatory effect [24], and the occurrence of osteogenesis promotion, when using taxifoline [25]. Taxifoline (31.25 µg / mL to 7.81 µg / mL) was not cytotoxic, but there was a reduction in cell viability at higher concentrations (39.2% - 67.8%) (Fig. 1A). Taxifoline provides a reduction in the expression of osteoclast-specific genes, leading to a decrease in bone loss, thus being considered a potential therapeutic agent for the treatment of osteoclast-related diseases [26].

Gallic acid (3,4,5-trihydroxybenzoic acid) is a phenolic compound found in vegetables from the Anacardiaceae, Fabaceae and Myrtaceae families. It includes beriberi leaves, pomegranate roots and bark, hardwood plants such as oak (Quercus robur) and chestnut (Castanea sativa L.), as well as in processed beverages such as red wines and green teas [27-31]. Antioxidant, anti-inflammatory and antimicrobial activities have been attributed to the molecule [27]. Regarding the gallic acid cytotoxicity (Fig. 1B), no statistical difference on cell viability from control were found only at concentrations of 15.62 µg / mL and 7.81 µg / mL showed no statistically significant difference from control (90.56% ± 5, 53 and 104.16 ± 13.17, respectively). Indeed, a modest cell proliferation could be observed ate the lowest concentration under evaluation. Concentrations from 1000 µg / mL to 31.25 µg / mL showed cell viability in the range of 49.02% ± 3.24 - 54.16% ± 1.82. In agreement with our findings, it has been demonstrated mild cytotoxicity and suppression of cell proliferation by gallic acid [32]. In order to improve its effect, the same authors modified the molecule structure through sulfonamide group introduction and, thus, were able to promote osteoblast growth, increase alkaline phosphatase activity, as well as the expression of osteogenesis-related genes. There has been reported the presence of gallic acid in the stem

of Bombax ceiba, which is traditionally used to treat bone disorders [33]. The combination of gallic acid and sulfadimoxine could promote an increase on osteogenic proliferation and differentiation of primary osteoblasts and, consequently, on bone matrix production and mineralization [34].

Resveratrol is polyphenol (4,3 ', 5'-trihydroxystilbene) extracted from plants such as Arachis hypogea, Cassia sp., Eucalyptus, Morus rubra, Vitis vinifera, Veratrum grandiflorum, Vaccinum sp., Artocarpus sp. Rhey rhaponticum, polygonum cuspidatum, Gnetum montanum, Picea sp. Bauhinea sp., Pinus sylvestris, Veratrum sp. The compound is widely used to combat aging-related diseases due to its antioxidant action [35-39], besides exhibiting anti-inflammatory action [40], neuroprotective [41], photoprotective [42], antiviral [43], among others. Some authors had suggested the resveratrol power to reduce bone loss by acting on osteoblasts and osteoclasts, but this application still needs further study [44]. In the present study, this compound reduced cell viability (38.08% ± 0.94 and 65.29% ± 4.04) at all concentrations tested and the effect does not appear to be dose dependent (Fig. 1C).

Beta-escine (polyhydroxiolean-12-ene 3-O-monodesmosides) is a natural mixture of triterpenoid saponins, isolated from Aesculus hippocastanum seeds, that comes in alpha or beta form. It has anti-inflammatory effect, protective in vascular, pulmonary and hepatic lesions, antitumor action, wound healing and angiogenesis [45]. Beta-escine (Fig. 1D) provided cell proliferation at concentrations of 31.25 µg / mL and 15.62 µg / mL (104.0% ± 8.3 and 117.5% ± 12.0, respectively). Among the other concentrations (1000-62.5 µg / mL) there was loss of cell viability (45.03% ± 0.8 - 46.40% ± 1.77). For this compound, there were no data available in the scientific literature about its action on bone cells, which demonstrates the unprecedented character of this research.

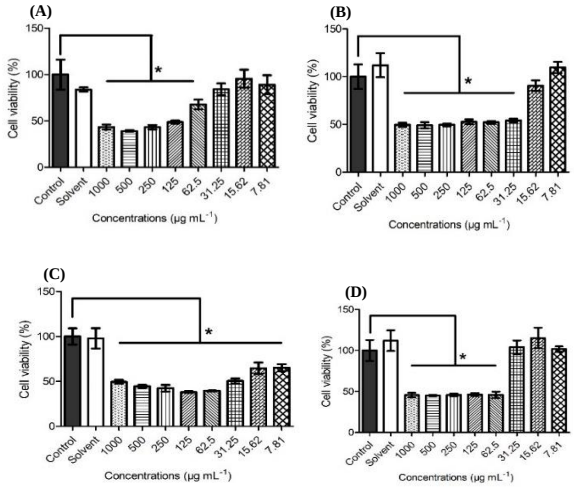

Fig. 1- Cell viability of murine osteoblast precursors (MC3T3) under different chemical treatments. A: Taxifoline; B: Gallic acid; C: Resveratrol; and D: Beta Escine. * p <0.05 (ANOVA followed by Bonferroni post-test).

IV. CONCLUSION

Altogether, the data analysis shows that both beta-escine and gallic acid were able to modulate, in vitro, the proliferation of murine osteoblast precursors (MC3T3). Thus, we suggest conducting new studies to confirm and broaden the findings presented here.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

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