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Nacre Compared to Aragonite as a Bone Substitute: Evaluation of Bioactivity and Biocompatibility

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Aragonite is a metastable polymorph of calcium carbonate found in mollusk's shells, appearing in tiles and prismatic columns, cemented in a protein matrix - mainly proteins - that acts as a framework on which the aragonite is nucleated forming nacre, besides selecting the morphology of the nucleated cristaline phase. The presence of the mineralyzing organic matrix may affect osteoinductive properties of biogenic aragonite, hypothesis tested by combinated tests, comparing viability and bioactivity of biomineralizated aragonite and nacre. Bioactivity was observed by deposition of Ca-P (presumably calcium phosphate) on the surface of samples immersed in Simulated Body Fluid; biocompatibility was verified by adhesion with VERO cells; cytotoxicity and alkaline phosphatase activity assays were performed with human adipose stem cells (hASC). Samples were characterized by scanning electron microscopy and X-ray diffraction. Both materials showed similar behaviour on bioactivity assay; in contrast, exhibited different behaviours in the presence of hASC.

Keywords: *aragonite, nacre, biocompatibility, bioactivity, osteogenesis*

1. Introduction

The main aim of tissue engineering – a branch of the sciences that unites Materials Engineering and Biomedicine – is the production of materials that can substitute and/or induce advanced regenerative processes in damaged tissue[1]. Mechanical, industrial, biological and clinical aspects are taken into consideration such as biocompatibility, bioactivity, recruiting capacity and induction of cellular differentiation. These materials cannot move on towards *in vivo* tests before a battery of *in vitro* tests[2-4], among which immersion assays and tests with cellular cultures can be singled out. The creation of protocols of flexible and reliable tests permits reduction of costs and time in their application, assisting in the optimization of this crucial stage in the conversion of a prototype into a product.

The need for biocompatible materials for clinical use has become of a growing concern, the focus of much of the recent biomedical research[4-9]. What would be the ideal material for the substitution or regeneration of damaged bone tissue? Does there exist, in fact, an ideal material?[10,11]

Various methodologies have been employed to check the bioactivity of materials, and the SBF (*Simulated Body Fluid*)[12,13] and the *in vitro* culture of cells, using the biomaterial as a substrate, deserve to be singled out[14-16]. Through the cultivation of specific cellular lines[17] in contact with the biomaterial, information can be obtained with respect to the biocompatibility of the sample, verified by means of the proliferation and viability of the cells and by the observation of characteristics such as cellular morfology after a certain period of cultivation, cellular adhesion to the substrate and the latter's degradation.

Biomineralization is a highly successful process that consists in the production, in an intra and/or extracellular environment, of mineral compounds that fulfill structural and ionic storage functions[18,19]. The ceramics obtained by this process possess varied characteristics, and metastable polymorphs are widely employed by organisms, in addition to the temporary amorphous phases[20,21].

In mollusc shells, the aragonite and the protein matrix form a composite material (mineral matrix + organic matrix) called nacre. Taking into account that the mechanisms behind the construction of the different biomineralized tissues occur in similar fashion, it seems coherent to seek out materials that can substitute or induce the regeneration of bone tissue in other biomineralizing organisms, and this explains the attention given to corals and molluscs with shells[5,6,20-36].

Thus, this work has sought, through the gathering and comparison of the characteristics of clinical interest, to find in the residues from malacoculture the potential for obtaining an industrial input of low ecological/economic impact. Nacre and biogenic aragonite are two materials of

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biological origin fully described in medical literature[2,6,22,37]. Both have been repeatedly analysed, by different authors, and present important osteogenic and mechanical properties. Raw nacre was continuously tested, and its osteogenic potential observed even in large bone defects[25], without inflammation and/or fibrous formations. *In vivo* and *in vitro* studies indicate the nacre as a biocompatible, biodegradable and osteocondutive material, which may attract and activate bone marrow stem cells and osteoblasts; the same was observed in biogenic aragonite[22-35]. The mechanical and tribological properties - as tensile, compression and bending strength, besides resistance to abrasion - of the nacre, comparatively better than that of pure aragonite, are due to its hierarchical structure of micrometric hexagonal tablets with mineral bridges and interlocking surfaces[36,38-42] and cemented in organic matrix. Considering that to this matrix are also attributed mineralizing and osteogenic properties[22,30-34], the hypothesis raised initially was that the nacre would present more pronounced osteogenic properties than the aragonite, disregarding this study any differences in the mechanical properties.

2. Materials and Methods

2.1. Preparation of samples

The shells of the golden mussel (*Limnoperna fortunei*) were cleaned in running water and any fouling removed manually. After drying at room temperature, the shells were ground in a ball mill for 7h at 70rpm, until fine particles were obtained. Half of the powder obtained was submitted to thermal treatment in an electric muffle at 400°C for 1h to remove organic material[36,38] The predominance of aragonite was confirmed by X-ray diffraction in Synchrotron Light at the National Synchrotron Light Laboratory - LNLS (Campinas/SP). The other half, with an organic matrix (nacre) was stored at room temperature.

The pastilles were produced with a manual pastillator and then cut into smaller pieces, approximately 5 × 5 × 5mm. The pastilles were maintained in shape during the tests by the application of a partial envelope of carnauba wax.

2.2. Characterization

The $CaCO_3$ phases, like the transitions, were observed by X-ray diffraction (DRX) in Synchrotron Light at the LNLS (Campinas/SP). The morphology of the samples before and after the *in vitro* tests was examined using Scanning Electron Microscopy - SEM (TESCAN) after metallizing with gold/palladium. The composition of the material deposited after immersion in SBF was checked by Energy Dispersive Spectroscopy (EDS) attached to the SEM.

2.3. In vitro tests

2.3.1. Bioactivity test

Bioactivity was checked through tests of immersion in SBF.

1000mL of SBF were prepared[13] by dissolving 5.403g of NaCl; 0.504g of $NaHCO_3$; 0.426g of Na_2CO_3; 0.225g of KCl; 0.230g of $K_2HPO_4 \cdot 3H_2O$; 0.311g $MgCl_2 \cdot 6H_2O$; 17.892g of HEPES (2-(4-(2-hydroxyethyl)-1-piperazinyl) ethane sulfonic acid) previously dissolved in 100mL of aqueous solution of 0.2M-NaOH; 0.293g of $CaCl_2$; 0.072g of Na_2SO_4 and 1.5mL of NaOH at 1mol/L^{-1} in bidistilled water. The pH was measured by a digital phmeter and maintained around 7.4.

After immersion (1g of sample – in the form of a pastille – in 2mL of SBF) the samples were maintained at 37°C in a heater and removed from the solution at different times, they being: 1, 5, 10, 17, 24 and 31 days. The solution was substituted every 48h. The tests of immersion in SBF were carried out in wellplates with 24 wells. All the samples were tested in quadruplicate.

2.3.2. Cellular tests

Two cellular types were used in the execution of the tests: VERO cells (isolated from kidney epithelial cells extracted from an African green monkey - *Chlorocebus sp*.) and hASC (human adipose stem cells). While the hASC were used in the tests of Viability and Cellular Proliferation (MTT) and Alkaline Phosphatase Activity, the VERO cells were used in the contact tests, a qualitative test, where the main parameter evaluated is cellular morphology – VERO cells are used as a standard because of the facility to observe changes in their morphology due to interactions with the substrate[42].

2.3.2.1. Contact test

The VERO cells were sown in the samples (obtained in the form of pastilles) in the proportion 3×10^5 cells/well and incubated for 24h at 37°C and 5% CO_2. In the exhaustion chamber 400uL of glutaraldehyde 2% in PBS 0.15M per well were added to fix the material. Were also added 3 pastilles of NaOH in other wells in the plate to neutralize the volatile toxic gases from the gluteraldehyde. The plate was sealed with parafilm and incubated for a further 16h at 37°C. After the fixing of the samples, the gluteraldehyde was aspirated into the exhaustion chamber and the wells were submitted to stages of increasing dehydration with 30, 50, 70 and 100% alcohols solutions for 30 minutes, for each solution, at room temperature. The fixed and dehydrated samples were finally metallized and taken to the scanning electron microscope for examination.

2.3.2.2. Isolation and cultivation of the stem cells from the human adipose tissue

The isolation and cultivation of the stem cells from the human adipose tissue (hASC) was carried out as described in the literature[14-16,43]. The hASC were obtained from the lipoaspirate of patients of the female sex, between 20 and 40, submitted to liposuction surgery in conformity with the standards approved by the Ethics Committee in Research of the Federal University of Minas Gerais - UFMG (Opinion nº ETIC - 0107.0.203.000-10). The hASC were expanded to the fourth passage to be used in the experiments.

The culture medium used was DMEM (Sigma-Aldrich) supplemented with 5mM of sodium bicarbonate (Cinética Química Ltda.), 10% of Fetal Bovine Serum (FBS - Cripion), 1000 units/mL of penicillin, 1mg/mL of streptomycin, 2,5µg/mL of amphotericin B (Sigma-Aldrich) and 60mg/L of gentamicin (Schering- Plough). The pH was adjusted

to 7.2 and the medium was filtered with a polyvinylidene difluoride membrane of 0.22µm (Millipore).

2.3.2.3. Cytotoxicity test

Cytotoxicity of the biomaterials was measured by cell viability and proliferation through MTT[44]. The hASC cells were plated in the fourth passage in a 24-well plates at a density of 1×10^4 cells per well. The cell populations were normalized with a DMEM medium for 24 hours. After this period the medium was changed and the samples placed in their respective wells.

The cells were incubated at 37°C in a humid atmosphere and 5% CO_2 for 24, 72 and 120 hours. At the end of each incubation period, the culture medium was removed and discarded and 210µL/well of DMEM medium added. Then 170µL/well of MTT solution (Invitrogen) (5 mg/mL) and the cells were incubated at 37°C, humid atmosphere and 5% CO_2, for 2 hours. The hASC were observed under an optical microscope to examine the formazan crystals. These were dissolved by the addition of 210µL/well of an SDS10% - HCl (0.01M hydrochloric acid - 10% of sodium dodecyl sulfate in water) solution followed by incubation at 37°C, humid atmosphere and 5% CO_2, for 18 hours. 100µL from each well were transferred to a 96-well plate (flat bottomed), in triplicate, and the optical density measured in the spectrophotometer (*Perkin Elmer Lambda 20*) at 595nm. During the experiment, all the steps involving the MTT reagent were carried out under minimal conditions of luminosity. The results obtained were analysed by GraphPad Instat 3 (GraphPad Software Inc., San Diego, USA) software and the means were compared by SNK test.

2.3.2.4. Alkaline Phosphatase Activity

Alkaline phosphatase activity (ALP) was evaluated by means of the NBT/BCIP (Gibco) test[45]. The hASC cells were plated in the fourth passage in 24 -well plates at a density of $1x10^4$ cells per well. The cell populations were normalized with a DMEM medium for 24 hours. After this period the medium was changed and the samples placed in their respective wells.

At the end of each incubation period, the culture medium was removed and discarded, the cells were washed with PBS 0,15M, pH 7.4 and incubated with 210µL/well of NBT/BCIP solution (prepared in accordance with the manufacturer's instructions) at 37°C, humid atmosphere and 5% CO_2, for 2 hours.

After the incubation period, the formation of a purple precipitate was observed by optical microscope. Following this, 210µL/well of SDS 10%-HCl were added to dissolve the purple precipitate. The plates were incubated at 37°C, humid atmosphere and 5% CO_2, for 18 hours. 100µL from each well were transferred, in triplicate, to a 96-well plate (flat bottomed) and the optical density was measured in the spectrophotometer (*Perkin Elmer Lambda 20*) at 595nm. During the experiment, all the steps involving the NBT-BCIP reagents were carried out under minimal conditions of luminosity. The results obtained were analysed by GraphPad Instat 3 (GraphPad Software Inc., San Diego, USA) software and the means were compared by SNK test.

3. Results

3.1. X-ray diffraction

The graphs obtained by DRX (Figure 1) showed a phase transition of the $CaCO_3$, which is converted, from about 300°C, from aragonite to calcite[38].

3.2. In vitro experiments

3.2.1. Immersion in simulated body fluid

The precipitation of calcium phosphate occurred in the same manner in the thermically treated samples as in those without treatment (Figure 2 and Figure 3). The appearance of a homogeneous covering was perceived initially. Subsequently, on this, points or agglomerates arise where the nucleation seems to occur preferentially, quite similar over the whole of the surface (Figure 4).

The composition of the material deposited was confirmed by EDS (Figure 5).

3.2.2. Cell tests

3.2.2.1. Contact test

Figure 6 presents the samples after immersion, for 24 hours, in a fibroblast cultural medium of VERO line. The slightly globular structures visible in the images are fibroblasts. The presence of filamentous structures of adhesion and anchorage, denominated pseudopodia (highlighted in Figure 6c), indicates the fixation process of the cells to the substrate, denoting a relationship of biocompatibility desired in materials for clinical use.

3.2.2.2. Cytotoxicity test

Figure 7 presents the results of the MTT assays in thermically treated and untreated material (NT: samples without thermal treatment; T: samples thermically treated

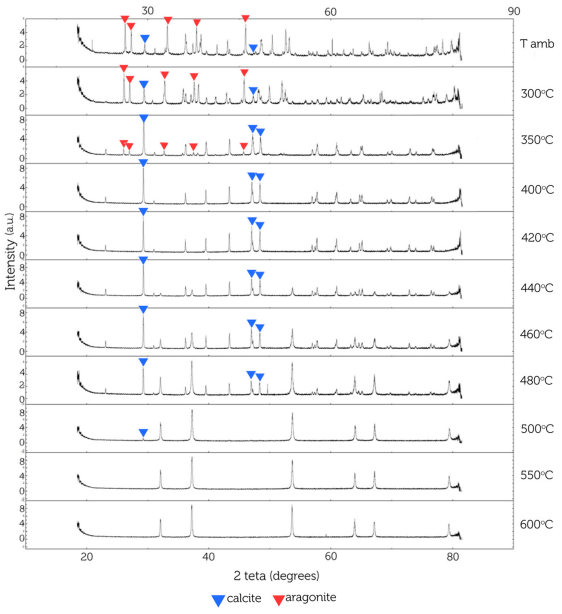

Figure 1. Graphs obtained by Synchrotron Light for samples of *L.fortunei* shells at different temperatures.

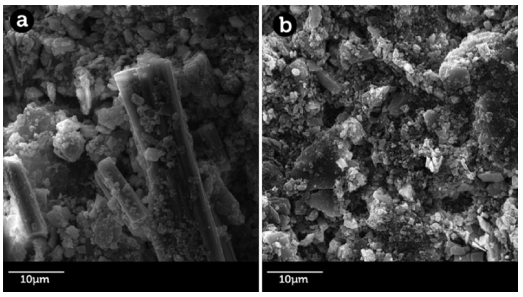

Figure 2. Pastilles immediately before soaking in SBF. a) NT Pastille. b) T Pastille. SEM.

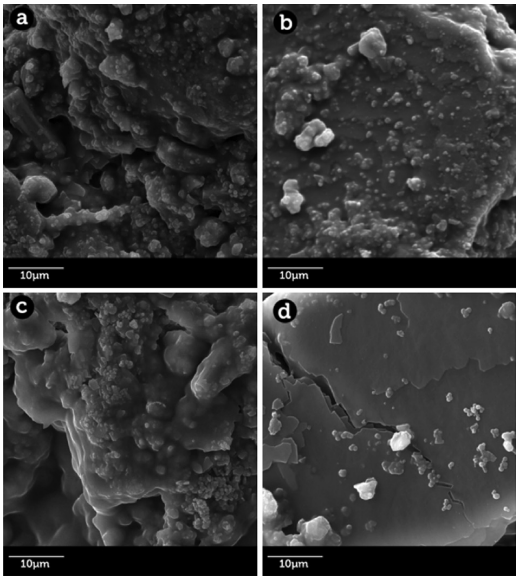

Figure 3. Immersion in SBF, day 1. a, b) Pastilles without treatment. c, d) Thermally treated pastilles. SEM.

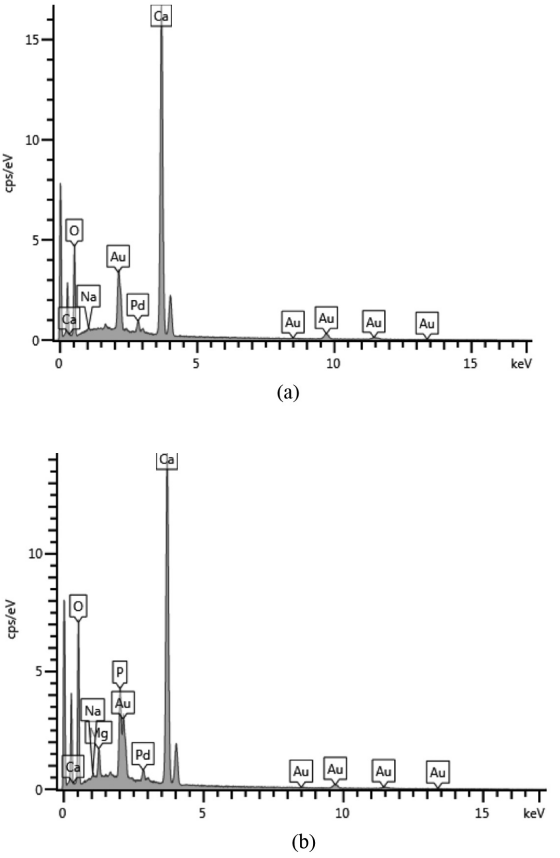

Figure 5. Results of the EDS (SEM) before immersion in SBF (a) and after immersion in SBF (b). Au and Pd relate to metallic cover applied over the sample for SEM preparation, while the presence of P in the post-immersion sample may indicate the presence of HA.

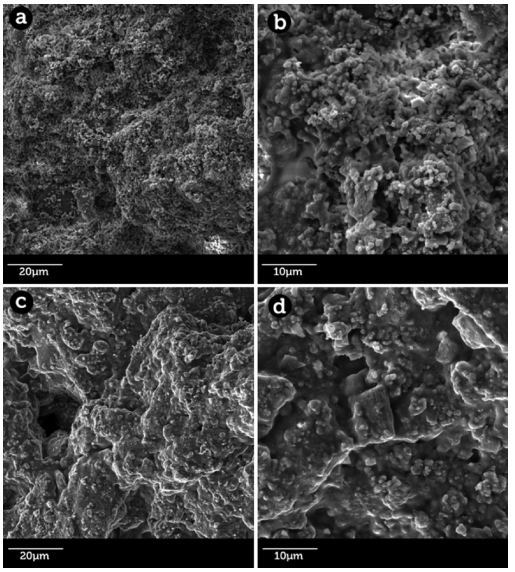

Figure 4. Micrograph of a pastille in the SBF Immersion assay, after the twenty-fourth day. a, b) Thermally treated pastilles. c, d) Untreated pastilles. SEM

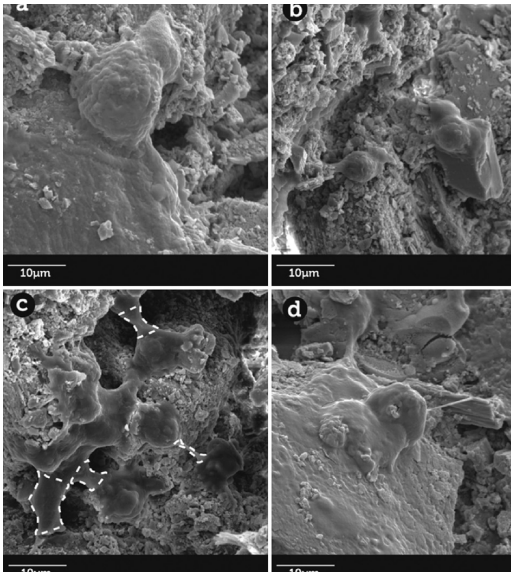

Figure 6. Pastilles exposed to the fribroblast culture of the VERO line. a, b) Pastilles without thermal treatment. c, d) Thermally treated pastilles. The arrows in c indicate pseudopodia.

at 400°C for 60 minutes), further compared were shell fragments *in natura*, washed in deionized water. DMEM refers to the culture medium appropriate for the cell type in question, having an appropriate pH and nutritional factors. The absence of a substrate allows it to be used as a positive control, while C- corresponds to the negative control, where all the cells are dead, guaranteeing the inexistence of metabolic activity. In the tests with powdered samples, calcite samples were further tested, proposed today as a mineral charge in bone implants[44-47].

Except for the T samples, which reached relative metabolic stability in 72 hours, the other samples maintained a growing level of metabolic stimulation in the period of 120 hours, mentioning especially the NT samples. It is known in the literature that the stem cells have high proliferative potential that gradually decreases as the cells commit to a cell lineage[48].

In the MTT assays with powdered samples (Figure 8), the calcite stands out in the periods 72 and 120 hours. The NT and T samples show similar behaviour, inducing metabolic reduction in the period of 72 hours followed by an increase in the period of 120 hours, while the DMEM shows linear growth over the duration of the experiment, staying below all the substrates tested. Besides, these results suggest that all biomaterials tested presented an induced behaviour on hASC viability and proliferation when compared to cell proliferation in DMEM media, evidencing no cytotoxicity from the samples and all of them can be considered suitable for cell culture.

3.2.2.3. Alkaline Phosphatase Activity

The graph presented in Figure 9 shows the results obtained in the ALP tests with the pastilles manually pressed (NT and T) and shell fragments *in natura*, in addition to the positive (DMEM) and negative (C-) control. While the ALP production increases over time in the NT samples, the T samples remained relatively stable, as well as the shell samples *in natura*. The T samples presented an improved production of ALP in 24 and 72 hours when compared to cells cultivated DMEM media, suggesting this biomaterial can be able to induce an inicial change in hASC phenotype, when compared to other samples. To prove this hypothesis, additional tests should be performed. Alkaline phosphatase is an enzyme present in undifferentiated cells, therefore it is used as a stem cell marker, however, this enzyme is produced at high levels in early osteogenic differentiation, and is considered an early marker of differentiation[49].

Taking into account that cells cultivated in T samples reached relative metabolic stability in MTT assay in 72 hours and produced high results of ALP at the same time, it can be suggested an inicial commitment of these cells towards an osteogenic phenotype.

In Figure 10, the graph shows the result of the tests with powdered samples. Taking into account that the NT samples are ground shells, the shell sample was substituted by calcite, also powdered, obtained by the heat treatment of the T sample in an electric muffle at 400°C[36]. The graph presented in Figure 10 presented very interesting results in respect to a potencial of these biomaterials related to osteogenic induction or differentiation. As mentioned before, only cells commited to osteogenic phenotype are able to

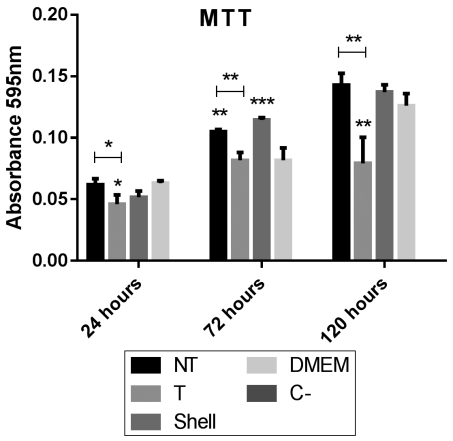

Figure 7. Results of the MTT assays in pastilles and fragments (Shell). Asterisks refer to the significance of the results, when compared to DMEM. Thus, *, ** or *** serve as a scale indicating how much the results differ from each sample DMEM.

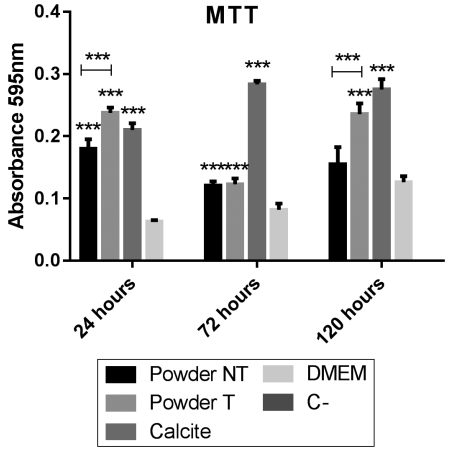

Figure 8. Results of the MTT assays in pulverized samples. Asterisks refer to the significance of the results, when compared to DMEM. Thus, *** serve as a scale indicating how much the results differ from each sample DMEM.

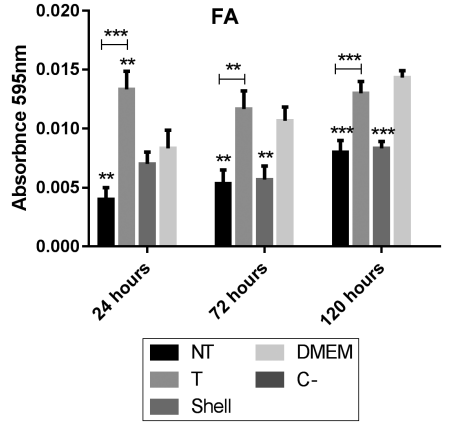

Figure 9. Results of the Alkaline Phosphatase tests with pastille or fragment (shells) samples. Asterisks refer to the significance of the results, when compared to DMEM. Thus, ** or *** serve as a scale indicating how much the results differ from each sample DMEM.

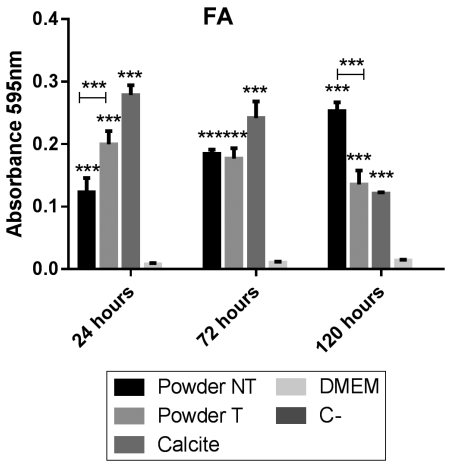

Figure 10. Results of the Alkaline Phosphatase tests with powdered samples. Asterisks refer to the significance of the results, when compared to DMEM. Thus, *** serve as a scale indicating how much the results differ from each sample DMEM.

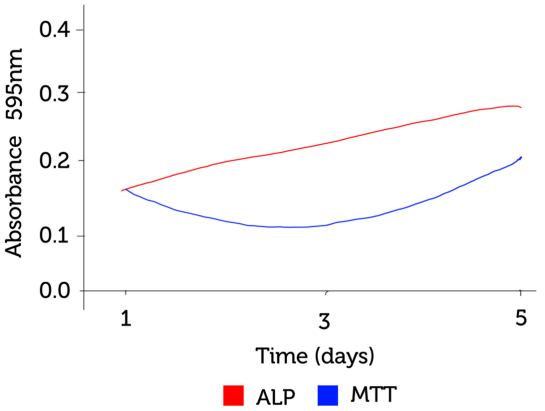

Figure 11. Graph showing the results of non-treated samples in Cytotoxicity tests (MTT) and Alkaline Phosphatase Activity (AP).

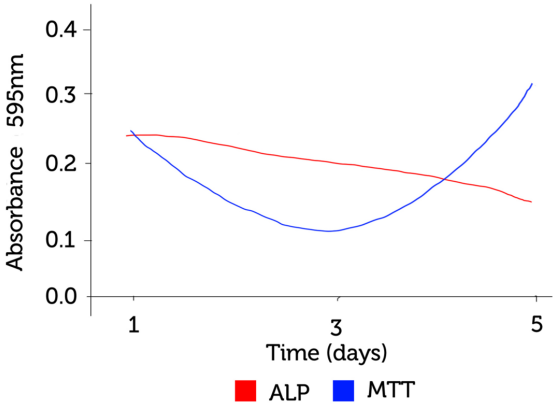

Figure 12. Graph showing the results of treated samples in Cytotoxicity tests (MTT) and Alkaline Phosphatase Activity (AP).

produce high concentrations of ALP enzime. These results are believed due to the shape of the biomaterial introduced, where the powder has a larger contact surface, which considerably increases the leaching of calcium in the media and favors and/or accelerate the possibility of osteogenic cell induction or differentiation. When compared to cells cultivated in DMEM media, all biomaterials evidenced a significative higher production of ALP, specially a high increase in concentration at first 24 hours in cells cultivated in presence of calcite and at 120 hours in cells cultivated in NT powder were observed.

4. Discussion

Taking into account the comparative nature of the proposal, the bioactivity and biocompatibility tests were carried out to observe the behaviour of the materials of interest for subsequent comparison.

As previously stated, it was expected that the presence of the organic matrix in the untreated samples (nacre) would affect the response of the material in the different tests, relative to the thermically treated samples (aragonite) where the organic matrix is absent, due to the osteogenic properties inherent to the organic matrix[22].

In the first experiment, immersion in SBF, followed by the scanning electron microscopy (SEM) of the samples, the bioactivity of the materials was confirmed. In accordance with similar experiments observed in the literature the quantity and size of the grains deposited act as somewhat quantitative indicators of the ability to induce precipitation of Ca-P (confirmed by EDS), and observing the images obtained by SEM, no perceptible difference appears in the comparison between the two materials, within these parameters. The results of the experiments with SBF approached, visually, those obtained with other ceramic materials such as α-tricalcium phosphate (α-TCP) and bi-phasic hydroxyapatite/α-tricalcium phosphate (HA/TCP)[50], and better than the results obtained with sintered Bioglasss® (BG)[51] and calcium phosphate monobasic monohydrate [$Ca(H_2PO_4)_2.H_2O$][52].

In the contact tests with VERO cells there was also no difference between the samples. The biocompatible character of the materials is reinforced, even if there are no conclusive indexes, considering the absence of *in vivo* tests. Comparing the morphology of the cells with tests found in the literature, the materials tested in this work presented results similar to those obtained with composite materials such as *34Bio_10Poly_5Pe* and *29Bio_12Poly* (polyurethane sponge employed as an organic skeleton for the scaffolds, which were realized using 45S5 Bioglass®), but they exceed the same materials in the cellular viability parameter after the first 24 hours[53], with results >60%, but lower than the 90% viability obtained with the hybrid Polyoxyethylene sorbitan laurate (TWEEN) plus graphene oxide (RGO)[54]. Also in relation to the morphology of the VERO cells, nacre and biomineralized aragonite exceed the results obtained with some titanium alloys, obtained by thermal and anodic oxidation and by the sol-gel technique, like cp-Ti and Ti1.5Al25V substrates[55], and match those results collected on chitin/nBGC and chitosan/nBGC scaffolds review[56]. The

use of osteoblastic cell lines can be a future step towards validation of the results in more specific cell lines.

However in the MTT and ALP assays considerable differences arose. Analyzing the results of the ALP tests, it can be seen that the calcite acts as a substrate with a high potential for cellular differentiation, nevertheless, this effect suffers a considerable reduction within the interval observed. This behaviour is repeated by the T samples (aragonite), on a smaller scale. Meanwhile, the NT samples (nacre) present the contrary behaviour, increasing the rates of differentiation of the bone tissue over the time interval.

Comparing the results of MTT, the cellular responses are inverted. While, over the interval observed, the calcite has reduced its capacity for cellular differentiation, its capacity to stimulate multiplication increases. The inversion of the rates is also observed in the NT samples.

NT: The production of Alkaline Phosphatase increases considerably with the passage of time, over the interval observed. In the corresponding period, the MTT reading suggests high rates of replication (in comparison with the positive control group, DMEM) at the start, followed by a small fall, coinciding with the increase in the PA readings (Figure 11).

T: Initially, the samples that underwent thermal treatment (aragonite) stimulate both the differentiation and cellular division, with very similar rates. The subsequent fall in the MTT and PA readings (Figure 12) may indicate a relative exhaustion of the cell culture.

Comparing the results of the MTT tests obtained with those found in the literature in relation to other bioceramics, the cellular viability of the nacre and the biomineralized aragonite, powdered, is close to the viability observed in akermanite ($Ca_2MgSi_2O_7$), as a Ca, Mg and Si containing bioceramic and β-TCP in the period observed, in a culture of human bone marrow-derived stromal cells (hBMSCs)[14].

5. Conclusion

Both biomaterials tested presented bioactivity and cytotoxicity rates compatible with the proposal to produce scaffolds for the substitution of damaged bone tissue, although *in vivo* tests are still indispensable for confirmation and advancing in this respect.

During the preparation of the hypothesis, the idea was broached that nacre, due to the presence of the organic matrix, would produce better osteogenic responses. However, throughout the constant bibliographical review and the adhesion assays, cytotoxicity and ALP activity, it could be seen that statements like "this or that material is the best" are superficial and do not take into account the varied factors that come together to obtain complex bone tissue. Thus, nacre and aragonite, together with other materials, form an increasingly large range of available materials, each with more or less marked properties, which can interact among themselves to obtain a composite material, capable of stimulating diverse responses in the body, in a chain of events, with the purpose of regenerating bone tissue preserving it's diverse characteristics.

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