









Preparation of Scaffolds of Amorphous Calcium Phosphate and Bacterial Cellulose for Use in Tissue Regeneration by Freeze-Drying Process

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Abstract: The elaboration of scaffolds for use in tissue regeneration processes plays an important role in the area of biomaterials. Natural and synthetic polymers, together with calcium phosphates, form suitable compounds for these studies because their combinations favor the union of the properties of both materials, such as their biocompatibility, biofunctionality, shape, porosity, and mechanical properties. The objective of this work was to develop a scaffold of amorphous calcium phosphate and bacterial cellulose, applying a freeze-drying process. The results demonstrated the feasibility of scaffolds elaboration applying the freeze-drying methodology. The formulation that presented the best results was the one that contained amorphous calcium phosphate (50%), bacterial cellulose gel (20%), and sodium alginate (30%). Cytotoxicity studies showed that the studied formulation did not present cytotoxicity, promoting cell viability.

Keywords: Biomaterials; Calcium phosphates, Scaffolds, Freeze-drying, Cytotoxicity study.

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1. Introduction

The aging of the population, together with the increase in the expectation and quality of life, has increased the development of biomaterials to be used in tissue regeneration. Bone tissue has a limited capacity for regeneration, depending on the size of the lesion, so the development of new types of treatments to be used in regeneration processes is an area of science that has a high social impact [1-3].

Among the methodologies currently applied in the field of tissue regeneration, is the development of Scaffolds made with biomaterials. Scaffolds are three-dimensional structures designed to support cell infiltration, growth, and differentiation, with the aim of improving the development and formation of new tissues [4-7].

These structures must be biodegradable over time, being one of the fundamental characteristics to be guaranteed during their development. That is why it is essential to define the biomaterials to be used during the manufacturing process [4, 5, 8, 9]. Biomaterials that have

good biocompatibility, an adequate rate of degradation, and physical, chemical, and mechanical properties are the optimal ones to guarantee the obtaining of a Scaffold with the quality necessary to be used in biological systems [8, 9, 10].

There are different methodologies for the elaboration of a scaffold. Some authors classify these methodologies into two main groups: Conventional (freeze-drying, leaching techniques, foam formation, phase separation, fiber formation, among others) and advanced (selective sintering by laser, stereolithography, deposition of molten material, inkjet printing and three-dimensional printing - 3D) [11-15]. Freeze-drying is a methodology conventional used for making scaffold and consists of a dehydration process where the material is frozen at low temperatures and subsequently removed by sublimation water using a technique known as lyophilization [16, 17].

Among the biomaterials used in tissue regeneration are calcium phosphates and bacterial cellulose [18-25]. The objective of this work was to develop a scaffold of amorphous calcium phosphate and bacterial cellulose, applying a freeze-drying process.

2. Materials and Methods

Amorphous calcium phosphate (ACP) was synthesized by applying the methodology described by Debone et al. [18]. Later it was ground and sieved. Material with a particle size of less than 74 μm was used in this study. Innovatecs Productos Biotecnológicos LTDA, São Carlos - São Paulo, Brazil, supplied bacterial cellulose gel.

2.1. Ink preparation procedure.

Two concentrations of sodium alginate (20% and 30%) were studied. The sodium alginate was dispersed in 100 mL of water. Subsequently, the amounts of bacterial cellulose gel (equivalent to 10%), amorphous calcium phosphate (equivalent to 50%), and 500 microliters of tween were added.

The mixture was homogenized for 30 minutes at a speed of 350 rpm using a mechanical stirrer. The inks obtained were divided into two parts. A part was evaluated by determining the viscosity at the beginning and 7 days after elaboration. The other part was placed in a mold (diameter of 1 cm and height of 5 cm) and freeze-dried for 48 hours. The scaffolds obtained were named Cart 1 and Cart 2. A study to determine the content of absorbed water and the loss of mass during a study of the influence of humidity was carried out. In parallel, they were evaluated through a cytotoxicity study. The scaffold that presented the best result was chosen to continue the studies.

Once the concentration of sodium alginate was established, the appropriate concentration of bacterial cellulose in the formulation was determined. Three concentrations of bacterial cellulose (10%, 20%, and 30%) were studied. The amorphous calcium phosphate concentration was maintained at 50%.

The elaborate inks were divided into two parts. With one of the parts, the viscosity was evaluated at the beginning and 30 days. While with the other part, the scaffold was made by freeze-dried and the Shore A hardness was determined.

With the selected variant, the Scaffolds were made by freeze-drying and characterized by Scanning electron microscopy (MEV) and Electron dispersive spectroscopy (EDS), determination of surface area, and porosity, mechanical test and cytotoxicity test.

2.2. Characterization.

The viscosity was determined on a Haake concentric cylinder viscometer (model RV20), which is coupled with the M5/NV system. The evaluation was made at 25 ± 0.1 °C. A velocity gradient between 0 and 100 s⁻¹ and shear stress of 0 to 200 Pa were used. The measurements were made in triplicate.

Shore A hardness was determined in accordance with ASTM D2240 (ASTM D2240 – 15. Standard Test Method for Rubber Property—Durometer Hardness). A Metrotokyo durometer (Japan) was used. The test was performed with a weight of 1kgf and a penetration time of 1 s. The experiment was carried out in three different points, calculating the mean and standard deviation.

The study of the influence of humidity was carried out according to ISO 6270-2 (ISO 6270-2:2017. Paints and varnishes — Determination of resistance to humidity — Part 2: Condensation in-cabinet exposure with heated water reservoir). A Hygrotherm 519 humidity chamber (ERICHSEN, Germany) was used. The samples were placed at 100% relative humidity and 37 °C for 24 h. They were weighed at the beginning (initial weight) and after 24 h (wet weight). Subsequently, they were dried at the constant weight at 80 °C and weighed (final weight). The determination of the absorbed water content and the loss of mass was calculated according to Sandonis [26].

The morphology samples were investigated by Scanning Electron Microscopy (SEM) in a JEOL microscopy model JSM-7500F equipped with an X-ray energy dispersive spectroscopy detector (EDS). The EDS counting time was 20000 s per analysis at an acceleration voltage of 10 kV.

The determination of surface area and porosity were determined according to Rodriguez Chanfrau et al., [19]. Nitrogen Absorption/desorption isotherms were obtained using a Micromeritics ASAP 2010 (Micromeritics Instrument Corp. USA). The measurements were performed to 76.8 K (Araraquara, Brazil), using or static volumetric method and relative pressure between 0.002 and 0,998 intervals. Prior to the experimental runs of N₂ sorption, each sample was degassed for 60 minutes under a vacuum of 10⁻⁶ Torr.

The mechanical compression resistance test (Emic DL2000, Tesc Program 384) was performed with a 2 kN Trd 28 load cell, using a speed of 1 mm/min, in accordance with ISO 5833 standards. Six samples were evaluated in the experiment. The compressive strengths of the samples were calculated as the maximum applied force divided by the loaded area. The reported compressive strength values are the average values of the six measurements, each taken with a single sample to capture variations in the manufacturing process.

2.3. Biological test.

2.3.1. Cell culture experiments.

Chinese hamster ovary cells (CHO-K1) were grown in 1:1 Ham-F10+D-MEM (Sigma®, St. Louis, MO) culture medium supplemented with 10 % (v/v) fetal bovine serum (FBS) (fetal bovine serum - Cultilab, Campinas, Brazil) and antibiotic antimycotic solution (A5955 Sigma®, St. Louis, MO) at 37 °C in a 5% CO₂ atmosphere. Cells were used between the third and eighth passages.

For cytotoxicity tests, eluates from Cart1, Cart2, and Cart nHA 20 were made according to ISO 10993-12 (ISO 10993-12 Biological evaluation of medical devices — Part 12: Sample preparation and reference materials, considering the area (1.25 cm²). The materials were

immersed in 1:1 Ham-F10 + D-MEM medium (Sigma®, St. Louis, MO) without fetal bovine serum (FBS) at 37 °C for 24 h, shaking at 133 rpm in an incubator (New Brunswick Scientific – Excella E24 Incubator Shaker Series). The eluate was filtered with a syringe filter (0.45 µm). Four different concentrations were used (100%, 75%, 50%, 25%).

2.3.2. Cytotoxicity tests

XTT assay. To perform these experiments CHO-K1 cells were used and the Cell Proliferation Kit II (Roche Applied Science). This test is based on the cleavage of tetrazolium salt (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate) to form an orange formazan dye. However, this conversion occurs only in viable cells due to the activity of mitochondrial dehydrogenases. Cells were seeded (2×10^4) in 24-well plates in a volume of 1 mL of HAM-F10: D-MEM medium (1: 1) supplemented with 10% FBS, and incubated at 37°C in a 5% CO₂ 24 hours. On the following day, the cells were treated with the eluates for 24 hours. Each well was supplemented with 10% FBS. Negative controls (NC) were wells with cells and culture medium supplemented with 10% FBS in the absence of any material (untreated controls). Positive controls (PC) were wells containing CHO-K1 cells treated with doxorubicin ($3 \mu\text{g}\cdot\text{mL}^{-1}$) for 24 hours. All treatments were carried out in duplicate. After treatment, the cultures were washed with PBS solution, and fresh medium was added. On the next day, the cultures were washed with PBS solution, and immediately 500 µL of DMEM without phenol red was added, followed by the addition of 60 µL of the XTT/electron solution (50:1) (Cell Proliferation Kit II – Roche Applied Science). After 3 h reaction, the supernatant was transferred to a 96-well culture plate, and the absorbance was measured by a Microplate Reader (VersaMax, Molecular Devices, Sunnyvale, CA) at 492 and 690 nm. The absorbance is directly proportional to the number of metabolically active cells (viable cells) in each treatment after 24 h of exposure. Cell viability was calculated from the absorbance. Three independent experiments were conducted.

Clonogenic assay. The clonogenic assay or colony formation assay is an in vitro assay based on the ability of a single cell to grow, multiply and form a colony, which is defined to be composed of at least 50 cells. Only mitotically viable cells are capable of producing progenitor cells; the number of colonies formed after treatment and an indicator of cell viability and proliferation [27]. After 24 h of seeding, cells (4×10^4 cells seeded) were exposed to the eluates for 24 hours. Negative controls (NC) were wells without material, and positive controls (PC) were cells treated with doxorubicin ($0.3 \mu\text{g}\cdot\text{mL}^{-1}$) for 4 h. All treatments were carried out in duplicate. After exposure, the cultures were washed with PBS solution, and fresh medium was added. Exponentially growing cells were seeded, after treatment, at a number of 150 cells per 25 cm² culture flasks, in duplicate for each treatment. The flasks were incubated at 37 °C, 5% CO₂, for 7 days without a media change. The colonies that formed were fixed with methanol: acetic acid: water (1:1:8 v/v/v) for 30 minutes and stained with Giemsa 1:20 for 20 minutes. The number of colonies counted in the NC was considered as 100%. From this, the calculations of survival fractions (FS) were performed: $\text{FS} = \text{number of colonies counted in each treatment} \times 100 / \text{Number of colonies observed in the negative}$. Three independent experiments were conducted.

2.4. Statistical analysis.

At least 3 experiments were conducted for each analyzed parameter. The results were expressed as mean and standard error (SE). The Shapiro-Wilk test was used to assess the normality of the data and Levene's test for homogeneity. A one-way ANOVA test followed by Tukey's test was applied to the data. In addition, the data from treated groups were compared with the negative control (Dunnett's test). Graphpad Prism 5.01 was used to perform statistic tests. Differences were considered statistically significant when $p < 0.05$.

3. Results and Discussion

The viscosity results of the inks made with concentrations of 20% and 30% sodium alginate showed that both formulations were stable at room temperature for 72 h, without undergoing organoleptic alterations or phase separation. This aspect is important to guarantee that during the manufacturing process, no variations occur in the quality and stability of the formulation.

The formulation with 20% sodium alginate presented viscosity values of around 7.8 Pa.S (77.8 and 78.1 Pa.S at the beginning and at 72 h, respectively), which represents approximately 48% lower than the viscosity values obtained. in the formulation made with 30% sodium alginate (150.3 and 150.8 Pa.S at the beginning, and 72 h, respectively).

The results of the absorbed water and loss of mass determinations of the Scaffolds made with the formulations containing 20% and 30% sodium alginate was shown in Table 1.

Table 1. Results of the determination of the content of absorbed water and the loss of mass of the Scaffolds during the study of the influence of humidity.

Scaffold	Absorbed water (%)	Loss of mass (%)
Cart 1	3.92	13.9
Cart 2	1.17	12.7

The results showed that under the established working conditions, the Scaffolds made with the formulation containing 30% sodium alginate, the amount of water absorbed was less than in the Scaffolds made with the other formulation studied. On the other hand, during the study, it was observed that the loss of mass was similar in each formulation. Based on the results, the Cart 2 formulation was selected to continue or work.

Table 2 shows the results of the viscosity study of the inks made with concentrations of bacterial cellulose at 10%, 20%, and 30%, as well as the results of the determination of the hardness of the Scaffolds made with these formulations.

Table 2. Results of the viscosity study of the inks made with concentrations of bacterial cellulose at 10%, 20%, and 30% and values of the determination of the hardness of the Scaffolds made with these formulations.

Formulation	Ink viscosity (Pa.S)		Scaffolds
	0 day	30 days	Hardness (N/mm ²)
CartnHa 10	149.2	149.1	490
CartnHa 20	522.4	521.9	1250
CartnHa 30	323.7	325.6	998

No variations in viscosity were observed at 30 days for any of the formulations evaluated, the ink being stable during that time. The highest viscosity values were observed in the formulation that contained 20% of bacterial cellulose, coinciding that it also showed a higher value of hardness in the scaffolds made with this formulation.

Based on these results, the formulation containing 30% sodium alginate and 20% bacterial cellulose was selected as the appropriate formulation to make scaffolds using the freeze-drying methodology. The amorphous calcium phosphate concentration was maintained at 50%.

Once the formulation to be used was defined, a 1L batch of ink was prepared. Subsequently, the corresponding Scaffolds were elaborated and characterized.

Figure 1 shows a superficial and transversal MEV image of the scaffold made with the studied formulation. The presence of abundant porosity was observed. The qualitative analysis of EDS showed the presence of calcium, sodium, and phosphorus, which corroborates the presence of calcium phosphate and sodium alginate in the formulation.

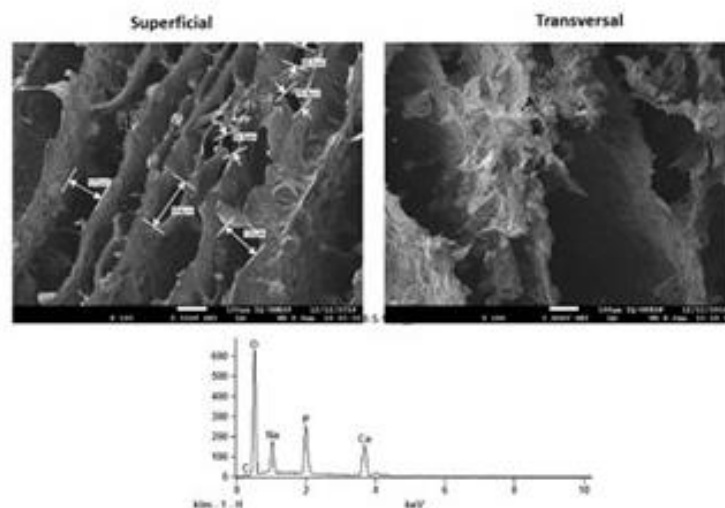


Figure 1. Superficial and transversal MEV image in 100X of scaffold made with the studied formulation and EDS analysis.

The determination of the surface area determined by BET was $39.71 \pm 0.69 \text{ m}^2/\text{g}$. The pore diameter size was in the range between 17 \AA and 6000 \AA , with 39.3% of the pore diameter being over 100 \AA . The average pore diameter was 267.33 \AA (equivalent to 0.027 \mu m).

Studies reported in the literature have recommended that the optimal porosity should be between 5 \mu m and 100 \mu m , while the interconnectivity between the pores should be between 30 \mu m and 50 \mu m [10] because when the pores are smaller, cell proliferation can be affected because mobility and penetration within the Scaffolds would be difficult. On the other hand, pores with very large dimensions, create space that makes it difficult to contact cells with a pore surface, which can cause cell death.

It is also known that the great difficulty of conventional methods, such as that used in this work to elaborate scaffolds, lies in difficulty in controlling the size of the pores, which justifies that the mean values of the pore size achieved in this work are below the recommended values in the literature [12],

The results of the test of the compressive strength in the sample showed that the scaffold undergoes a process of deformation after the rupture of the piece. This phenomenon is caused by the presence of polymers (sodium alginate and cellulose bacterial) in the formulation. It has been reported that during the application of a load at a constant speed, the polymers respond efficiently to tension applied, absorbing energy, and dissipating it, will cause cracks in the piece [28], The compressive strengths of the samples were $66.76 \pm 0.09 \text{ MPa}$.

MEV (Figure 2) of the samples before performing the compression test shows the morphology of layers in leaf forms, with abundant pores. This morphology changes after

carrying out the test, observing a decrease in the size of the pores and a deformity in the structure of the sample.

3.1. Cytotoxicity test.

As part of the biological studies, the cytotoxicity of the Scaffold Cart 1 and Cart 2 was evaluated, as well as the scaffold CartnHa 20.

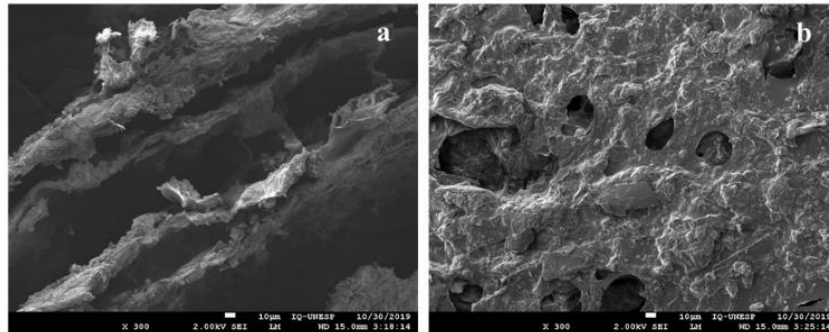


Figure 2. MEV of the sample before (a) and after (b) performing the compression test.

3.2. XTT assay.

Figure 3 shows the mean and standard error (mean \pm SE) for cell viability obtained by XTT assay for Cart1 and Cart2 eluates. Figure 4 presents CartnHa 20 XTT results. Cell viability is related to the absorbance, and Negative Control (NC) was considered as 100% cell viability.

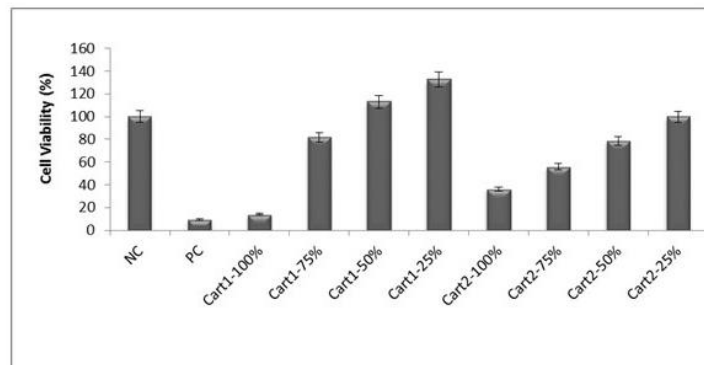


Figure 3. Cytotoxicity (cell viability) assessed by XTT assay. Columns indicate the mean value of cell viability. NC was considered 100% cell viability. Bars indicate the standard error. *** = $p < 0.0001$, ** = $p < 0.001$, * = $p < 0.05$ in relation to NC. Dunnett's test.

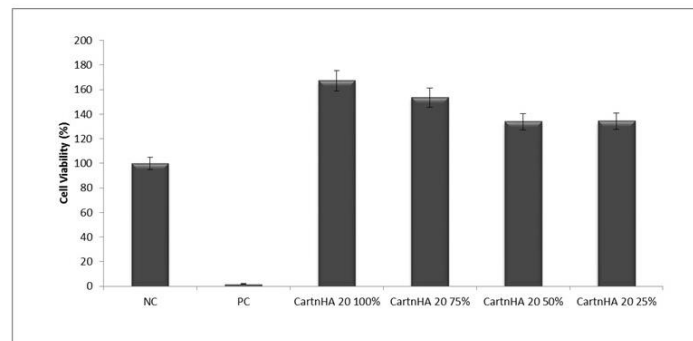


Figure 4. Cytotoxicity (cell viability) assessed by XTT assay. Columns indicate the mean value of cell viability. NC was considered 100% cell viability. Bars indicate the standard error. *** = $p < 0.0001$, ** = $p < 0.001$, * = $p < 0.05$ in relation to NC. Dunnett's test.

Cart1-100% revealed a high cytotoxic effect compared to NC ($p < 0.0001$; Dunnett's). No cytotoxic effects were verified in Cart1 75%, 50%, and 25%. Cart2-25% presented no statistically significant difference compared to NC. Although Cart2 100%, 75%, and 50% decreased cell viability.

If we consider the score of cell viability according to the method of Sjogren et al. and Atay et al. [29, 30], if cell viability exceeded 90%, the material was deemed non-cytotoxic. For cell viability at 60–90% range, the material was regarded as slightly cytotoxic. For cell viability at 30–59% range, the material was regarded as moderately cytotoxic. For cell viability below 30%, the material was considered severely cytotoxic. The concentrations of 100% and 75% of Cart2 were moderately cytotoxic (36% and 56%, respectively), and Cart2 50% was slightly cytotoxic (79%).

CartnHa 20 did not present cytotoxicity by XTT assay. On the contrary, wells treated with the CartnHa 20 eluates demonstrated greater cell viability compared to NC. The highest eluate concentrations increased cell viability (Figure 4).

3.3. Clonogenic assay.

Figure 5 presents the mean and standard error (mean \pm SE) for the surviving fraction obtained by clonogenic assay for Cart1 and Cart2 eluates. Negative Control (NC) was considered as 100% surviving fraction.

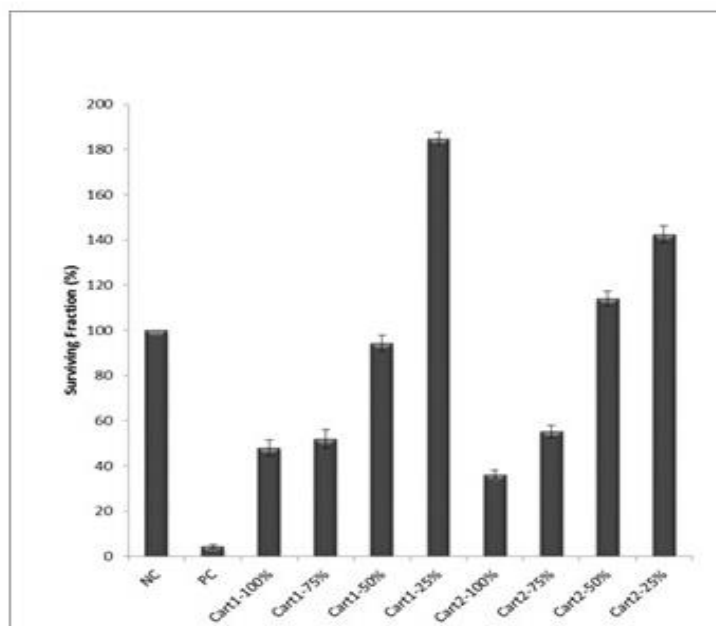


Figure 5. Cytotoxicity (cell's proliferation capacity) assessed by Clonogenic assay. Columns indicate the mean value of the surviving fraction. NC was considered a 100% surviving fraction (100% of the proliferation capacity of the cells). Bars indicate the standard error. *** = $p < 0.0001$ in relation to NC. Dunnett's test.

Clonogenic assays, or colony formation assays, measure clonogenic potential, i.e., the proliferative ability of single cells to form a colony [31]. Besides colony formation, this assay measures cell survival and is routinely used as a sensitive model for assessing long-term cytotoxicity.

Cart1-100%, Cart1-75%, Cart2-100%, and Cart2-75% presented surviving fraction values statistically different compared to NC ($p < 0.0001$; Dunnett's), indicating a compromise in the proliferative capacity of the cells.

It was verified that the cell surviving fraction of Cart1-50% was similar to NC ($p > 0.05$; Dunnett's). Cart1-25%, Cart2-50%, and Cart2-25% presented a significant increase in the proliferative capacity of the CHO-K1 cells compared to NC ($p < 0.0001$; Dunnett's).

XTT cell viability assay shows the immediate cytotoxic effect on the cells, while the clonogenic assay shows if other damages may have occurred to cells that stop their proliferative capacity at a later time. According to Saotome et al. [32], a single in vitro experiment shows only one aspect of the action of chemicals. Because of this, a short-term cytotoxicity method (XTT) and a long-term cytotoxicity test (Clonogenic assay) were made.

Analyzing together the two cytotoxicity tests (XTT and Clonogenic Survival), it can be observed agreement in the results of both tests regarding the concentrations of Cart1 eluates. The lower concentrations (50% and 25%) were not cytotoxic. Besides, Cart1-25% increased cell viability and improved the proliferative capacity of the cells. Similar results were observed in Cart2 eluates. The lower concentrations showed the best responses related to cytotoxicity. Although Cart2-50% slightly decreased the cell viability (XTT), it did not promote late cytotoxicity (clonogenic assay). On the contrary, it increased the proliferative capacity of the cells by 14% compared to NC. Cart2-25% presented similar cell viability (XTT) compared to NC, and increased the proliferative capacity of the cells by 43%.

It can be concluded that the lower concentrations of Cart1 and Cart2 presented no cytotoxic effects in CHO-K1 cells, and increased the cell viability and the proliferative capacity of the cells. CartnHa 20 presented no cytotoxicity. On the contrary, it promoted high cell viability.

4. Conclusions

The results of this work demonstrated the feasibility of Scaffolds elaboration, applying the freeze-drying methodology. The formulation that presented the best results was the one that contained amorphous calcium phosphate (50%), bacterial cellulose gel (20%), and sodium alginate (30%). Cytotoxicity studies showed that the studied formulation did not present cytotoxicity, promoting cell viability.

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Conflicts of Interest

The authors declare no conflict of interest.

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