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Chitosan and hydroxyapatite scaffolds with amoxicillin for bone repair

Scaffolds de quitosana e hidroxiapatita com amoxicilina para reparação óssea

Andamios de quitosano e hidroxiapatita con amoxicilina para la reparación ósea

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Abstract
The bone repair process, among other physiological mechanisms, occurs in a harmonious manner throughout the body. However, the presence of some deleterious factors such as surgery, trauma or pathology, can interfere with the physiology of bone remodeling. It´s Known that the synergistic antimicrobial action between drugs and biomaterials can help and favor osteogenesis after grafting surgery. Therefore, this study aims to develop chitosan / hydroxyapatite scaffolds with amoxicillin for bone repair in the oral cavity. For this, raw materials were selected and characterized and scaffolds were made by the processes of solubilization, dispersion and lyophilization. The characterizations were made by optical microscopy, scanning electron microscopy, eenergy dispersive spectrometer, swelling potential, degradation analysis, apparent porosity test and *in vitro* cell cytotoxicity. The scaffolds produced in this research showed to have not only adequate physical characteristics but also chemical and biological ones. It was as well as detected a reproducible methodology that resulted in a biomaterial with morphology that provided a high degree of swelling, porosity, satisfactory degradation and the presence of amoxicillin which was confirmed by the eenergy dispersive spectrometer. Nevertheless, the scaffolds with 30% hydroxyapatite showed the best results for *in vitro* cell viability. So, it is possible to conclude that the scaffolds produced show successful characteristics for bone repair in the oral cavity.
Keywords: Chitosan; Hydroxyapatite; Scaffolds; Amoxicillin; Drug delivery systems.

Resumo
O processo reparatório ósseo, entre outros mecanismos fisiológicos, ocorre de forma harmoniosa em todo o corpo. No entanto, a presença de alguns fatores deletérios, como cirurgia, trauma ou patologia, pode interferir na fisiologia da remodelação óssea. Sabe-se que a ação antimicrobiana sinérgica entre fármacos e biomateriais pode auxiliar e favorecer a osteogênese após cirurgia de enxerto. Assim, este estudo tem como objetivo desenvolver scaffolds de quitosana / hidroxiapatita com amoxicilina para reparo ósseo na cavidade oral. Para isso, as matérias-primas foram selecionadas e caracterizadas e os scaffolds foram confeccionados pelos processos de solubilização, dispersão e liofilização. As caracterizações foram feitas por microscopia ótica, microscopia eletrônica de varredura, espectrômetro dispersivo de energia, potencial de entumecimento, análise de biodegradação, teste de porosidade aparente e citotoxicidade celular in vitro. Os scaffolds produzidos nesta pesquisa mostraram características não apenas físicas adequadas, mas também químicas e biológicas. Foi também detectada uma metodologia reprodutível que resultou em um biomaterial com morfologia que proporcionou um alto grau de intumescimento, porosidade, degradação satisfatória e a presença de amoxicilina que foi confirmada pelo espectrômetro dispersivo de energia. Dentre as amostras, os scaffolds com 30% de hidroxiapatita apresentaram os melhores resultados para viabilidade celular in

vitro. Assim, é possível concluir que os scaffolds produzidos apresentam características de sucesso para reparação ósseo na cavidade oral.
Palavras-chave: Quitosana; Hidroxiapatita; Scaffolds; Amoxicilina; Sistemas de liberação de medicamentos.

Resumen

El proceso de reparación ósea, entre otros mecanismos fisiológicos, se produce de forma armoniosa en todo el organismo. Sin embargo, la presencia de algunos factores deletéreos, como cirugía, traumatismo o patología, puede interferir con la fisiología de la remodelación ósea. Se sabe que la acción antimicrobiana sinérgica entre fármacos y biomateriales puede ayudar y favorecer la osteogénesis tras la cirugía de injerto. Por lo tanto, este estudio tiene como objetivo desarrollar andamios de quitosano / hidroxiapatita con amoxicilina para la reparación ósea en la cavidad oral. Para ello, se seleccionaron y caracterizaron las materias primas y se confeccionaron los andamios mediante los procesos de solubilización, dispersión y liofilización. Las caracterizaciones se realizaron mediante microscopía óptica, microscopía electrónica de barrido, espectrómetro de dispersión de energía, potencial de hinchamiento, análisis de biodegradación, prueba de porosidad aparente y citotoxicidad celular in vitro. Los andamios producidos en esta investigación mostraron no solo características físicas adecuadas, sino también químicas y biológicas. También se detectó una metodología reproducible, lo que resultó en un biomaterial con morfología que aportó un alto grado de hinchamiento, porosidad, degradación satisfactoria y la presencia de amoxicilina lo cual fue confirmado por el espectrómetro de dispersión de energía. Entre las muestras, los andamios con 30% de hidroxiapatita mostraron los mejores resultados para la viabilidad celular in vitro. Así, es posible concluir que los andamios producidos tienen características exitosas para la reparación ósea en la cavidad bucal.
Palabras clave: Quitosano; Hidroxiapatita; Andamio; Amoxicilina; Sistemas de liberación de medicamento.

1. Introduction

Thousands of bacteria live in the human body and thus are everywhere. In the oral environment, for example, around 500 species that are part of the oral microbiota coexist. The normal bacterial flora is beneficial to the body, for example, of the commensal bacteria that play a role of prevention and protection of the mucosa against infections. However, surgery, trauma, diseases, poor hygiene, inadequate diet, among other circumstances can cause imbalance in this habitat. In such situations, a selection occurs for certain opportunistic pathological microorganisms and the biofilm becomes virulent and may cause local and systemic lesions that trigger deleterious effects throughout the organism (Nicolas & Lavoie, 2011; Sixou, Diouf, & Alvares, 2007).

In equilibrium conditions, absence of virulence, the bones of the jaws function as an active tissue that undergoes remodeling in a kinetic, continuous and balanced process; involving formation, resorption and bone maintenance that are based on the activity of osteoblasts, osteoclasts and osteocytes, respectively (Anamarua, Rico, Ferrer, Ivanković, & Ivanković, 2016; Khanna et al., 2017; Shu et al., 2018). On the other hand, after infections, injuries due to surgeries with loss of bone structure, trauma or disease, repair is initiated in response to the physiological regulatory mechanisms associated with inflammation and the patient's immune response. Thus, the control of virulence biofilm has such a great importance for the treatment, prevention and reestablishment of altered normal functions. In this sense, many biomaterials have been investigated in an attempt to aid and protect, for example, bone repair in injured or surgery regions (Khanna et al., 2017; Shu et al., 2018).

Combinations of biomaterials as an alternative to autogenous and heterologous grafts have been developed and applied in tissue engineering, with desirable properties: adequate mechanical resistance to support the tension exerted, physical-chemical fixation avoiding migration of the region of surgical or injured action, favoring bioactivity in cell differentiation and proliferation, in addition to microstructures with interconnected pores and dispersed in their volume , enabling wetting, blood swelling and consequently the blood supply of the reparably restrain region. The presence of pores influences cellular adhesion and activity and angiogenesis (neovascularization), among other factors (Atak et al., 2017; Callister & Rethwisch, 2012; Zhang et al., 2014) (Zhang et al., 2014).

Among these materials, the choice for hydroxyapatite is due to its similarity and affinity with bone tissue, high ability to adsorb and/or absorb molecules. Thus, this phosphate has been used as a support in bone lesion treatments, to increase

surface reactivity, expanding integration with the adjacent tissue stimulating cells adhesion, migration and proliferation. But hydroxyapatite has some limitations that may compromise its biological activity in some applications, such as mechanical fragility and migration of particles from the implant region. However, when associated with chitosan in sscaffolds, the hydroxyapatite can improve its individual properties (Almeida et al., 2019; Chaves, 2015; Heidari et al., 2018; Luna-Domínguez et al., 2018; Shao, Zhao, & Yang, 2014; Zainol, Ghani, Mastor, Derman, & Yahya, 2009).

Hydroxyapatite can be produced by several synthetic routes. Among these, coprecipitation as an important method to ensure an economically desirable, single-phase, nanocrystalline phosphate. This method also presents a regular morphology with fine powders, linked by weak physical forces and with interparticular porosity that favors their dispersion (Çanakçı, 2021; Gecim, Dönmez, & Erkoc, 2021).

Such studies show the efficacy of the union between hydroxyapatite and chitosan in bone repair (R. S. Almeida et al., 2019; Heidari et al., 2018). This biopolymer is a linear polysaccharide, derived from chitin, with high load density, reactive groups and hydrogen bonds, with characteristics such as biocompatibility, biodegradability and ability to accelerate bone formation. However, there is still a search for an ideal material that is based on reducing the number of pathogenic microorganisms to control and prevent infections in bone receptors sites through the incorporation of some drugs such as antibiotics (Rebelo et al., 2015; Xu, Strandman, Zhu, Barralet, & Cerruti, 2015).

Antibiotics are molecules that bind to specific proteins that are essential to the function of bacteria. This mechanism of action leads to apoptosis (death) of these microorganisms. Among the main antibiotics used in dentistry, there is amoxicillin that has therapeutic action against Gram-positive and Gram-negative bacteria, the most likely pathogens, particularly implicated in the etiology of oral diseases. Amoxicillin can be administered in a systemic or local manner. It is possible to number some advantages of the local administration such as: longer drug remains in the region and reduction of possible toxic or subtherapeutic levels. Thus, minimizing undesirable side effects and decreasing the number of doses required when they are administered in a conventional systemic manner. Drugs such as amoxicillin can also be associated with biomaterials in scaffolds (Birt, Anderson, Toby, & Wang, 2017; Cai et al., 2017; Dowd, Yagiela, Johnson, Mariotti, & Neidle, 2010; Yagiela, Dowd, Johnson, Mariotti, & Neidle, 2010).

Scaffolds are porous and temporary three-dimensional supports with adequate biophysical and biochemical conditions, both for cell propagation and to remain integrated to the host tissue, without risk of rejection, with synergistic action in the acceleration of angiogenesis, favoring the differentiation of mesenchymal cells. These properties have aroused the interest of several researchers (Al-Namnam et al., 2017; Heras et al., 2019; Sharipova, Gotman, Psakhie, & Gutmanas, 2019; Xu et al., 2015).Therefore, several studies have been done with scaffolds for application as biomaterial for bone repair, among these: calcium phosphate scaffolds (Batista, 2016; Lett, Sagadevan, Prabhakar, & Latha, 2019); hydroxyapatite and magnetite scaffolds (Chaves, 2015; Xu et al., 2015); bioscaffold hydroxyapatite doped with MgFe2O4 (John, Janeta, & Szafert, 2017); scaffold with strontium/hydroxyapatite and chitosan (Lei; et al., 2017); chitosan scaffold and hydroxyapatite (R. S. Almeida et al., 2019; Anamarua et al., 2016; Atak et al., 2017; Dantas, 2016; Lei; et al., 2017). These and other researches have proven the potential of scaffolds' properties in biomedical applications.

Therefore, the combination of chitosan and hydroxyapatite in scaffolds associated with an antibiotic, in particular, amoxicillin can potentiate and improve the properties of these biomaterials, especially biological ones. Knowing that the Federal University of Campina Grande, through CERTBIO (Laboratory of Evaluation and Development of Biomaterials of the Northeast) has infrastructure to produce a certified chitosan with a high degree of purity LabSMac (Laboratory of Synthesis of Ceramic Materials) synthesizes, by viable and low-cost routes, monophase and crystalline hydroxyapatites, and that amoxicillin is the most indicated antibiotic for the control of oral infections, this work has the goal to develop

chitosan/hydroxyapatite/amoxicillin scaffolds and to study its morphological, structural, chemical and biological properties, aiming at application in tissue repair in the oral cavity.

2. Methodology

Regarding this nature qualitative research, the scientific method was based on the work carried out by (Aranaz, Gutiérrez, Ferrer, & Del Monte, 2014; Shahbazarab, Teimouri, Chermahini, & Azadi, 2018); Whang, Thomas, Healy, and Nuber (1995); (Zhang, Zhang, Ma, Yang, & Nie, 2015). Chitosan from the exoskeleton of *Lipopenaeus vannamei* shrimp with average molecular weight between 50-150 KDa, determined by viscometry (PSL Rheotek, São Paulo, Brazil), and degree of deacetylation of 85.7% to 90%, determined by the infrared spectroscopy (Perkin Elmer, Beaconsfield, U. K.) was produced by CERTBIO - (Northeastern Biomaterials Evaluation and Development Laboratory). Hydroxyapatite was produced by LabSMac - (Laboratory of Synthesis of Ceramic Materials), Sodium hydroxide (NaOH) was purchased from Neon® (São Paulo, SP, Brazil). Phosphate buffer saline (PBS, pH 7.4) was acquired from Sigma Aldrich® (St. Louis, USA), glacial acetic acid P.A 99.9% and Sodium Hydroxide supplied by Vetec® Química Fina Ltda.

Initially, 3 g of chitosan was solubilized in 100 mL of acetic acid solution at 1% (v/v), under mechanical agitation at 22 °C for 2 hours at 346 rpm. This methodology was used for all chitosan solutions obtained. After obtaining the solution, the pH 4.15 was verified with Phmetro Quimis®.

Preparation of scaffolds, 30, 50 e 70% of hydroxyapatite synthesized by coprecipitation were immersed in three solutions containing 100 mL of chitosan at 3% (m/v). Subsequently, the dispersions were subjected to agitation for 30 min. After dispersing the hydroxyapatite, the drug (1.5 g of amoxicillin) was added to the dispersion. This remained for 30 min in immersion ultrasound to promote the dispersion of particles and removal of air bubbles. Then 30 mL of each solution was poured into Petri dishes 9 cm in diameter.

The Petri dishes containing the dispersions were placed in an ultra freezer at a temperature of -86 °C for 24 hours and submitted to the lyophilization process for 72 hours. After this stage, the scaffolds were immersed in sodium hydroxide solution at 1 mol/L for 1 hour for neutralization and then washed with distilled water to remove excess sodium hydroxide, swelled, refrozen and again lyophilized to maintain the porous structure. They Were then characterized.

The morphology of the surfaces of the scaffolds, distribution, and agglomeration of hydroxyapatite and amoxicillin particles were analyzed under a Hirox Digital Optical Microscope (KH 13000, Tokyo, Japan) operating in reflection mode using magnifications of 60X and 120X. The measurements were performed using ImageJ (Java 1.8.0.112 Version, National Institutes of Health and Laboratory for Optical and Computational Instrumentation, Wisconsin, WI, USA).

The surface morphology of the scaffolds was evaluated by Scanning Electron Microscopy (SEM, TESCAN VEGA3 SBH with dry EDS detector from Oxford X-ACT IE150). It was used to highlight the morphological aspects as well as the surface, size, shape, pores distribution, particles and also the analysis of the chemical composition of the raw materials present in the matrix of the scaffolds. In this analysis magnifications of 100X and 500X were used and the surface of the scaffolds was covered with conductive metallic material.

The swelling was carried out in order to investigate the dimensional behavior of the biomaterial when immersed in an aqueous solution with neutral pH. For this, samples with diameter and thickness of 1.0 x 1.0 mm were prepared and divided into groups. Initially the samples were dried at 40 ºC for 24 hours, weighed an analytical scale of digital precision, immersed in 7.5 mL of phosphate buffer solution (PBS, pH 7.4) and conditioned at 37 ºC. After 24 hours, they were removed from the solution, dried slightly on absorbent paper to eliminate the excess of the solution and the wet weight was obtained on the same measured scale.

In vitro degradation of scaffolds was evaluated in 7.5 mL phosphate buffer solution (PBS, pH 7.34) at 37 °C. The samples were dried at 40 °C for 24 hours, weighed and conditioned at 37 °C in PBS solution that was changed weekly. At each biodegradation period (14 and 21 days), the samples were taken from the solution, washed in distilled water, dried at 40 °C for 24 hours and then weighed. ty test.

To calculate apparent porosity of all scaffolds, samples with a thickness of 1.0 x 1.0 mm were prepared and conditioned at 40 ºC for 24 hours. After this time, the samples were weighed on a precision scale, immersed separately in 7.5 mL of distilled water, then covered and left immersed for 24 hours. Subsequently, the immersed sample weight was measured. Then, the samples were dried slightly on absorbent paper to eliminate excess of water and had the wet weight measured on the same previous scale. The test was performed in quintuplicate.

The evaluation of *in vitro* cell cytotoxicity of scaffolds was performed by the test of evaluation of cell viability and proliferation of fibroblasts by MTT 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide according to ISO 10993-5:2009 (Standard for Biological Evolution of Medical Devices) using L929 mouse fibroblast cell lines, and the direct method of contact between the substrate of the material and the cells was used. The cell line was L929, acquired from the Cell Bank of Rio de Janeiro. MTT is a quantitative, sensitive and reliable colorimetric assay, which mediates the viability, proliferation and activity of cells based on the ability of the enzyme dehydrogenase, which is found in living cell mitochondria, to convert the water-soluble yellow substrate MTT into a purple-colored product, resulting from the formation of formazana crystals that are insoluble in water and can then be detected by absorbance reading in a spectrophotometer. The amount of formazana produced is directly proportional to the number of viable cells.

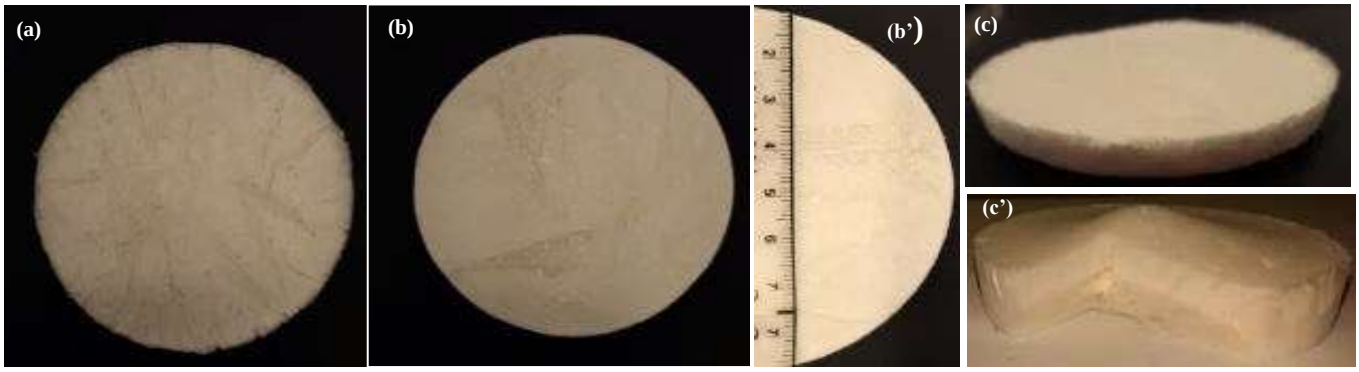
The analyzes were carried out at the Northeast Biomaterials Evaluation and Development Laboratory - CERTBIO, an accredited laboratory by the ABNT ISSO / IEC 17025: 2005 Standard, CRL 0799 for Chemical and Biological Tests, LabSMac (Laboratory of Synthesis of Ceramic Materials)

3. Results and Discussion

The Figure 1 illustrates the macroscopic aspect of chitosan/hydroxyapatite/amoxicillin scaffolds and their interfaces. Figure (1a) shows the outer surface, in Figure (1b and 1b') it is possible to observe the base (surface in contact with the Petri dish) and in Figure (1c and 1c') observe the cutting surface with lamelar formation perpendicular in the horizontal direction and parallel in the vertical direction. The shape of the scaffolds was determined by the chitosan matrix that maintained its morpho structural characteristics even after the dispersion of hydroxyapatite and amoxicillin particles. Similar result was observed by Rosendo (2016), Almeida (2013) and Cruz, Catão, Barbosa, and Fook (2016) when they developed and characterized chitosan scaffolds by the lyophilization process for drug delivery system.

Macroscopically delimited regions are also observed that are attributed to the molecular rearrangement of polymer chains during the freezing and lyophilization process. Rapid freezing induces high nucleation rate and low crystal growth rate which leads to the formation of small crystals. These, when sublimated give rise to interconnected pores that are geometrically similar to lyophilized crystals. Result also reported by D. Zhang et al. (2014) and Shamloo, Kamali, and Fard (2019) when they produced scaffolds by the freeze-drying method.

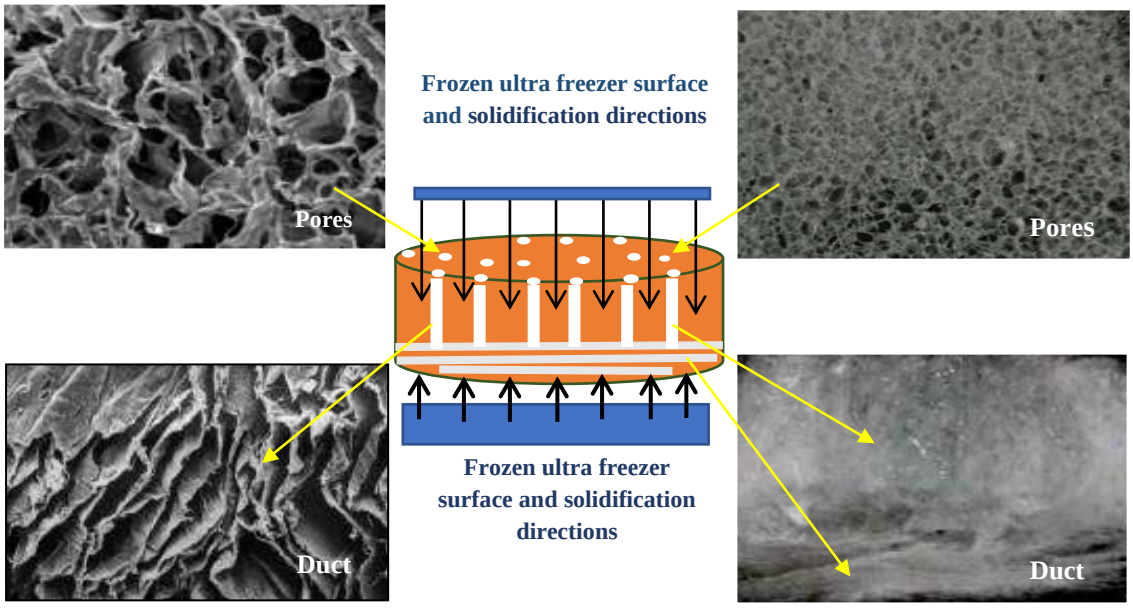
Figure 1 - Macroscopic morphology of scaffolds, (a) Outer surface, (b and b') base and (c and c') Cutting surface.



Source: Authors.

As seems in Figure 2, on the outside outer surface pores were formed that interconnect through axial multichannels that are formed parallel to the direction of solidification in the upper region and perpendicular to the direction of solidification in the lower region. Similar result was reported by Q. Zhang et al. (2012). These morphological characteristics are extremely necessary in surgery to repair the bone of the jaws.

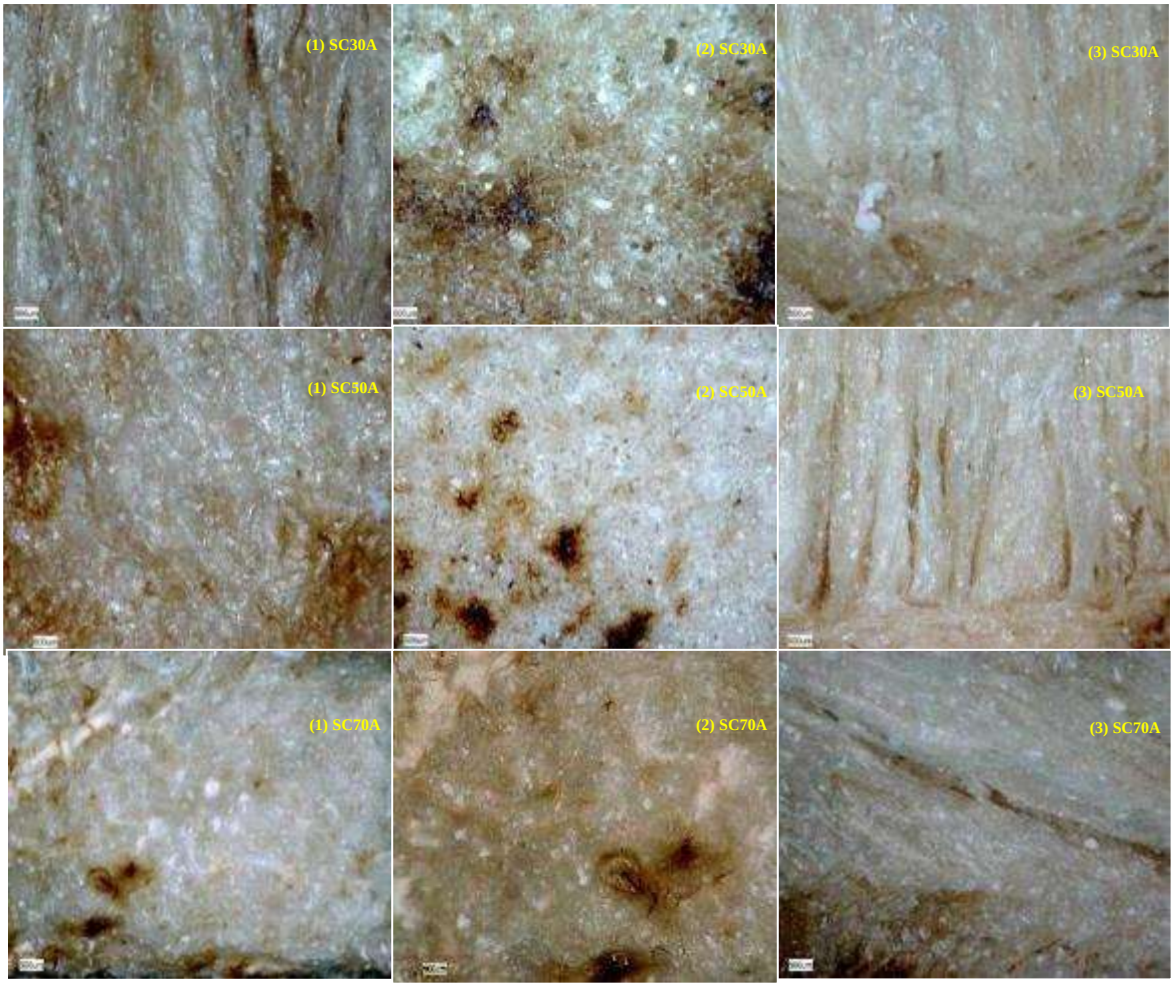
Figure 2 - Direction of solidification and formation of the ducts and pores of the structure of the scaffolds.



Source: Authors.

Successful graft surgeries are influenced by factors such as age, disease, positioning and morphology of biomaterial and bone. Thus, the graft needs to be inserted in the appropriate position that favors its rapid blood swelling and revascularization of the repaired lap (Ávila Souza, Borrasca, Aranega, & Ponzoni, 2014; Caneva et al., 2017; Carvalho et al., 2020).

Figure 3 - Optical microphotographs of scaffolds (SC30A, SC50A e SC70A), (1) base, (2) outside surfaces and (3) cross cutting surfaces.



Source: Authors.

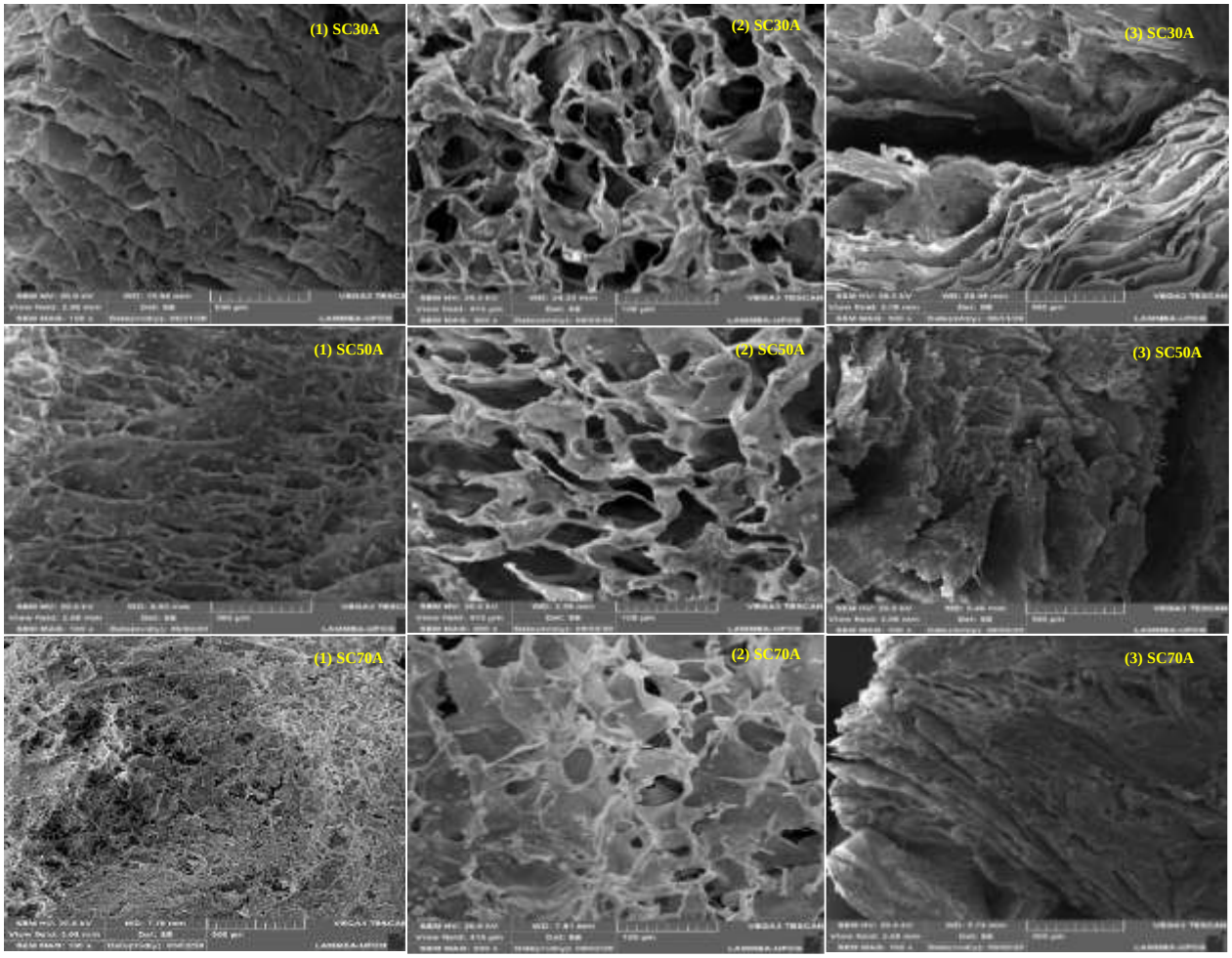
In Figure 3 can be visualized the images obtained by OM of SC30A, SC50A e SC70A (scaffolds with 30%, 50% and 70% of hydroxyapatite with amoxicillin), respectively.

The morphological characteristics of the scaffold have the potential to favor and assist in surgeries after tooth extraction and, for this, the external surface with the presence of pores must be positioned in contact with the recipient bone that needs to be decorticalized (perforated) to stimulate bleeding. In this way, the blood will be absorbed, conducted through the parallel axial channels and kept in the channels at the base of the scaffold and will be in contact with the gums that will be vascularized. Therefore, this research stands out and innovates in the prospect of success in bone repair surgeries.

The Figure 3 (1) obtained by OM illustrates the base of the scaffold that presents parallel rugosity and shallow grooves, Figure 3 (2) shows the outer surface that presents with pores of various sizes and shapes, distributed throughout of this surface and Figure 3 (3) illustrates the cross-cutting surface with lamelar formation. It can be also observed hydroxyapatite and amoxicillin particles dispersed in the chitosan matrix without significant change in its conformation. However, the presence of the drug brought about a visible change to the naked eye and also to the OM in the color of the matrix. These results were confirmed by SEM, as illustrated in Figure 4.

As it can be observed in Figure 4, the freeze-drying methodology in all samples was an efficient and reproducible process. Similar result was also cited by Silva, Nascimento, Ribeiro, and Fook (2016), Duman and Bulut (2021) and Shamloo et al. (2019) who produced chitosan scaffolds with similar morphological structures and presence of pores.

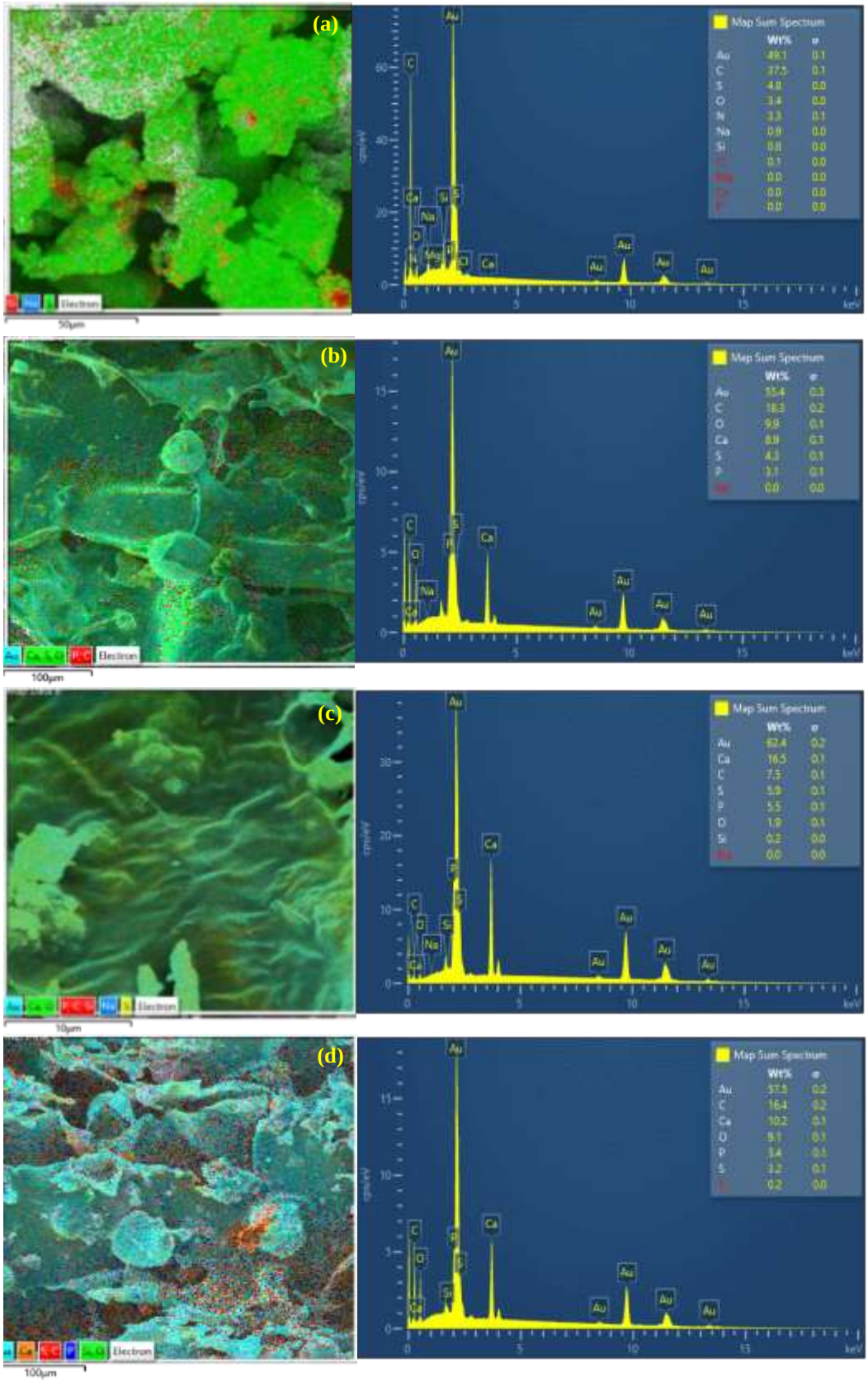
Figure 4 - Microphotographs of chitosan scaffolds (SC30A, SC50A e SC70A), (1) the basis, (2) outside surfaces and (3) cross cutting surfaces.



Source: Authors.

Figure 5 illustrates the results of energy dispersive spectroscopy of amoxicillin and the SC30A, SC50A e SC70A (scaffolds with 30%, 50% and 70% of hydroxyapatite with amoxicillin) before the neutralization with sodium hydroxide. This technic shows that dispersion, distribution and percentages of chemical elements in the chitosan matrix which are observed through in the Figure 6 and obtained tables. Principally, the presence of the elements: Gold (Au), Sulfur (S), Oxygen (O), Silicon (Si), Calcium (Ca), Potassium (P), Carbon (C) and Nitrogen (N), related to the raw material of chitosan, hydroxyapatite and Amoxicillin were observed, thus showing that, there was no evidence of contaminants during the preparation of scaffolds.

Figure 5 - EDS: (a) Amoxicillin, (b) SC30A, (c) SC50A e (d) SC70A.

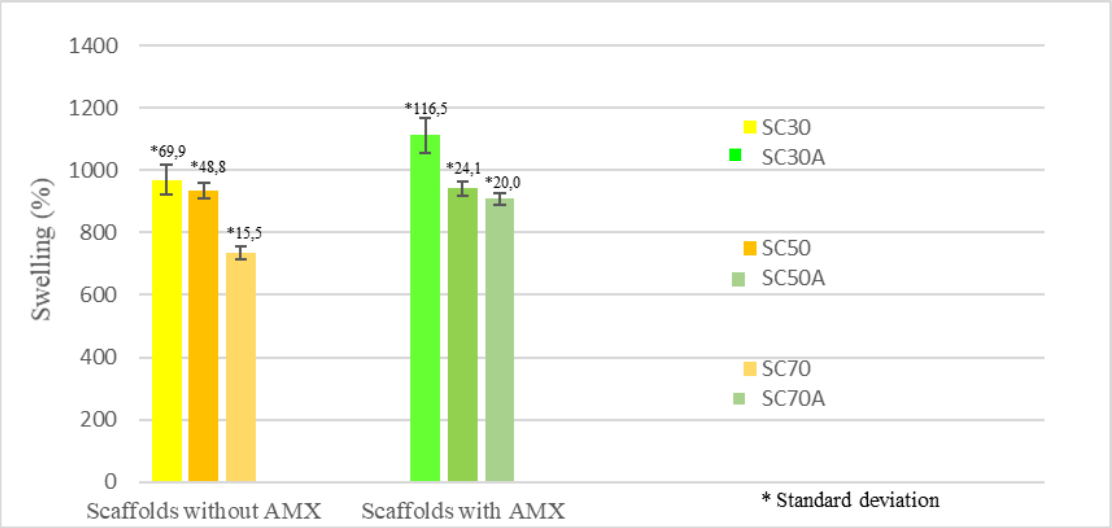


Source: Authors.

According to Anamarua et al. (2016) and Öfkeli, Demir, and Bölgen (2021) swelling is one of the main factors that influences biocompatibility and the absorbed water content confers physical-chemical properties to biomaterials. According to some studies (Liu et al., 2019; Rebelo et al., 2015) the hydrophilicity of a porous structure facilitates the penetration of nutrients into its interior, influencing cell adhesion, division and proliferation. Furthermore, the absorption of fluid by the material can be an important factor in drug release, neovascularization and consequent bone neoformation.

The histogram in Figure 6 illustrates the swelling and standard mean deviation of the SC30A, SC50A e SC70A and the SC30, SC50 e SC70 (scaffolds with 30%, 50% and 70% hydroxyapatite without amoxicillin) and the Table 1 presents the degree of crystallinity of these scaffolds.

Figure 6 - Histogram the swelling of chitosan scaffolds (SC30, SC50 e SC70) without AMX and (SC30A, SC50A e SC70A) with AMX.



Source: Authors.

In the histogram of Figure 6, it is noted that the scaffolds presented a considerable absorption and retention capacity of phosphate buffer solution with mass gain, once they absorb more liquid than their own weights, in the simulated physiological conditions. Considerable mass gain is observed in all scaffolds. It was observed that by increasing the percentage of hydroxyapatite, they presented a decrease in swelling that can be justified by the increase in the crystallinity of the scaffold as a result of the increase in the hydroxyapatite/chitosan ratio. Thus, it was noticed in Table 1 that the degree of crystallinity of the samples is inversely proportional to the swelling. Results also observed by Öfkeli et al. (2021). Another hypothesis is a probable interaction of groups Ca^{2+} and PO_4^{3-} with the groups -COOH and NH_2 of chitosan, reducing the hydrophilia of the biopolymer. In addition, the hydroxyapatite creates a barrier preventing the permeability of water in the matrix, thus causing less spacing between the chains of chitosan (Hoffman, 2012).

Table 1 - Degree of crystallinity of the scaffolds without AMX and with AMX.

Samples (DC %)			
	30%	**50%**	**70%**
Scaffolds without AMX	28,9	29,8	31,8
Scaffolds with AMX	29,2	31,0	34,6

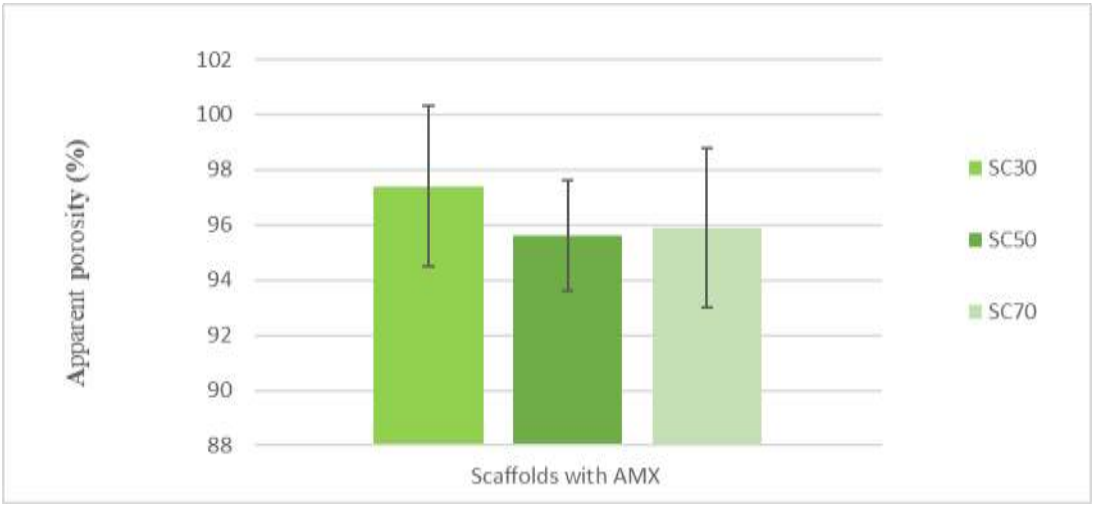
Source: Authors.

It was also observed that there was an increased swelling in scaffolds SC30A, SC50A and SCA70A in relation to SC30, SC50 and SCA70 scaffolds without the drug. Result that can be attributed to the hydrophilicity of amoxicillin incorporated in chitosan/hydroxyapatite dispersion.

Thus, SC30 and SC30A with the lowest percentage of hydroxyapatite and degree of crystallinity showed the highest swelling, followed by the scaffolds SC50 and SC70. It was also observed that the SC30A presented the highest standard deviations 69.9 and 116.5, respectively. However, among the other samples the standard deviation was not significant. All these results show that the microstructural characteristics of the scaffolds produced in this research are similar and reproducible according to the results found in OM, SEM and porosity test.

Figure 7 shows that the scaffolds presented a morphology control that resulted in an apparent porosity of around 96%. Scaffolds for tissue engineering must have porosity close to or bigger than 90%, being considered minimum values around 70% and maximum close to 95% (Öfkeli et al., 2021; Oliveira et al., 2018; Zhang, Wu, Jing, & Ding, 2005).

Figure 7 - Histogram of apparent porosity of scaffolds (SC30A, SC50A e SC70A) with amoxicillin containing 30%, 50% and 70% hydroxyapatite.



Source: Authors.

The presence of pores and lamelar spaces in the microstructure of the scaffolds, as observed by the OM and SEM, favored the swelling of the polymeric network of the chitosan matrix. The permeability of scaffolds depends on a combination of factors, including porosity, pore size, porogeometry and porous distribution. Any change to one of these parameters affects directly the structural integrity of scaffolds (Dai et al., 2019; Freitas, 2015).

According to D. Zhang et al. (2014), the hydroxyl group, in the acetoamide group and the covalent bonds of the amino group (N-H) have electronegativity that generate polarity favoring the rearrangement of water molecules around these sites. Thus, the hydrophilicity of chitosan keeps liquids in the porous space, as observed in the histogram in Figure 7.

According to Anamarua et al. (2016), scaffolds are extremely important as a biomaterial for bone repair, because it helps in bone neoformation, neovascularization, produces wettability that allow the penetration of biological fluids, facilitates adhesion and improves cell activity, properties that are indispensable to grafts.

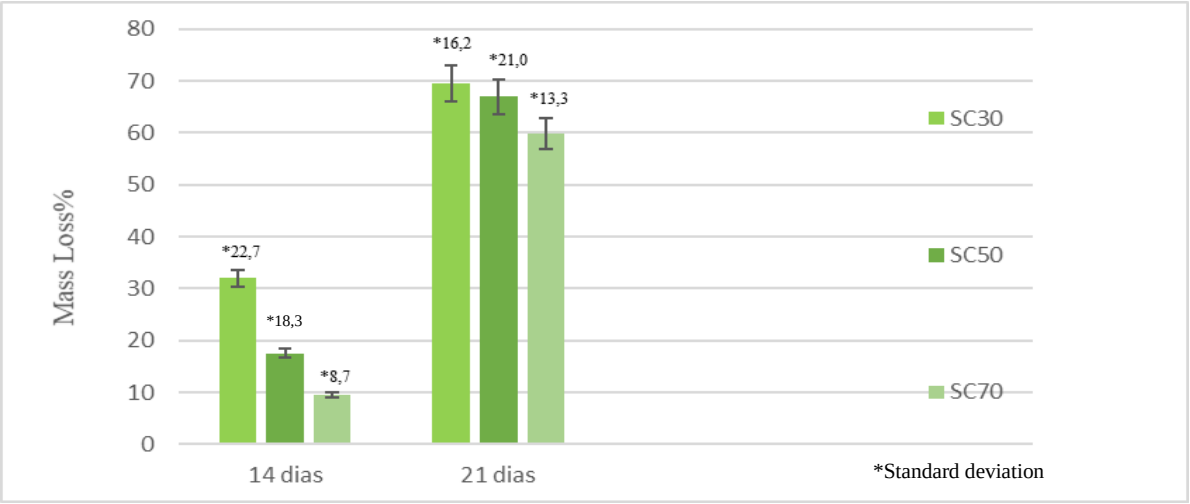
According to Dorozhkin (2010), porous structures increase the surface area, increase the space for fixation of cells and favors the chemical bond with adjacent tissue. Moreover, a high degree of porosity is responsible for regulating bioactivity, as it directly influences structural permeability, responsible for controlling not only the initial speed of tissue regeneration, but also allows great potential in the development of materials for the slow release of drugs, as the scaffold is degraded.

The degradation of a biomaterial in biological environment is one of the variables of great relevance, once this property is directly related to the length of permanence of the material after implantation in the patient.

According to Materials (2010) a hydrolysable device under hydrolytic conditions is challenging at 37 °C and buffered saline solution is a common means to obtain an approximation of the degradation profile of an absorbable material or device. This condition does not necessarily represent the actual conditions *in vivo*.

The Figure 8 illustrates the degradation test carried out in order to investigate the behavior of the biomaterial during immersion and permanence in aqueous phosphate buffer solution. It was observed that there was a loss of variable mass between the samples in the period of 14 days and 21 days and that the SC30A presented greater mass loss in relation to SC50A, which lost more mass than the SC70A. As noted in Table 1 the scaffold SC70A presented a higher degree of crystallinity among the scaffolds and consequently lower mass loss. This does not imply the degradation of chitosan, but the fragmentation and loss of the inorganic phase during the *in vitro* assay. Result also reported by Nazeer et al. (2020).

Figure 8 - Histogram of the scaffolds (SC30A, SC50A e SC70A) degradation test.



Source: Authors.

It is also observed that the higher the amount of hydroxyapatite, the lower the degradation of the biomaterial, which can be attributed to the amount of chitosan in each system, that is, the higher the quantity of hydroxyapatites, the lower the amount of chitosan, since the total volume is equal for all scaffolds. Regarding the degradation of samples with drug, it was

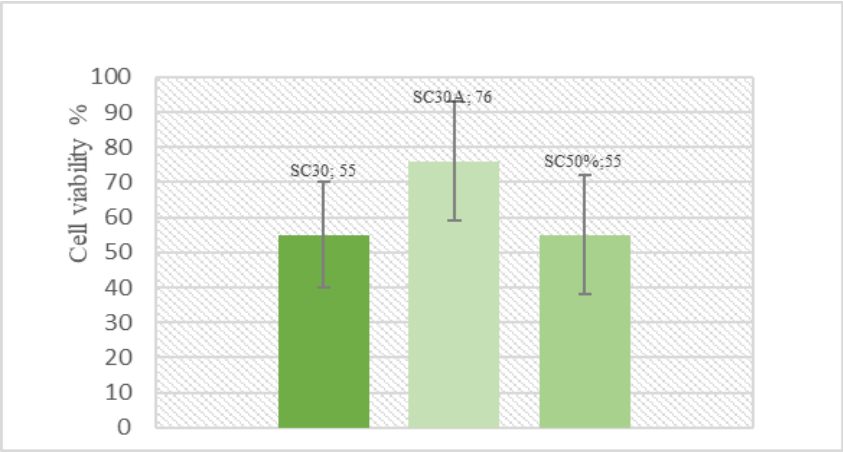
observed that there was greater mass loss when compared to the other samples and this fact may be related to drug release, chitosan matrix degradation and hydroxyapatite fragmentation.

In vivo studies have shown that hydroxyapatite only begins to be gradually reabsorbed after 4 or 5 years of implantation, being the most frequent and thermodynamically more stable phase at physiological pH. Scaffolds containing chitosan and hydroxyapatite lose more than 1% of weight during the first week and 5-6% at the end of the 15th week. This is probably due to the loss of chitosan and hydroxyapatite deposited on the surface, as well as in the pores. A slow degradation is desirable in bone tissue regeneration applications, since the bone takes much longer to regenerate, so that the degradable matrix would be replaced by bone. For example, spongy bone takes 3 to 6 months, while cortical bone takes 6 to 12 months to reshape. However, scaffolds degrade very quickly, the structural architecture to promote cell proliferation and functionality are lost, resulting in cell death (Almeida et al., 2019; Dumont, 2017).

Chitosan is a polysaccharide that suffers degradation when in aqueous solution. In this case the connections of the polymer complex are broken, which join the H^+ and OH^- ions of the water. The degradation of chitosan in the body leads to the release of amino sugars that can be incorporated and metabolized or simply excreted by the body. The products of the enzymatic degradation of chitosan are N-acetyl-D-glucosamines oligomers, which have healing, antimicrobial properties and are fully reabsorbable by the body. That is, they are nontoxic. Scaffolds should promote bone regeneration as they are reabsorbed, and both the material and their degradation products should be adequately tolerated by the body and should not provoke foreign body reaction (Shu et al., 2018) (Lei et al., 2017; LogithKumar et al., 2016).

According to Figure 9, the cell viability test demonstrated that the SC30A presented viability of 76% (±15), significantly higher than that presented by SC30 (55/±15) and SC50 (55/±17) without amoxicillin.

Figure 9 - Histogram of fibroblast viability of the scaffolds SC30, SC30A and SC50.



Source: Authors.

According to Nurfuadi (2019) scaffolds that have viability greater than 75% have indicative of cell nontoxicity. Thus, it can be observed that the scaffolds studied did not cause any cytotoxic effects and the fibroblasts could grow and proliferate on their surface, reflecting that the biomaterial were non-toxic and may be biologically acceptable as scaffold.

4. Conclusion

Chitosan and hydroxyapatite scaffolds with amoxicillin were successfully prepared by freeze-drying methodology. All samples obtained showed interconnected micropores that correspond to the porosity conditions required to allow their uses as

scaffolds in the biomedical field. Hydroxyapatite, produced by coprecipitation, and amoxicillin microparticles were homogeneously dispersed in the scaffold matrix. The swelling of the scaffolds showed that they have a high capacity of absorption and retention in simulated biological fluids. This capacity probably helps in bone neoformation, neovascularization, adhesion and improves cell activity. All scaffolds suffered relative degradation in contact with phosphate saline buffer solution during the evaluation periods. This degradation occurred in the polymer matrix and possibly in the drug. The presence of amoxicillin in the scaffolds was confirmed by the Energy dispersive spectroscopy. The teste of cell viability revealed that fibroblast could grow and proliferate on the scaffolds, reflecting that the biomaterial were non-toxic and may be biologically acceptable as scaffold. In view of the characteristics obtained, can be concluded that the scaffolds have potential for bone repair in the oral cavity.

Concerning future work, it is suggested to study the release of the drug *in vitro and* conduct pre-clinical assessment of the scaffolds *in vivo*, aiming to confirm their effectiveness for bone repair.

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