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Biocompatibility and biodegradation analysis of Nile Tilapia gelatin and apatite membranes[1]

Análise de biocompatibilidade e biodegradação de membranas de gelatina e apatita de tilápia do Nilo

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ABSTRACT - Fish farming by-products could represent large-scale raw materials for xenogeneic implants that could be used for bone regeneration. The objective of this research was to analyze the biocompatibility and biodegradation of gelatin (G) and apatite (A) membranes from Nile tilapia. Adult male Swiss mice underwent subcutaneous implantation of biocomposites derived from skins and scales in different concentrations: 60%G:40%A (G1), 70%G:30%A (G2) and 80%G:20%A (G3). Commercial collagen membrane (C+) or implantless surgery (C–) were used as controls. Euthanasia was performed at 1, 3 or 9 weeks for histological analysis of the inflammatory and repair criteria as well as the integrity of each material. The statistical analysis of non-parametric data was performed using the Kruskal-Wallis test and post-hoc Dunn test, with $p < 0.05$. In vivo results during the experimental period demonstrated progressive improvement in biocompatibility, with G1 and G2 being slightly irritating and G3 non-irritating, just like C+. There were significant differences between test and control groups regarding the presence of neutrophils, lymphocytes, macrophages, foreign body giant cells, neovascularization and connective tissue. There was also a decrease in the integrity of the implants, where G1 maintained greater stability than G3 and G2, but less than C+. All biocomposites proved to be biocompatible and partially biodegradable. G1 suggests greater potential for use as an osteopromoting membrane, with its biological performance associated with higher mineral concentration compared to the organic phase. Future long-term orthotopic studies will be conducted to investigate its osteopromoting action.

Key words: Gelatin. Apatites. Biocompatible materials. Absorbable implants.

RESUMO - Subprodutos da piscicultura poderiam representar matéria-prima em larga escala para implantes xenógenos úteis à regeneração óssea. O objetivo desta pesquisa foi analisar a biocompatibilidade e a biodegradação de membranas de gelatina (G) e apatita (A) de tilápia do Nilo. Camundongos Swiss machos adultos foram submetidos a implante subcutâneo de biocompósitos derivados de peles e escamas em diferentes concentrações: 60%G:40%A (G1), 70%G:30%A (G2) e 80%G:20%A (G3). Como controles, foram usados membrana colágena comercial (C+) ou cirurgia sem implante (C–). Eutanásia foi realizada em 1, 3 ou 9 semanas para análise histológica de critérios inflamatórios, de reparo e integridade de cada material. A análise estatística dos dados não paramétricos foi realizada pelo teste de Kruskal-Wallis e pós-teste de Dunn, com $p < 0,05$. Resultados in vivo durante o período experimental demonstraram melhora progressiva na biocompatibilidade, com G1 e G2 suavemente irritantes e G3 não irritante, tal como o C+. Houve diferenças significativas entre grupos teste e controles quanto a presença de neutrófilos, linfócitos, macrófagos, células gigantes do tipo corpo estranho, neovascularização e tecido conjuntivo. Ainda, houve decréscimo da integridade dos implantes, onde G1 manteve maior estabilidade do que G3 e G2, porém inferior ao C+. Todos os biocompósitos se mostraram biocompatíveis e parcialmente biodegradáveis. O G1 sugere maior potencial de uso como membrana osteopromotora, com seu desempenho biológico associado à maior concentração mineral frente à fase orgânica. Futuros estudos ortotópicos a longo prazo serão conduzidos para investigar sua ação osteopromotora.

Palavras-chave: Gelatina. Apatitas. Materiais biocompatíveis. Implantes absorvíveis.

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INTRODUCTION

Brazil is a prominent player in global agribusiness and one of the largest suppliers of animal protein, with national prevalence for the production of million tons (Mt) per year of chicken meat (13 mt) compared to pork (4 mt) and fish (1 mt), despite the country being the fourth largest producer of tilapia in the world (CASTRO-SILVA *et al*., 2018). The Nile Tilapia (*Oreochromis niloticus*) is the most representative specie in Brazil, with an increase in production of 22.3% per year (MARTINS *et al*., 2018). However, the fish industry's growth generates waste on a large scale and it is estimated that up to 62.5% of the raw material used in tilapia filleting goes to waste, which reveals a major environmental problem as this can contribute to the pollution of water resources, soil and air (MARTINS *et al*., 2018).

On the other hand, the skin and scales could represent biomimetic components to the human body if properly collected and processed (CALDATO; NAVES; ZATTA, 2019; JEONG *et al*., 2019). The use of these natural by-products for the development of composite implants could be useful in the technological roadmap of new biomedical devices from the perspective of global sustainability and the circular economy, using waste recovery technologies to obtain high value-added products (CALDATO; NAVES; ZATTA, 2019; MARTINS *et al*., 2018).

Gelatin can be extracted from tilapia skin and it contains collagen type I (ALVES *et al*., 2018; OUYANG *et al*., 2018). Tilapia scales are a source of collagen and hydroxyapatite, with a high concentration of calcium and phosphate (CALDATO; NAVES; ZATTA, 2019; MARTINS *et al*., 2018). Such a combination, in the form of a tissue scaffold, may be a good biomaterial candidate for application in bone regeneration (JEONG *et al*., 2019; OUYANG *et al*., 2018).

Williams (1999) defined a biomaterial as one or more substances in combination, of natural or synthetic origin, used during any time, to partially or completely replace tissues, organs or bodily functions. There is a range of biomaterials with great variation in their composition, size, structure, origin and applications (ARAÚJO *et al*., 2020). The use of biomaterials in humans dates back centuries, but with the biotechnological advances since the second half of the twentieth century, there has been an acceleration in innovations and the development of new biocompatible and biodegradable products for clinical use, providing a greater quality of life (HARRIS; LU; GABRIELE, 2018).

Membranes and grafts for guided bone regeneration are promising alternatives capable of assisting in the morphofunctional recovery of injured tissue in orthopedics and oral surgeries (ARAÚJO *et al*., 2020). Materials of natural human origins (both autogenous or allogenous) have given way to xenogeneic ones due to the greater availability and commercialization, in addition to alloplastics as these dispense of a donor (ARAÚJO *et al*., 2021). Polymer-ceramic composite biomaterials have been increasingly studied, with emphasis on organic and inorganic combinations for bone tissue regeneration, favored by the biomimicry and synergism of the natural apatite and collagen of various animal species, such as cattle, pigs and poultry (JACOB *et al*., 2018).

Biomaterials used in bone regeneration should be able to provide appropriate mechanical support and present favorable surface properties for the regeneration of soft and hard tissue, with the modulation of the acceleration of biodegradation being a challenge for researchers (AL-MAAWI *et al*., 2017; ASGHARI *et al*., 2017). The biocompatibility of materials can be influenced by their intrinsic characteristics, by the interaction with the organism and by the implant site, and it is expected that there is no release of local or systemic toxic substances that elicit an exuberant inflammatory process, but that the repair *in situ* is favored instead (AL-MAAWI *et al*., 2017; ASGHARI *et al*., 2017). An innate and adaptive immune response is predicted after the implant of a biocompatible material, progressing with a decrease in the density of neutrophils, lymphocytes, macrophages and, later, of multinuclear giant cells, closely associated with the biodegradation of the implant (Al-MAAWI *et al*., 2017).

The objective of this study was to biologically characterize three biocomposites derived from Nile Tilapia, with different concentrations of gelatin and hydroxyapatite, evaluating their biocompatibility and biodegradability.

MATERIAL AND METHODS

Ethical-legal Aspects

This study was developed in compliance with the regulations of the Brazilian Ministry of Agriculture, Livestock and Supply and the Brazilian Management System of Genetic Heritage and Associated Traditional Knowledge (registration number A6BF297). The *in vivo* phase of this study was approved by The Ethics in Animal Use Committee of the Federal University of Ceará (CEUA UFC Sobral, registration number 01/19) and the 3R principles (reduction, refinement and replacement) were adopted to minimize the use of specimens, safeguarding animal welfare.

Fish Biocomposites

The natural raw material was derived from the skins and scales of fresh *Oreochromis niloticus* from a fish processing plant in Fortaleza, Ceará, Brazil. The

processing of the material, including the extraction of gelatin and apatite and the subsequent synthesis of hybrid membranes, was based on the protocols of Martins *et al*. (2018), Souza Filho *et al*. (2017) and Kongsri *et al*. (2013). Three different formulations or experimental groups were produced: 60% gelatin: 40% apatite (G1), 70% gelatin: 30% apatite (G2) and 80% gelatin:20% apatite (G3). All samples were crosslinked with 1% riboflavin and were subsequently lyophilized.

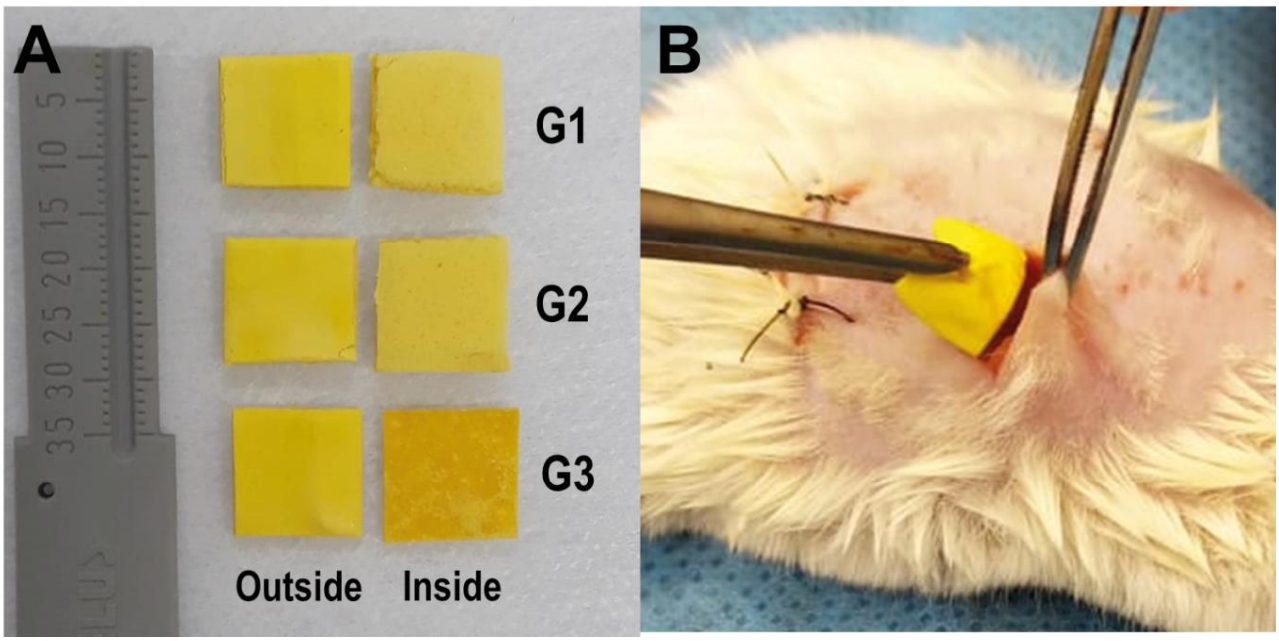
In Vivo Biological Characterization

The experimental design was based on ISO 10993-6. Thirty healthy animals *Mus musculus*, a laboratory lineage of outbred albino Swiss mice, adult males and weighing 30 g, were randomly divided into groups of 5 animals per experimental condition (1, 3 and 9 weeks). The animals were anesthetized intramuscularly with a 10% ketamine solution (Dopalen®, Sespo Indústria e Comércio Ltda, SP, Brazil) at a dose of 100 mg/kg and 2% xylazine (Anasedan®, Sespo Indústria e Comércio Ltda, SP, Brazil) at a dose of 12 mg/kg. Each animal had the trunk-dorsal region trichothomized with antisepsis being accomplished with 0.5% aqueous chlorhexidine. Linear incisions of 1 cm were performed, followed by tissue divulsions for the formation of subdermal pockets. Subcutaneous implants of test materials (G1, G2 and G3) standardized with an area of 10 mm^2 were performed in 15 animals, while the other 15 mice were used as the positive (C+: bovine collagen membrane LuminaCoat Double Time, Criteria Ltda®, SP, Brazil) and negative (C-: subdermal pouch without implant, filled with the surgery's own blood clot) control groups. At the end of each surgery, the operated regions underwent simple sutures with a mononylon thread (Figure 1).

All animals were monitored throughout the experimental period and did not present any local or systemic morbidity. The animals were euthanized by anesthetic overdose and an immediate excisional necropsy of the area compatible with each surgery was performed, fixed in 4% buffered formaldehyde solution (Allkimia, RJ, Brazil), pH 7.0, for 48 h. After fixation, the necropsies were cleaved, decalcified with a fast decalcifying acid solution (Allkimia, RJ, Brazil) for 12 h, washed in running water for 1 h, dehydrated in growing baths of ethanol 70% to 100% (Allkimia, RJ, Brazil), bathed in xylol (Allkimia, RJ, Brazil), impregnated and embedded in paraffin (Allkimia, RJ, Brazil). The paraffin-embedded samples were microtomized in cuts with 4µm thickness and stained in Hematoxylin-Eosin (HE) (Allkimia, RJ, Brazil).

A qualitative and quantitative analysis was performed for the characterization of biocompatibility and biodegradation. Histological slides were examined under the supervision of an experienced pathologist. Five images from each sample were captured in adjacent, non-overlapping fields on a Cybershot DSC-W300 Super Steady Shoot camera (Sony, Tokyo, Japan), coupled to a FWL-1000 optical microscope (Feldman Wild Leitz, AM, Brazil), using a 40x lens and 4x digital zoom, for a final magnification of 1600X. For the qualitative analysis,

Figure 1 - Three biocomposites derived from Nile Tilapia: G1 (60% gelatin: 40% hydroxyapatite), G2 (70% gelatin:30% hydroxyapatite) and G3 (80% gelatin: 20% hydroxyapatite) (**A**). Implantation of a sample in subcutaneous tissue of mice (**B**)



the slides of each experimental group were selected and described morphologically to represent the observed events. The quantitative analysis of the biocompatibility or irritation pattern employed the recommendations of ISO 10993-6, considering the presence of neutrophils, lymphocytes, macrophages and foreign body giant cells as inflammatory criteria, while the presence of neovascularization and connective tissue were considered as reparative criteria. The standard evaluates these criteria through presence scores defined as 0 (absent), 1 (rare), 2 (moderate), 3 (intense) or 4 (overcrowding), generating a numerical system capable of determining the irritation pattern. Three equations were used to arrive at the irritation pattern of each experimental condition (25 results, considering quintuplicates of animals and images of each test or control group): (1) inflammatory pattern equation (I_x); (2) repair pattern equation (R_x); (3) total irritation score equation.

$$I_x = 2\sum\left(Nt + L + M + FBGC\right) \quad (1)$$

Where I_x = inflammation pattern by group; x = test group (t) or control (c); Nt = mean neutrophils score; L = mean lymphocytes score; M = mean macrophages score; $FBGC$ = mean foreign body giant cells score.

$$R_x = \sum\left(N\upsilon + CT\right) \quad (2)$$

Where R_x = repair pattern by group; x = test group (t) or control (c) subindex; $N\upsilon$ = mean neovascularization score; CT = mean connective tissue score.

$$IP_x = \left(I_x + R_x\right) - \left(I_{c-} + R_{c-}\right) \quad (3)$$

Where IP_x = irritation pattern; x = test group (t) or positive control (c +) subindex; I_x = total inflammation score by group; R_x = total repair score by group; I_{c-} = total inflammation score in the negative control; R_{c-} = total repair score in the negative control. After the general calculations, the ISO 10993-6 standard adopts the negative control as the standard for the other groups, through subtraction. As such, its irritation pattern is scored as zero and the experimental conditions follow the patterns: non-irritating (0.0-2.9), slightly irritating (3.0-8.9), moderately irritating (9.0-15.0) or severely irritating (> 15); with the negative result being considered standard 0.

For analysis of the biodegradation in the photomicrographs obtained in each test and control group, a similar standard of scores based on ISO 10993-6 was developed to grade the integrity or presence of the biomaterial by quartiles, defined as 0 (absent), 1 (minimal: up to 25%), 2 (light: up to 50%), 3 (moderate: up to 75%) or 4 (predominant: above 75%).

For the statistical analysis, all raw data were tabulated in an Excel spreadsheet (Microsoft Office, USA) and then statistically analyzed with GraphPad InStat 3.10 (GraphPad Software, USA) for intergroup comparisons according to the described criteria and experimental times. The Kruskal-Wallis test and post-hoc Dunn test were applied to the data with non-normal distribution (non-parametric), with a significance level of 5% ($p < 0.05$).

RESULTS AND DISCUSSION

In Vivo Biological Analysis

The qualitative analysis of the biocompatibility and biodegradation after implant in groups G1, G2, G3, C+ and C- at 1, 3 and 9 weeks is represented in Figure 2.

At 1 week, G1, G2, G3 exhibited an intense mixed inflammatory infiltrate, with discrete neovascularization, rare presence of giant cells and moderate integrity of the implants, while C+ and C- showed slight presence of inflammation and loose connective tissue. After 3 weeks, G1, G2, G3 and C+ exhibited mild mixed inflammatory infiltrate, moderate presence of foreign body giant cells and slight reduction in the initial size

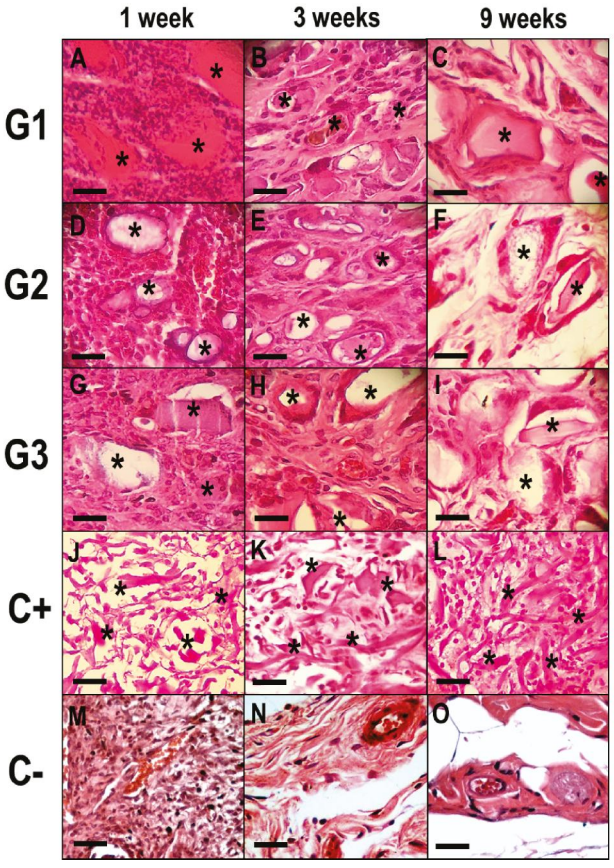


Figure 2 - Qualitative analysis of G1, G2, G3, C+ and C - in the subcutaneous tissue of mice at 1, 3, 9 weeks. *Implanted Material. Staining: Hematoxylin-Eosin, Magnification: 1600x. Scale bar: 20 µm

of the implants, while C- presented the discreet presence of neovascularization and dense connective tissue. At 9 weeks, there were no inflammatory cells, a slight presence of neovascularization and an increase in dense connective tissue in all groups. For G1, G2, G3 and C+, there was a persistence of foreign body giant cells and a continuous process of reducing the integrity of the initial implant.

The quantitative analysis of the biocompatibility after implant in groups G1, G2, G3, C+ and C- at 1, 3 and 9 weeks it is represented in Tables 1 and 2.

Table 1 reveals that all test groups showed progressive improvement in biocompatibility or a decrease in the irritation pattern, according to the progression of experimental time. Between 1 and 9 weeks, G1 ranged from moderately to slightly irritating, G2 remained constant as slightly irritating, and G3 ranged from slightly irritating to non-irritating, while C+ was non-irritating at the beginning and end of the experiment.

Table 2 showed significant differences ($p < 0.05$) between biocomposites and controls according to the inflammation and repair criteria. There was a greater presence of: neutrophils in G1 throughout the experimental period; lymphocytes in G3 at 1 week and C+ at 3 and 9 weeks; macrophages in G1, G2 and G3 at 1 week and C+ at 3 weeks; foreign body giant cells in G1 and G2 at 1 week and G1, G2 and G3 at 3 and 9 weeks; neovascularization in G3 at 1 week, G2 at 3 weeks and G1 and G2 at 9 weeks; and connective tissue in G1 and G2 at 1 week, G1, G2 and G3 at 3 weeks, and G1 and G2 at 9 weeks. C- had the lowest means for the inflammatory variables compared to the test and C+ groups, while for the repair variables, it exceeded the results of the C+.

Table 1 - Biocompatibility according to ISO 10993-6 of biocomposites (G1, G2 and G3) and controls (C+ and C-) in the subcutaneous tissue of mice during the experimental times (T) of 1, 3 and 9 weeks (mean±standard deviation of 25 fields per treatment)

Criteria		Experimental groups					
		T	G1	G2	G3	C+	C-
INFLAMMATION	Neutrophils	1s	2.96 ± 0.68	2.00 ± 0.76	2.36 ± 0.76	0	0.24 ± 0.44
		3s	0.84 ± 0.69	0.32 ± 0.56	0.08 ± 0.28	0.56 ± 1.00	0.08 ± 0.28
		9s	0.28 ± 0.46	0.08 ± 0.28	0.04 ± 0.20	0	0
	Lymphocytes	1s	1.76 ± 1.01	1.32 ± 0.80	1.88 ± 0.93	1.28 ± 0.46	0.72 ± 0.46
		3s	0.64 ± 0.49	0.40 ± 0.50	0.52 ± 0.51	2.04 ± 0.73	0.60 ± 0.50
		9s	0.36 ± 0.49	0.44 ± 0.58	0.44 ± 0.51	0.88 ± 0.33	0.44 ± 0.51
	Macrophages	1s	0.92 ± 0.49	0.88 ± 0.33	0.88 ± 0.33	0.04 ± 0.20	0.40 ± 0.58
		3s	0.20 ± 0.41	0.52 ± 0.51	0.32 ± 0.48	0.68 ± 0.48	0.40 ± 0.50
		9s	0.20 ± 0.41	0.32 ± 0.48	0.20 ± 0.41	0.12 ± 0.33	0.12 ± 0.33
	FBGC	1s	0.56 ± 0.92	0.64 ± 0.86	0.20 ± 0.50	0.28 ± 0.61	0.04 ± 0.20
		3s	2.00 ± 0.87	2.08 ± 0.70	1.80 ± 0.58	0.68 ± 0.63	0.04 ± 0.20
		9s	1.36 ± 0.76	1.28 ± 0.94	1.48 ± 0.92	0.24 ± 0.44	0.16 ± 0.37
REPAIR	Neovascularization	1s	1.24 ± 0.88	1.28 ± 0.61	1.56 ± 0.77	0.44 ± 0.51	1.00 ± 0.82
		3s	0.92 ± 0.49	1.36 ± 0.57	1.20 ± 0.41	0.84 ± 0.80	1.16 ± 0.47
		9s	1.12 ± 0.67	1.00 ± 0.50	0.64 ± 0.57	0.68 ± 0.95	1.00 ± 0.65
	Connective tissue	1s	1.16 ± 0.37	1.12 ± 0.44	0.76 ± 0.60	0.72 ± 0.46	1.64 ± 0.49
		3s	3.04 ± 0.35	2.36 ± 0.91	1.80 ± 0.82	0.92 ± 0.49	1.28 ± 0.46
		9s	2.88 ± 0.78	2.76 ± 0.97	1.96 ± 1.10	1.40 ± 0.82	1.88 ± 0.67
Irritation pattern		1s	9.12 (MI)	6.64 (SI)	7.52 (SI)	0.00 (NI)	-
		3s	6.64 (SI)	5.68 (SI)	3.76 (SI)	5.00 (SI)	-
		9s	4.08 (SI)	3.68 (SI)	2.60 (NI)	0.24 (NI)	-

Test groups: **G1** (60% gelatin: 40% apatite), **G2** (70% gelatin: 30% apatite) and **G3** (80% gelatin: 20% apatite). Positive control: commercial collagen membrane (**C+**). Negative control: subdermal pouch without implant (**C-**). *Scores*: non-irritating (**NI**), slightly irritating (**SI**) or moderately irritating (**MI**). **FBGC:** Foreign body giant cells

Table 2 - Statistical analysis of the biocompatibility criteria of biocomposites (G1, G2 and G3) and controls (C+ and C-) in the subcutaneous tissue of mice during the experimental times (T) of 1, 3 and 9 weeks (s)

Criteria		Experimental groups and p value*						
		T	G1	G2	G3	C+	C-	ρ
INFLAMMATION	Neutrophils	1s	G2, G3, C-	C-	G1, C-	-	G1, G2, G3	G1
		3s	G2, G3, C+, C-	G1	G1, C+	G1, G3, C-	G1, C+	G2, G3, C-
		9s	G2, G3, C+, C-	G1	G1	G1	G1	G2, G3, C+, C-
	Lymphocytes	1s	C-	G3, C-	G2, C+, C-	G3	G1, G2, G3	G2, G3, C+, C-
		3s	C+	C+	C+	G1, G2, G3, C-	C+	C-
		9s	C+	C+	C+	G1, G2, G3, C-	C+	C+
	Macrophages	1s	C+, C-	C+, C-	C+, C-	G1, G2, G3	G1, G2, G3	C+
		3s	G2, C+	G1	C+	G1, G3, C-	C+	C+, C-
		9s	-	-	-	-	-	G2, C+
	FBGC	1s	C-	G3, C-	G2	-	G1, G2	-
		3s	C+, C-	C+, C-	C+, C-	G1, G2, G3, C-	G1, G2, G3, C+	C-
		9s	C+, C-	C+, C-	C+, C-	G1, G2, G3	G1, G2, G3	C+, C-
REPAIR	Neovascularization	1s	C+	C+	C+, C-	G1, G2, G3, C-	G3, C+	C+, C-
		3s	G2	G1, C+	C+	G2, G3, C-	C+	C+
		9s	G3, C+	G3, C+	G1, G2	G1, G2, C-	C+	G2
	Connective tissue	1s	G3, C+, C-	G3, C+, C-	G1, G2, C-	G1, G2, C-	G1, G2, G3, C+	G3, C+
		3s	G2, G3, C+, C-	G1, G3, C+, C-	G1, G2, C+	G1, G2, G3	G1, G2	G3, C+, C-
		9s	G3, C+, C-	G3, C+, C-	G1, G2	G1, G2	G1, G2	G2, G3, C+, C-

Test groups: **G1** (60% gelatin: 40% apatite), **G2** (70% gelatin: 30% apatite) and **G3** (80% gelatin: 20% apatite). Positive control: commercial collagen membrane (**C+**). Negative control: subdermal pouch without implant (**C-**). **FBGC:** Foreign body giant cells. *Statistically significant values between test and control groups per experimental period through the Kruskal-Wallis test and post-hoc Dunn test

The general findings for biocompatibility according to the ISO 10993-6 standard of this study using materials of fish origin are similar to Souza *et al*. (2021) who tested poultry implants and demonstrated a higher irritation pattern in the bioapatite and nanokeratin group (slightly irritating) over 9 weeks compared to the chicken collagen (non-irritating). This could explain the improvement in biocompatibility or the decreasing irritation pattern observed in G1, G2 and G3 at 9 weeks associated with the bioceramic concentration of the groups.

Intense and mixed inflammatory infiltrate at 1 week for all materials, with the exuberant presence of polymorphonuclear cells and lymphocytes, was also reported by Jardelino *et al*. (2012) when they tested the biocompatibility of an alginate-capsule alloplastic membrane. Muñoz, Cardona-Ramirez and Silva (2019) reported an initial increase in polymorphonuclear cells for a bovine hydroxyapatite-zinc xenograft. Based on these findings, the expected presence of these cells is confirmed, which appear in the first days after the surgical implant and tend to gradually decrease in 3 weeks until practically disappearing in 9 weeks (FRANZ *et al*., 2011).

The presence of multinucleated giant cells was generally moderate and, later, at 3 weeks, expressive in the tested composites. The expression of this cell profile varies, with observations in 2 weeks by Salgado, Teixeira and Monteiro (2019) when testing nano-hydroxyapatite and collagen biocomposites, or in 30 days by Pereira *et al.* (2019), when evaluating an alloplastic hydroxyapatite implant. According to Herrera-Vizcaíno *et al*. (2020), giant cells are formed through the fusion of macrophages, stimulated by cross-linked collagenous biomaterials and local body fluids. Al-Maawi *et al.* (2018), on the other hand, reported that giant cells are more common in synthetic materials, with the physicochemical properties of polymers, inducing cellular reactions related to the resorption of biomaterials.

The discrete neovascularization and the gradual growth of connective tissue were similar to the findings of Sena *et al.* (2014) for an alloplastic composite at the three experimental times. Salgado, Teixeira and Monteiro (2019) obtained a gradual increase in neovascularization and connective tissue at 1, 2 and 4 weeks, both favored

by the infiltration of endothelial cells and fibroblasts through the porous structure of the employed scaffolding. Giant cells surrounding the biomaterial express vascular endothelial growth factor, which also indirectly stimulates neovascularization (AL-MAAWI *et al*., 2018; HERRERA-VIZCAÍNO *et al*., 2020).

The quantitative analysis of biodegradation after implant in groups G1, G2, G3, C+ and C- at 1, 3 and 9 weeks is represented by Tables 3 and 4.

The data presented in Tables 3 and 4 showed significant differences ($p < 0.05$) between biocomposites and controls regarding the integrity of each material. All test groups showed progressive partial degradation, characterized by a decrease in the integrity of the implanted material, according to the progression of experimental time. Between 1 and 9 weeks, G1 maintained a higher mean integrity than G3 and G2, respectively, despite showing a lower performance than that found in the C+. For the controls, C+ remained the most stable among all tested conditions, while C- did not present an implant, which justifies its absence.

Thus, the test materials showed a good rate of biodegradation balanced with the invasion of inflammatory cells and repair tissue, similar to the findings of Souza *et al*. (2021), where the bioceramic load could explain the higher stability of tested implants compared to the rapid degradation of biopolymers.

Commercial membranes of bovine, porcine or equine xenogeneic collagen have a minimum integrity time of 30 days (ARAÚJO *et al*., 2021). This reinforces the candidacy of innovative fish biocomposites within the new generation of resorbable membranes for guided bone regeneration (GBR), which can already count on collagenous biopolymers with enzymatic degradation, with rare reports of apatite composites, reabsorbed by multinucleated foreign body giant cells (DANIELETTO-ZANNA *et al*., 2020; JUNG *et al*., 2020).

Gelatin has the ability to integrate well into any internal environment, whether animal or human, reducing chronic inflammation (OGAWA *et al*., 2017). In the GBR of the calvaria of rabbits using tuna skin gelatin, Jung *et al.* (2020) observed the preservation of the material's mechanical properties and inhibition of soft tissue invasion in the area for up to 4 weeks of the experiment. Sbricoli *et al*. (2020) further reinforce the biomimicry of collagen membranes with the native type I molecule in periodontal connective tissue itself. Sancilio *et al.* (2018) stated that collagen gelatin in combination with hydroxyapatite assists in the formation of calcium and phosphate, important components for bone

Table 3 - Biodegradation of biocomposites (G1, G2 and G3) and controls (C+ and C-) in the subcutaneous tissue of mice during the experimental times (T) of 1, 3 and 9 weeks (s) (mean±standard deviation of 25 fields per treatment)

Criterion	Experimental groups					
	T	G1	G2	G3	C+	C-
Integrity	1s	2.20 ± 0.87	1.28 ± 0.54	2.16 ± 1.11	3.96 ± 0.20	0
	3s	2.04 ± 0.79	1.36 ± 0.49	1.76 ± 0.72	3.88 ± 0.33	0
	9s	1.32 ± 0.48	0.84 ± 0.47	1.20 ± 0.65	2.48 ± 1.81	0

Test groups: **G1** (60% gelatin: 40% apatite), **G2** (70% gelatin: 30% apatite) and **G3** (80% gelatin: 20% apatite). Positive control: commercial collagen membrane (**C+**). Negative control: subdermal pouch without implant (**C-**)

Table 4 - Statistical analysis of the biodegradation of biocomposites (G1, G2 and G3) and controls (C+ and C-) in the subcutaneous tissue of mice during the experimental times (T) of 1, 3 and 9 weeks (s)

Criterion	Experimental groups and p value*						
	T	G1	G2	G3	C+	C-	p
Integrity	1s	G2, C+	G1, G3, C+	G2, C+	G1, G2, G3	-	4,807xE^-13
	3s	G2, C+	G1, C+	C+	G1, G2, G3	-	1,234xE^-13
	9s	G2, C-	G1, C+, C-	C-	G2, C-	G1, G2, G3, C+	1,466xE^-11

Test groups: **G1** (60% gelatin: 40% apatite), **G2** (70% gelatin: 30% apatite) and **G3** (80% gelatin: 20% apatite). Positive control: commercial collagen membrane (**C+**). Negative control: subdermal pouch without implant (**C-**). *Statistically significant values between test and control groups per experimental period through the Kruskal-Wallis test and post-hoc Dunn test

mineralization. Salgado, Teixeira and Monteiro (2019) pointed out that hydroxyapatite crystals in subcutaneous tissue are degraded more slowly and induce a small chronic inflammation, but that they would be ideal for bone filling, since they retain their stability for longer.

Jardelino *et al*. (2010) reported that biodegradation is a desirable feature in biomaterials for dental application to avoid a second surgical procedure, although a six-week integrity is recommended to ensure the regeneration of lost bone. Based on the time variables found in the literature, negatively modulating the speed of biodegradability of a biomaterial with high organic content remains a big challenge (JARDELINO *et al*., 2010, 2012).

Wang *et al*. (2017a) observed total degradation in 28 days for a chicken keratin hydrogel, while Jardelino *et al.* (2010) demonstrated partial degradation at 3 weeks and total degradation at 9 weeks for a porcine collagen membrane. Despite this, the studies of Wang *et al*. (2017b) proved that crosslinking in collagenous materials derived from freshwater fish improves their resistance to degradation, transforming their linear chains into three-dimensional polymers with a high molar mass and greater thermal stability.

For the reasons presented, the natural crosslinked composites of gelatin and apatite tested show potential for development as GBR membranes. Given the limitation of the ectopic site used in this preliminary preclinical test, future biological studies, including orthotopic tests, are recommended to evaluate the osteopromoting potential of these membranes (DANIELETTO-ZANNA *et al*., 2020).

CONCLUSIONS

All tested gelatin and apatite composites derived from Nile tilapia were biocompatible and partially biodegradable. G1 suggests greater potential for use as an osteopromoting membrane, with its biological performance associated with higher mineral concentration compared to the organic phase in the biocomposite.

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REFERENCES

AL-MAAWI, S. *et al*. In vivo cellular reactions to different biomaterials-Physiological and pathological aspects and their consequences. **Seminars in Immunology**, v. 29, n. 1, p. 49-61, 2017.

AL-MAAWI, S. *et al*. In vivo implantation of a bovine-derived collagen membrane leads to changes in the physiological cellular pattern of wound healing by the induction of multinucleated giant cells: an adverse reaction? **Frontiers in Bioengineeering and Biotechnology**, v. 6, n. 104, p. 1-13, 2018.

ALVES, A. P. N. N. *et al*. Study of tensiometric properties, microbiological and collagen content in nile tilapia skin submitted to diferente sterilization methods. **Cell and Tissue Banking**, v. 19, n. 1, p. 373-382, 2018.

ARAÚJO, L. K. *et al.* Brazilian dentists' perceptions of using bone grafts: an inland survey. **Acta Odontológica Latinoamericana**, v. 33, n. 3, p. 165-173, 2020.

ARAÚJO, L. K. *et al*. Membranas osteopromotoras em odontologia: tendências científicas e análise do mercado brasileiro. *In*: FADEL, C. B.; MARTINS, A. S.; PINHEIRO, J. C. **Odontologia**: pesquisa e práticas contemporâneas. São Paulo: Editora Científica, 2021. cap. 9, p. 103-120.

ASGHARI, F. *et al*. Biodegradable and biocompatible polymers for tissue engineering application: a review. **Artificial Cells, Nanomedicine, and Biotechnology**, v. 45, n. 2, p. 185-192, 2017.

CALDATO, K.; NAVES, F. K. S.; ZATTA, L. Gelatina extraída de escamas da tilápia do Nilo (*Oreochromis niloticus*) produzidas no município de Pato Branco: caracterizações e comparações com amostras comerciais. **Revista Brasileira de Tecnologia Agroindustrial**, v. 13, n. 1, p. 2730-2751, 2019.

CASTRO-SILVA, I. I. *et al.* Biotechnological potential of by-products of the Brazilian animal protein industry in the generation of xenogeneic biomaterials for bone regeneration. **Trends in Research**, v. 1, n. 3, p. 1-2, 2018.

DANIELETTO-ZANNA, C. F. *et al*. Osteopromotion capacity of bovine cortical membranes in critical defects of rat calvaria: histological and immunohistochemical analysis. **International Journal of Biomaterials**, v. 2020, n. 1, p. 1-9, 2020.

FRANZ, S. *et al.* Immune responses to implants: a review of the implications for the design of immunomodulatory biomaterials. **Biomaterials**, v. 32, n. 28, p. 6692-6709, 2011.

HARRIS, J. J.; LU, S.; GABRIELE, P. Commercial challenges in developing biomaterials for medical device development. **Polymer International**, v. 67, n. 1, p. 969-974, 2018.

HERRERA-VIZCAÍNO, C. *et al*. Modification of collagen-based sponges can induce an upshift of the early inflammatory response and a chronic inflammatory reaction led by M1 macrophages: an in vivo study. **Clinical Oral Investigation**, v. 24, n. 10, p. 3485-3500, 2020.

JACOB, J. *et al.* Piezoelectric smart biomaterials for bone and cartilage tissue engineering. **Inflammation and Regeneration**, v. 38, n. 2, p. 2-11, 2018.

JARDELINO, C. *et al*. Biocompatibility analysis of a novel reabsorbable alloplastic membrane composed of alginate-capsul. **Revista Gaúcha de Odontologia**, v. 60, n. 4, p. 419-423, 2012.

JARDELINO, C. *et al*. Porcine peritoneum as source of biocompatible collagen in mice. **Acta Cirúrgica Brasileira**, v. 25, n. 4, p. 332-336, 2010.

JEONG, J. *et al*. Bioactive calcium phosphate materials and applications in bone regeneration. **Biomaterials Research**, v. 23, n. 4, p. 1-11, 2019.

JUNG, S. *et al*. Effect of gellan gum/tuna skin film in guided bone regeneration in artificial bone defect in rabbit calvaria. **Materials**, v. 13, n. 6, p. 1-9, 2020.

KONGSRI, S. *et al*. Nanocrystalline hydroxyapatite from fish scale waste: preparation, characterization and application for selenium adsorption in aqueous solution. **Chemical Engineering Journal**, v. 215/216, p. 522-532, 2013.

MARTINS, M. E. O. *et al*. Thermal and chemical properties of gelatin from tilapia (Oreochromis niloticus) scale. **Journal of Aquatic Food Product Technology**, v. 27, n. 10, p. 1120-1133, 2018.

MUÑOZ, L. C.; CARDONA-RAMIREZ, S.; SILVA, R. F. Comparison of subcutaneous inflammatory response to commercial and engineered zinc hydroxyapatite implants in rabbits. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 71, n. 6, p. 1873-1879, 2019.

OGAWA, Y. *et al*. Preparation and biocompatibility of a chitin nanofiber/gelatin composite film. **International Journal of Biological Macromolecules**, v. 104, n. 1, p. 1882-1889, 2017.

OUYANG, Q. Q. *et al.* Chitosan hydrogel in combination with marine peptides from tilapia for burns healing. **International Journal of Biological Macromolecules**, v. 112, n. 1, p. 1191-1198, 2018.

PEREIRA, L. C. *et al*. In vitro physico-chemical characterization and standardized in vivo evaluation of biocompatibility of a new synthetic membrane for guided bone regeneration. **Materials**, v. 12, n. 1186, p. 1-12, 2019.

SALGADO, C. L.; TEIXEIRA, B. I. B.; MONTEIRO, F. J. M. Biomimetic composite scaffold with phosphoserine signaling for bone tissue engineering application. **Frontiers in Bioengineeering and Biotechnology**, v. 7, n. 206, p. 1-16, 2019.

SANCILIO, S. *et al*. Alginate/hydroxyapatite-based nanocomposite scaffolds for bone tissue engineering improve dental pulp biomineralization and differentiation. **Stem Cells International**, v. 2018, n. 1, p. 1-13, 2018.

SBRICOLI, L. *et al*. Selection of collagen membranes for bone regeneration: a literature review. **Materials**, v. 13, n. 3, p. 1-16, 2020.

SENA, L. A. *et al*. Biocompatibility of wollastonite-poly(N-butyl-2-cyanoacrylate) composites. **Journal of Biomedical Material Research B: Applied Biomaterials**, v. 102, n. 6, p. 1121-1129, 2014.

SOUZA FILHO, M. S. M. *et al*. **Obtenção e caracterização de gelatina de pele de tilápia-do-nilo (Oreochromis niloticus) em escala piloto**. Fortaleza: Embrapa Agroindústria Tropical, 2017. 19 p.

SOUZA, F. F. P. *et al.* Poultry by-products as source of collagen, nanokeratin and bioapatite for biomedical use. **Revista Ciência Agronômica**, v. 52, n. 4, e20207565, 2021.

WANG, J. *et al.* Feather keratin hydrogel for wound repair: preparation, healing effect and biocompatibility evaluation. **Colloids and Surfaces B: Biointerfaces**, v. 149, n. 1, p. 341-350, 2017a.

WANG, J. K. *et al*. Fish scale-derived collagen patch promotes growth of blood and lymphatic vessels *in vivo.* **Acta Biomaterialia**, v. 63, n. 1, p. 246-260, 2017b.

WILLIAMS, D. F. **The Williams dictionary of biomaterials**. Liverpool: Liverpool University Press, 1999. 42 p.