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Influence of Hydrogen Peroxide on Composition, Thermostability, Porosity and Swelling of Collagen Matrices of Demineralized Porcine Cortical Bone

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HIGHLIGHTS

- Demineralized porcine bone is an alternative for generating scaffolds for tissue regeneration.
- Increasing H_2O_2 concentration directly modulated physicochemical changes in cortical bone.
- The single treatment with H_2O_2 was more structurally conservative than the double treatment.
- Future biocompatibility, reabsorption and osteopromotion in vivo studies should be carried out.

Abstract: Porcine source attracts the biotechnological development of natural xenogeneic materials, due to the availability of collagen in soft tissues, but the use of the demineralized organic matrix of cortical bone remains little explored. This study developed a demineralized porcine cortical bone matrix and analyzed the interference of peroxidation on the composition, thermostability, porosity and swelling of the material. Fresh ribs were processed with 3%-30% hydrogen peroxide under single treatment (G1-G4) or double treatment (G5-G8), using native bone (NB) as a control. Physicochemical characterization, porosity histomorphometry and swelling evaluation were performed. There were infrared bands indicative of the prevalence of collagen and residual phosphate groups even after demineralization, mass loss events by thermogravimetry ranging from 94 to 590 °C, being more accelerated in G7 and G8, with all groups below the thermal stability of the NB, as well as similar endothermic events between treatments in differential calorimetry. Changes in lamellar bone morphology occurred in all tested groups, being more conservative in G1 and G2 and more aggressive in G7 and G8. The single treatment dilated pores and kept the matrix closer to the original, while the double treatment significantly enlarged and fused the bone canals mimicking a mesh. The permeable areas in the bone matrix were larger and the hydrophilicity was greater and more persistent for G5-G8 compared to G1-

G4 and NB. Single exposure and at lower concentrations of hydrogen peroxide suggest a better modulation of the tested determinants, for the generation of collagen biomaterial applied to bone regeneration.

Keywords: Cortical Bone; Bone Matrix; Hydrogen Peroxid; Biocompatible Materials; Guided Tissue Regeneration.

INTRODUCTION

The multidisciplinary science of dental materials seeks cost-effective and safe options for the development of products applicable to guided bone regeneration (GBR) [1, 2, 3, 4, 5]. In the last four decades, Brazil has increased interest in research with composite or ceramic bone grafts compared to biodegradable polymeric barrier membranes, preponderating the level of preclinical evidence [2]. The dental market corroborates the prevalence of greater availability and use of bone grafts than membranes, both from xenogeneic sources and foreign origin, identifying that the financial factor can be a barrier to popularization among patients [3, 4]. From the clinical point of view, dentists prefer autogenous or xenogeneic biomaterials over alloplastic and allogeneic ones, due to operational costs or the lower acceptability of the human cadaveric source, respectively [4]. Such findings indicate the unexplored potential of the Brazilian industry in the innovation of implantable and accessible devices for GBR, especially xenogeneic membranes [3, 4].

Soft tissues from porcine sources, such as skin, intestine or pericardium, are already well used for the elaboration of regenerative membranes, due to their abundance of collagens I and III whose biomimicry minimizes the inflammatory response and favors neovascularization when implanted in vivo [5]. About a decade ago, it appeared on the market a new membrane made of collagenized porcine bone called cortical lamina, which proved to be reliable and easy to handle for horizontal and vertical defects [6]. Porcine bone can be considered an interpenetrating composite, given its mineral and organic composition and its corticomedullary arrangement [7]. The pig's bone density is like that of humans, despite its biochemical constitution having less collagen and more other extractable proteins, also surpassing other animals such as dog, cattle, sheep, chicken and rat [8]. Similar to the human model, mature porcine femurs consist of plexiform bone with dense bone organized in a Haversian system, as well as goat, sheep, cattle and horses [9]. However, the roundness of osteons in humans is closer to that of the pig compared to the deer or dog [10]. In particular, the pork rib body has a more flattened shape and a more cortical histological constitution, with a minimal intermediate trabecular portion [11]. Flat bones are characterized by less thickness, about 5.16 mm for the human parietal bone with the external and internal compact layers [12], when compared to the cortical thickness of long bones (man: 4.90 mm and animals: 3.35 mm) [13]. Interestingly, pigs, sheep and kangaroos have body dimensions closer to humans and greater similarity in cortical bone thickness compared to dogs, with less thickness, or cattle, with greater thickness [14]. Such a laminated design would be interesting for generating a membrane. Therefore, demineralized cortical bone indicates a little explored perspective, but very attractive for the development of a xenogeneic biomaterial with the hypothesis of generating a membrane for GBR.

Good processing reduces the risk of xenozoonosis, with a greater association of potential morbidities derived from bacterial agents in untreated pork products [15] than from prion disease, more associated with products of bovine origin [16]. Chemical modifications in cortical bone can alter the behavior of Haversian and Volkmann vascular channels interconnected by a lacunar-canalicular system with osteocytes, their filipodial extensions and surrounding pericellular matrix, increasing hydraulic permeability, influencing the transport of nutrients, metabolites and signaling molecules (ATP, PGE2 and NO) and on the metabolic functions of osteocytes and osteoblasts [17, 18]. Increased porosity in cortical bone may occur naturally with age in humans and rodents, as an effect of hormonally mediated osteoclastic remodeling action [19]. Hydrogen peroxide is a chemical agent that modifies bone, whose oxidative stress generates negative effects on bone mineral density and stiffness [20]. For the manufacturing proposal, peroxidation can remove debris in hard tissues, and as a result of unobstructing the canalicular systems, modulate the increase in porosities in the native bone and achieve a desirable biodegradability of the final product [21, 22]. The literature varies in isolated protocols involving hydrogen peroxide, from 3% [21] to 30% [20, 22, 23, 24], with a persisting lack of knowledge about a possible optimal concentration that preserves the organic matrix, producing a prototype of natural membrane for GBR. Valuing the dynamic importance of the extracellular matrix in bone regeneration, with collagen and non-collagen proteins regulating cell adhesion, proliferation, responses to growth factors, bone differentiation and maturation [25, 26], the aim of this pilot study was to develop a demineralized matrix of porcine cortical bone and analyze the interference of the degree of peroxidation on composition, thermostability, porosity and swelling of the material.

MATERIAL AND METHODS

Laboratory development of matrices

The handling of animal samples, obtained from parts of cadavers after slaughter for food purposes, with a traceable origin from a commercial establishment, complies with current bioethical regulations. In accordance with Brazilian legislation on non-experimental interventions related to agricultural practices, there was no need for institutional assessment by the Ethics Committee on Animal Use [27, 28].

Fresh refrigerated pork ribs (*Sus scrofa domesticus*, Landrace breed), intended for human consumption, were obtained from a local market in the city of Sobral, CE, Brazil (3° 40' 58'' South, 40° 21' 4'' West). All ribs were carefully cleaned of meat residues, washed abundantly in running water and kept in a freezer at 4°C until the moment of material processing. One rib was reserved for each experimental protocol, as well as for the control group or native bone (NB).

The experimental protocol was adapted from Kamadjaja and coauthors [21] and Zhang and coauthors [24]. The groups were delineated following single or double treatment protocols with hydrogen peroxide at different concentrations, before or after the demineralization step.

The single treatment consisted of 3 hours of immersion in hydrogen peroxide (Millipore Sigma, USA) at concentrations of 3% (G1), 10% (G2), 20% (G3) or 30% (G4), with increasing whitening activity in the bone according to the concentration. After this exposure, there was washing in distilled water until the pH was neutral, followed by demineralization in a 0.45% hydrochloric acid solution (Allkimia, Brazil) for 14 days, with changes every 3 days. The double treatment consisted of immersion in hydrogen peroxide for 3 hours before and 3 hours after washing and demineralization, maintaining the same initial and final concentrations of exposure, 3% (G5), 10% (G6), 20% (G7) or 30% (G8), which promoted a more pronounced bubbling effect in all dual treatment groups than single treatment. The sample from the control group (NB), in order to preserve the native bone structure, did not undergo a single or double treatment, only followed by washing in distilled water and demineralization in the acid solution as previously described.

All pieces were washed in distilled water until the pH was neutral, manually cleaved with a histological razor in dimensions of 10 mm × 10 mm × 1 mm to select only the cortical bone portion, dried in an electric dehydrator (Bentec, Brazil) at 50 °C for 3h and sterilized in UV Box Pro equipment (ELG Health Tech., Brazil) for 15min in ultraviolet light, with UV-A for antibacterial action and UV-C for antiviral action, remaining stored in 15mL polypropylene tubes (Kasvi, Brazil) sealed with Parafilm M (Pechiney, France) at room temperature until analysis. From the customized samples for each group, three went on to the physical-chemical characterization steps, five for microscopic analysis while five were destined for the swelling test.

Figure 1 summarizes the stages of laboratory development of the porcine cortical matrix previously described.

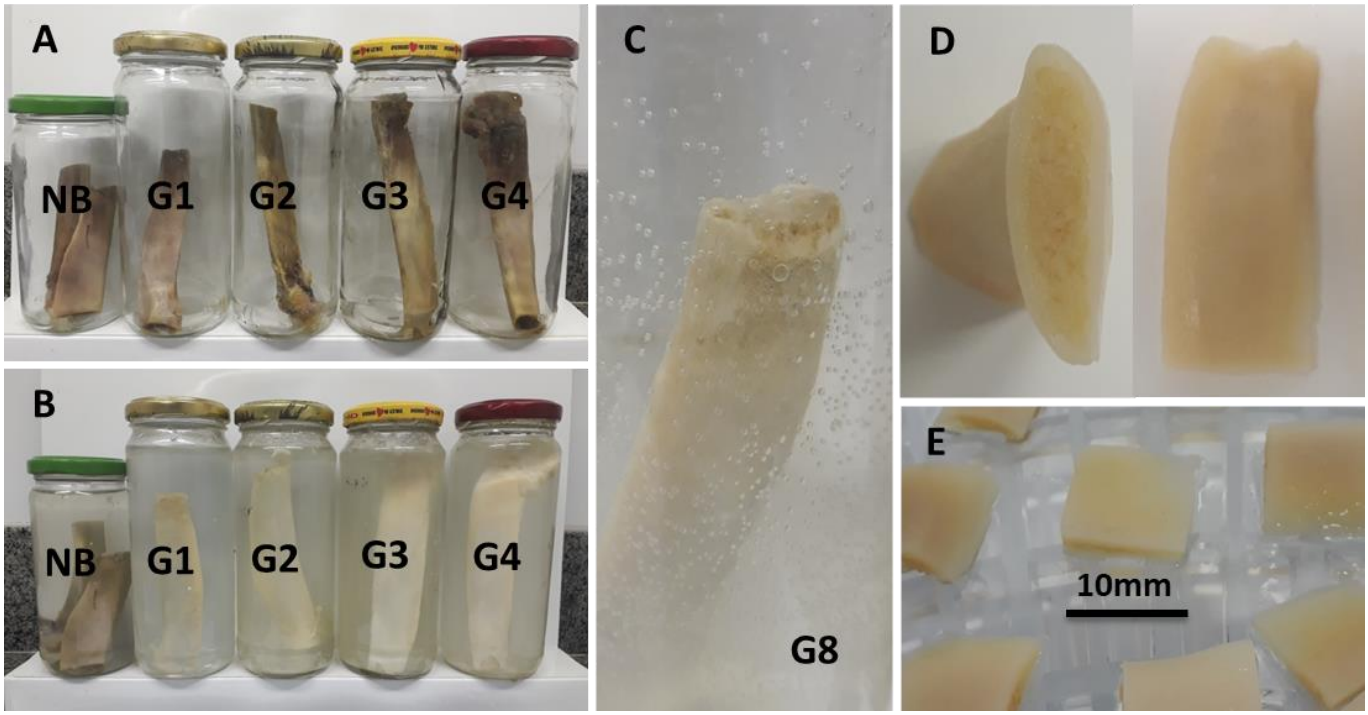

Figure 1. Development of the porcine cortical matrix. A: Clean pork ribs. B: Whitening with a single treatment. C: Bubbling in the double treatment. D: Cleavage after demineralization. E: Drying of customized samples.

Physical-chemical characterization

Samples were characterized using Fourier Transform Infrared Spectroscopy (FTIR) in FTLA 2000-102 equipment (ABB-BOMEM, Canada) in attenuated total reflectance (ATR) mode in the range from 500–4000 cm^{-1}, Thermogravimetric Analysis (TGA) and derivative thermogravimetry (DTG) in a STA 6000/8000 equipment (PerkinElmer, USA) with a 50 mL/min synthetic air atmosphere, at 25–800 °C and a heating rate of 10 °C/min and Differential Scanning Calorimetry (DSC) in Q20 V24.9 Build 121 equipment (TA Instruments, USA) in an atmosphere of nitrogen gas 50 mL/min at 25–250 °C and heating rate of 10 °C/min.

Histological processing and histomorphometric analysis

All samples were fixed in 10% buffered formalin for 48 hours and followed by conventional histological processing, including dehydration in progressive concentrations of 70-100% ethyl alcohol (Allkimia, Brazil), diaphanization in xylene baths (Allkimia, Brazil), impregnation and inclusion in paraffin (Allkimia, Brazil) in the longitudinal direction. The histological blocks were microtomized in a Leica RM 2125 RTS equipment (Leica, Germany) and a Leica 818-High profile razor (Leica, Germany) using 5 µm in thickness. The sample sections were colored by the Hematoxylin-Eosin kit (Allkimia, Brazil).

Four images of each sample, distributed in quintuplicate for each of the nine groups analyzed, totaled 180 images that were captured in adjacent, non-overlapping fields using a Cybershot DSC-W300 Super Steady Shoot digital camera (Sony, Japan) coupled to a Nikon optical microscope Eclipse E200 (Nikon, Japan) using 10x eyepiece lens, 10x objective lens and 3x digital zoom, achieving a final magnification of 300x. For qualitative analysis, representative images of each experimental and control group were selected and morphologically described.

Histomorphometric analysis was performed using Image J software version 1.52a (National Institutes of Health, USA), calibrated in micrometers/pixel Initially, a microscopic image with a millimeter ruler was opened in the Image J software for measuring calibration using the “Straight” tool to select the 1mm interval, followed by the options “Analyze - Set scale - know distance” to assign the record of 1mm and “Global” for standardizing the measurement of subsequent images. For each image followed, a sequence was followed: color standardization in the option: “Image - Type - 8bit”, segmentation of the binarized image to delimit the analysis regions in the option “Process - Subtract background”, adopting 50 pixels for NB, G1–G3 and 1000 pixels for G4–G8, unchecking the “Light background” option and selecting the “Image - Adjust - Threshold - Default: Black & White - Apply” option followed by “Process - Binary - Fill Holes”, which transformed the physical structure of the native bone or biomaterial for white color and their nutrient channels, porosities or gaps for black color. After these adjustments, it was possible to automatically measure the delimited areas using the “Analyze - Measure” tool. The database of images for each group was graphically presented using Prism 7.0 software (GraphPad, USA).

Data were tabulated in Excel software (Microsoft Office, USA), expressed graphically as mean±standard deviation and analyzed statistically using the Prism 7.0 software (GraphPad, USA) for comparisons of groups. Analysis of variance (ANOVA) with Tukey’s post-test was applied to analyze the normal/parametric distribution of the means of each parameter between the eight experimental groups and the control. They were considered 95% confidence level and significant differences if $p < 0.05$.

Swelling degree

For the swelling degree analysis, quadrangular samples were weighed at time 0 (dry weight) and then immersed in distilled water, at pH 7.1 and temperature of 25 °C and weighed during 14-time intervals up to 60 minutes (1, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 minutes). Then, the membranes were removed from the water and excess water was removed using filter paper and weighed at the defined times. The swelling degree was expressed as the percentage of weight gain compared to dry weight, according to Eq. (1):

$$D_s = \left(\frac{W_s - W_d}{W_d}\right) 100\% \quad (1)$$

where Ds is the degree of swelling, Ws is the wet sample weight and Wd is the dry sample weight. All measurements were performed in quintuplicate.

Data were tabulated in Excel software (Microsoft Office, USA), expressed graphically as mean±standard deviation and analyzed descriptively using the Prism 7.0 software (GraphPad, USA) for comparisons of groups.

RESULTS

FTIR analyzes demonstrated a homogeneous pattern of bands in all tested groups, regardless of the H_2O_2 treatment protocols (G1–G8). The bands stand out: 3290 cm^{-1} and 2918 cm^{-1} corresponding to symmetric bonds of N-H and C-H, respectively, of amide groups A and B common in secondary structures of the α-helix type of proteins; 1628 cm^{-1} corresponding to the stretching of C=O bonds of carbonyl groups of amide I; 1548 cm^{-1} and 1238 cm^{-1} corresponding to the stretching of N-H/CH_3 and N=C bonds, respectively, present in type II and III amides; 1080 cm^{-1} corresponding to the stretching of C-OH bonds and 600 cm^{-1} indicating the presence of phosphate groups, with a slightly more expressive peak in NB than in the other groups. Additionally, the NB group showed bands that indicate a more preserved original protein load of the bone not treated with H_2O_2 in 1740 cm^{-1} referring to the stretching of C=O bonds of carboxylic amide groups I, and 1158 cm^{-1}, referring to the stretching of C-N bonds of amine groups, both chemical groups being characteristic of protein structures. Such spectra point to a more expressive presence of organic collagen content, present in groups independent of single or double treatment or H_2O_2 concentration, and a lower presence of phosphate mineral content, residual even after the demineralization process (Figure 2).

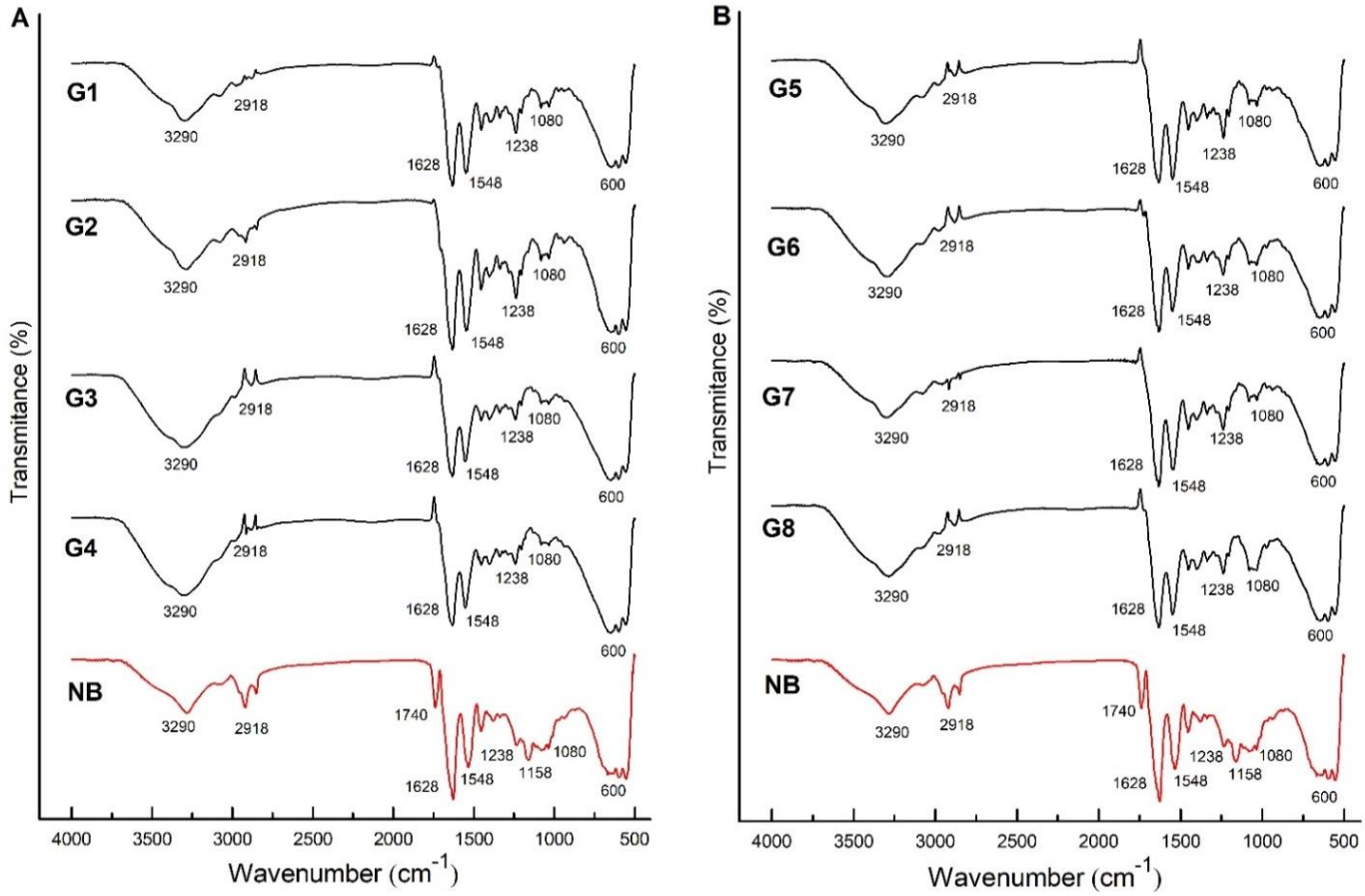

Figure 2. FTIR analysis of single (A) and double (B) H_2O_2 treatment showing chemical bands similar to NB.

The thermogravimetric analysis showed three well-defined thermal mass loss events and in similar temperature ranges between the samples, as can be seen in the TGA curves and respective DTG. For the single treatment groups, the first event occurred at ~70 °C for G4 and ~120 °C for G1–G3, with a mean mass loss of 10%; and for the double treatment groups, the event occurred at ~84 °C with a mass loss of 13% for G5–G6 and 32% for G7–G8. The second event occurred in the range of ~250 °C to 306 °C with a mass loss of ~60% (G1 and G2), ~70% (G3 and G4), and 75% (G5–G8), with a faster decay for G7 and G8 compared to the other treatments. The third event occurred in the range of 582 to 590 °C and culminated in a total mass loss at ~650 °C for all samples, except for NB, which presented a residual mass percentage of ~36% even after 800 °C. It was observed that, in general, the second thermal event was more determinant for mass loss in most experimental groups (G1–G6), except for G7 and G8, very evident from the first event. As expected, the NB group showed greater thermal stability in relation to the groups treated with H_2O_2 (Figure 3A–D). DSC analysis showed two very expressive endothermic events, the first at ~120 °C for G1–G4 and in the range of 94 to 100 °C for G5–G8, and the second event occurred in the range of 130 to 146 °C for all groups. NB

showed more expressive peaks at 135 °C and 188 °C, corroborating the greater stability observed in the analysis (Figure 3E–F).

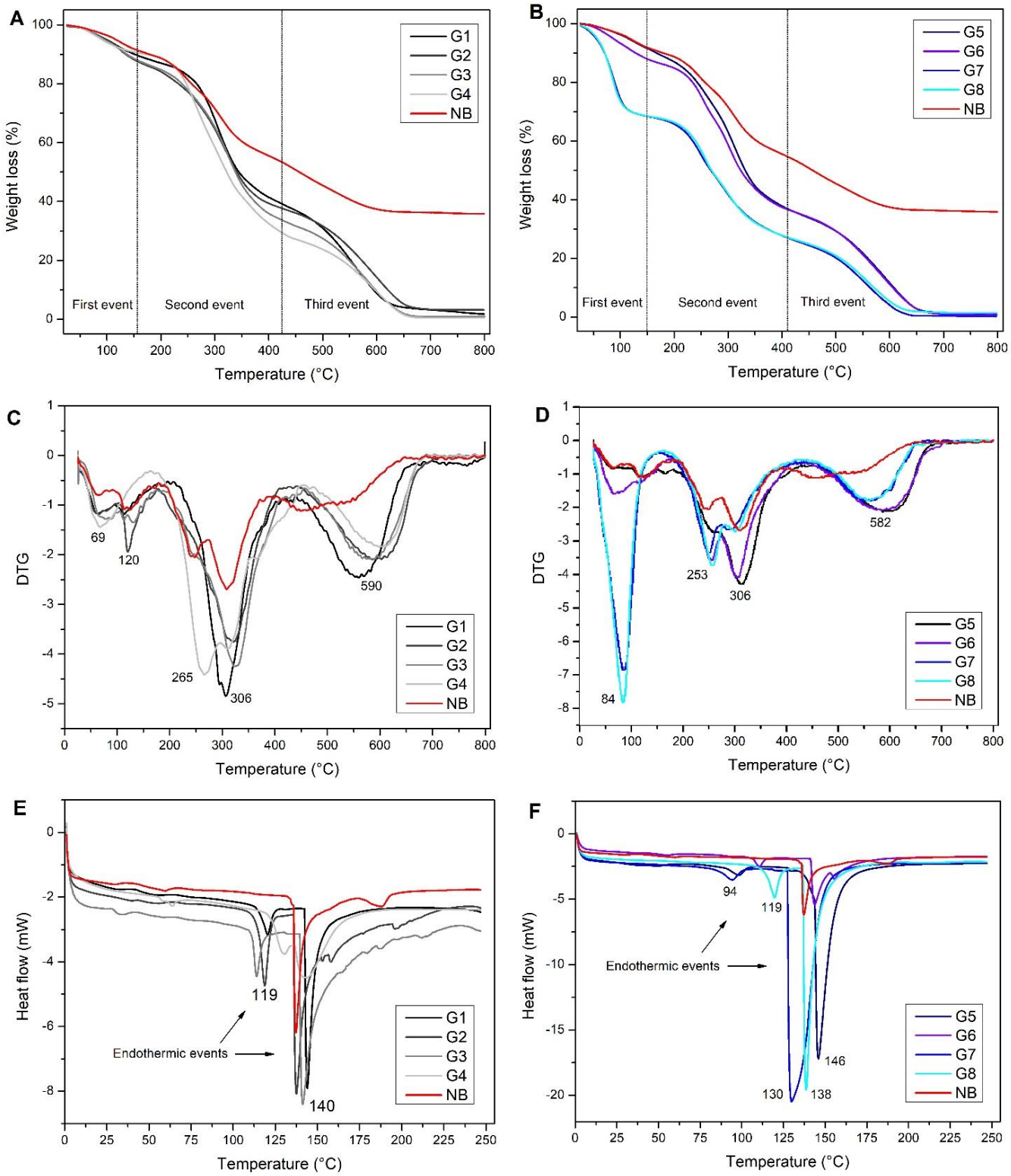

Figure 3. Analysis of single and double H_2O_2 treatment by TGA (A and B), DTG (C and D) and DSC (E and F), showing different thermal behavior of NB.

There were changes in typical lamellar bone morphology in all tested groups. The single treatment with H_2O_2 (G1–G4) generated an increase in porosity (<250µm), with areas increasing as the concentration increased, but maintaining the architecture of the matrix closer to the original (NB). In the double treatment with H_2O_2 (G5–G8), there was a much more evident increase in permeable spaces in the matrix (>250µm),

with progressive fusion of bone channels mimicking a mesh, especially under the two highest concentrations of H_2O_2 (G7 and G8) (Figure 4).

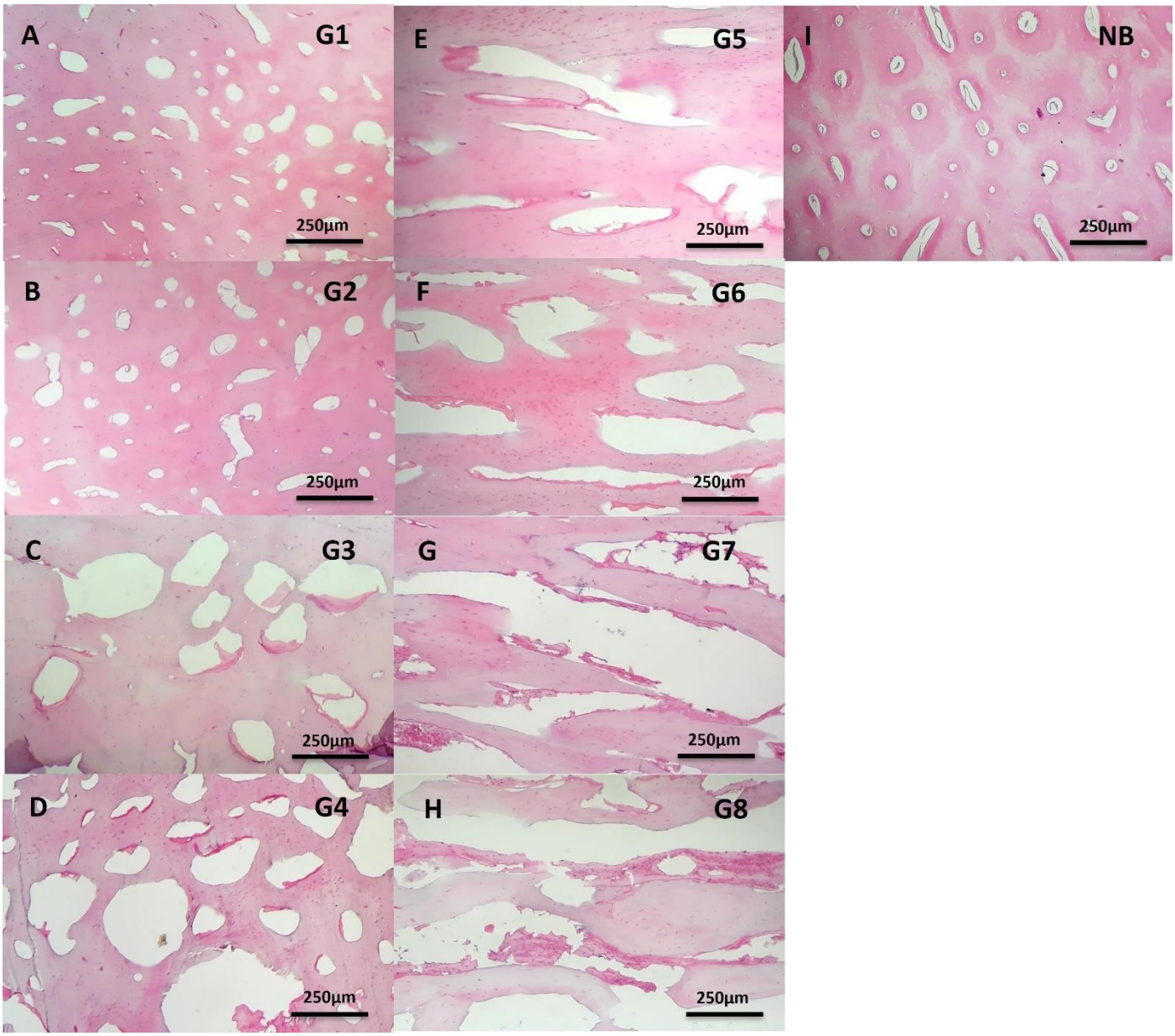

Figure 4. Histological analysis of porcine cortical matrix. A-D: Single treatment increased porosity (<250µm) according to concentration. E-H: Double treatment increased porosity (>250µm), fused bone channels and originated mesh morphology, depending on the concentration. I: Typical lamellar bone morphology preserved in NB.

Histomorphometric analysis of porosities showed that G1 was similar to NB, while all other groups significantly increased porosities. The double treatment promoted a larger total area than the single one and the highest concentrations of each respective protocol (G4, G7 and G8) display more significant results (Figure 5).

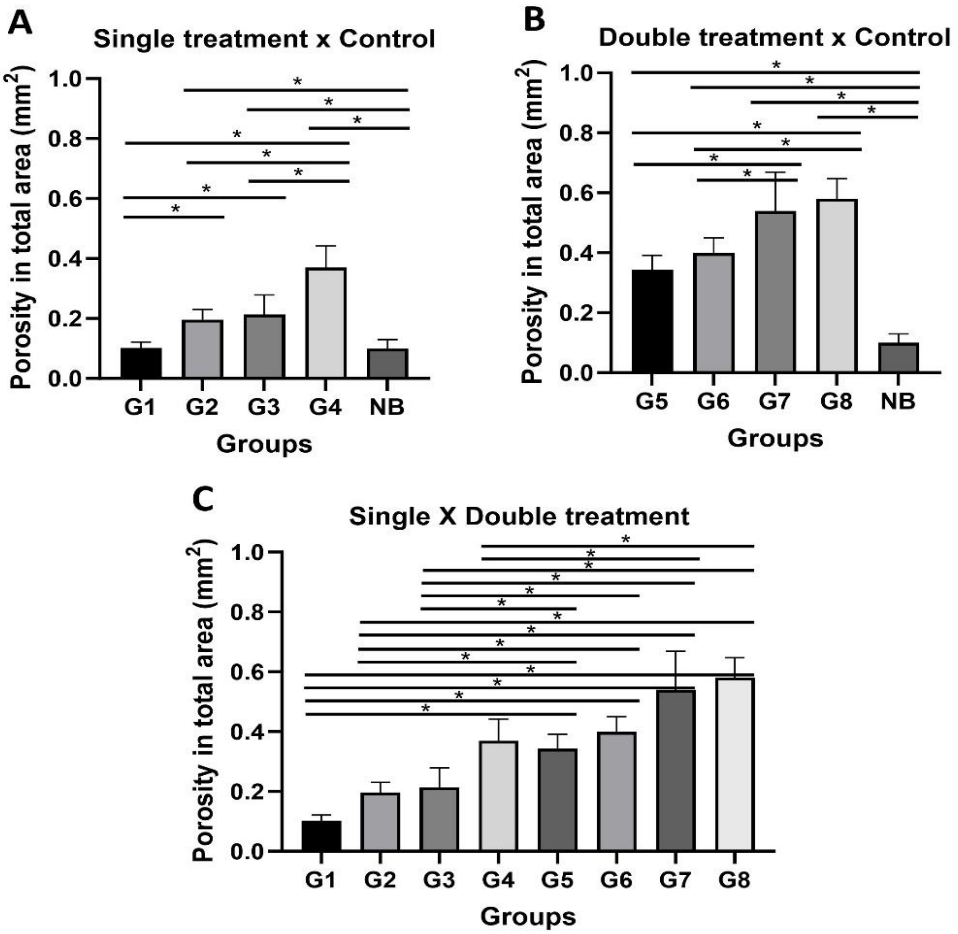

Figure 5. Histomorphometric analysis by total area of porcine cortical matrix porosities in G1–G4 (A) and G5–G8 versus NB (B), with double treatment promoting greater increase in total area than single treatment and control. *Significant differences, $p < 0.05$.

Corroborating these findings, in the swelling degree analysis, hydrophilicity was greater and slightly more persistent in the double treatment (20–30 minutes) than in the single treatment (10–15 minutes), with the two highest concentrations of each protocol (G3, G4, G7 and G8) as more expressive (Figure 6).

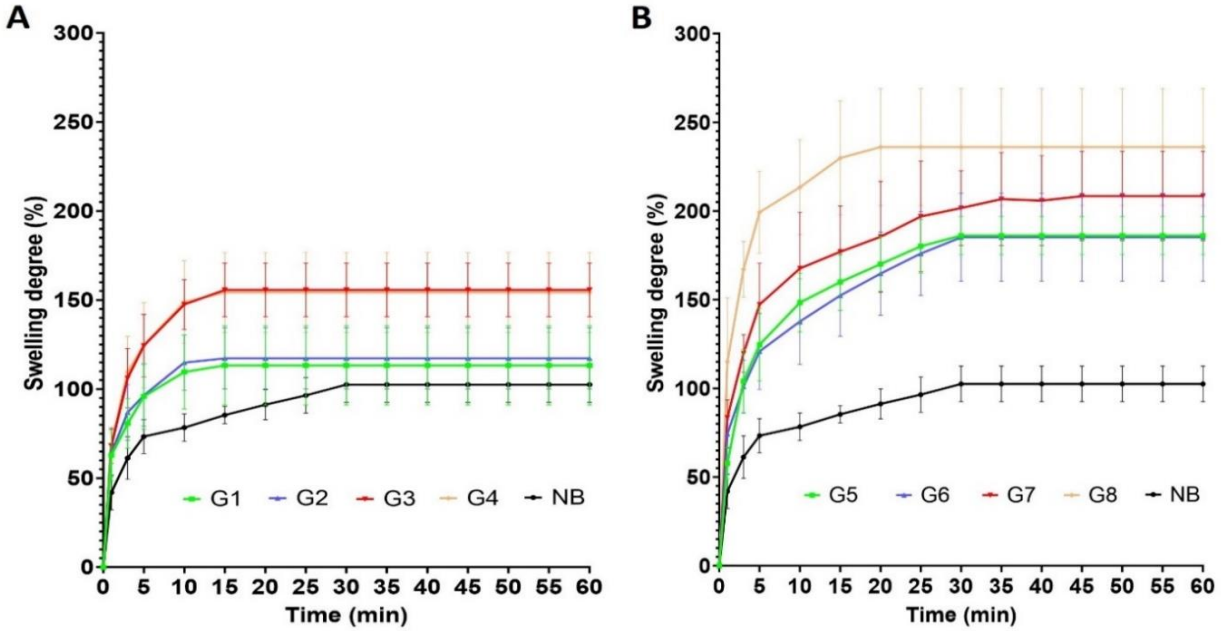

Figure 6. Analysis of the swelling degree of porcine cortical matrix in single treatment (A) and double treatment (B), showing more permeability in G5–G8 than G1–G4 and NB.

DISCUSSION

Biomimicry with bone matrix composition is a prerogative for the development of GBR membranes [2, 4, 5]. The different protocols tested and evaluated physicochemically perceived that it was possible to obtain natural collagen scaffolds, with a slight mineral expression, which could also contribute to the research of its bioactivity in the repair of bone defects [5, 29, 30]. A high maximum denaturing temperature contributes to good thermal stability of materials, and knowing the thermal behavior of a material makes it possible to predict possible applications of the same [29, 30, 31]. The materials achieved greater physical stability than expected for purely collagen matrices [29, 30], which could be explained by a more conservative demineralization [32, 33]. The double treatment and with high concentrations of H_2O_2 were determinant to weaken the structure of the test materials, as suggested in the literature on the peroxidation phenomena [20, 21, 22, 23, 24].

Morphometric diagnosis by microscopy or microtomography, two most common techniques in bone investigation, does not demonstrate differences between them in terms of bone volume or cortical thickness of porcine femur bones, although details of the microstructure require conventional histological examination [34] and the counting of cortical portion per area in mm^2 [35], which enhances the study option using standard microscopy with paraffin-embedded samples. Porcine bone demonstrated a morphophysiological analogy to human bone, as it is formed by an osteoid matrix, with osteocytes in a lamellar arrangement in the cortical portion, which confers resistance and adequate nutrition to the bone tissue [9, 11, 13, 36]. On the other hand, other animals, such as birds and reptiles, do not have the typical arrangement of the Haversian systems of mammals, exhibiting compact bone with wide variability in the amount and shape of vascular channels, as well as a more random or non-concentric orientation of osteocytes [37].

In long bones, human beings have an average of 14.57 osteons/μm^2 and 50.05µm in diameter in each Haversian canal, values close to twice that found in small to large mammals (7.45 osteons/μm^2 and 32.48µm, respectively) [13]. The diameter of the human Haversian canal can reach 100µm, with the swine presenting a third of this average [9]. In tissue scaffolds, however, the existence of macropores (>100 µm) is considered, which favor osteogenesis, angiogenesis and support cell migration to the implantation site, with some pores (150–800 µm) forming networks for growth vascular and nutritional transport that stimulate secretion from bone tissue, or micropores (<10 µm), which play an essential role in osteoinductivity [25]. When comparing treatment with porcine bone-derived membranes, greater bone formation was observed in those composed of the medullary or cortical-medullary portion than the cortical portion in critical defects in rabbits after 8 weeks of implantation [38]. However, it is important to emphasize that membranes with many porosities may have lower osteopromotive activity evolved non-porous [5], being therefore recommended that the design of a cortical bone membrane does not alter its native shape already with inherited porosity.

Regarding the degree of swelling, the highest values of the porcine bone groups in the double treatment approach those of poultry collagen hydrogels [29] and fall short of bacterial cellulose polymers [31]. In these cases, what may have reduced the volume was the incorporation of keratin, whose hydrophobicity could explain the slow hydration of the hydrogels, reaching a plateau in 80 minutes [29]. On the other hand, cellulosic matrices oxidized with sodium periodate, a crosslinking agent of the polysaccharide chemical structure, can influence the rate of its degradation and exhibit high and rapid hydrophilicity, reaching a plateau within just 12 minutes [31].

Analyzing the sanitization protocols for organic samples, to inactivate prions and minimize the risk of variant Creutzfeldt-Jakob disease due to contact with contaminated bovine derivatives, there is a recommendation for immersion of medical devices in 1N sodium hydroxide (NaOH), 20000 ppm sodium hypochlorite (NaOCl), phenolic disinfectant, alkaline bleach, combination of enzymatic bleach with vaporized hydrogen peroxide or sterilization in autoclave at 134 ^oC [39]. However, in this study, none of the previously mentioned decontamination solutions were used for the sample processing, due to the possible interference in the microstructure of the material and because the pig does not represent the animal source of this pathology. The risk of transmission of xenozoonosis from human contact with contaminated pig fragments, such as taeniasis, trichinellosis, salmonellosis, campylobacteriosis and toxoplasmosis, is very small and its prevention is based on rigorous disinfection [15]. Therefore, an easier and short-term form of sterilization such as UV light would be sufficient to maintain the porcine bone matrix aseptically free of pathogens until the time of the experiments. As the research is still at the level of conceptual technological maturity, more detailed studies are needed from basic to applied research up to scale production [40]. In fact, future microbiological tests should be conducted to ensure biological safety in commercial packaging and storage time for use, which may include gamma radiation, a well-used sterilization source in post-manufacturing of biomaterials for GBR [4, 21, 41].

To use tissues derived from animals, decellularization is important to avoid immunoreactivity [21], which was observed in the protocols tested due to the lack of cell nuclei and vital tissue in the bone treated with

H_2O_2. In this way, partial digestion of the organic extracellular matrix is also relevant and better achieved by deproteinization of porcine cortical bone with 0.35M NaOCl alone than a mixture of 2M H_2O_2, 1M NaOH and 1M of potassium hydroxide (KOH), being more effective in treatments from 4 days at 37 °C, due to the absence of collagen bands (proline, hydroxyproline, amide I and CH_2 deformation). However, up to 14 days, it still leaves some organic residue (amide III bands), without altering the phosphate and carbonate bands and the crystallinity of the mineral phase, even if combined with prior and/or subsequent delipidation for 1 day in solution 2:1 of chloroform–methanol [7]. An initial cleaning with 30% H_2O_2 and 75% ethanol for 2 hours and concentrations of 0.5% to 3% NaOH for 30 minutes promote good decellularization of bovine bone matrix and reduction of the immunogenic response by removing protein residues [42]. Furthermore, trabecular bone from deer corn exhibits efficiency in removing the organic portion with 2% NaOH for 12 hours and 30% H_2O_2 for 24 hours, followed by washing and delipidation in a 3:1 chloroform-methanol solution for 1 hour [24]. Very long duration protocols would be more in line with the generation of mineralized bone grafts [24, 42], not being approved for the proposal of a collagenic framework [25].

In addition to treatment aimed at cellular and protein components, the literature points to some protocols containing the removal of other organomineral residues from the bone. Sequential treatment of canine cortical bone demonstrated that removal of hydrophobic cues from porous compartments by treatment in alcohol-methanol solution at 4 ^oC for 3 days increased permeability by 21-fold, digestion of pericellular matrix collagen into bacterial collagenase a 37 ^oC overnight increased by 16 times the average of the previous step, while the removal of mineral salts from non-porous compartments by decalcification in EDTA at 4 ^oC for 2 to 3 weeks increased by 5 times the average of the previous step [17]. NaOH and KOH are also saponifiers, which react with vitamins by hydrolysis to create a salt and an alcohol, whereas NaOCl does not dissolve lipids [7]. However, such protocols were not adopted, in order to preserve the native organic structure as much as possible and not impact the quality of the collagen matrices generated.

The color change in the porcine bone sample treated with H_2O_2 may imply that the amount of protein is altered in relation to the untreated bone. However, the bone sample treated with H_2O_2 experienced that the bone microstructure did not change completely, while the study with NaOCl, NaOH or KOH, formed cracks in the longitudinal direction close to the vascular compartments, expanding the volume of the treated bone [7]. The H_2O_2 bleaching pattern was also used as a macroscopic standard for the initial chemical treatment of bovine cortical bone, removing blood, fat and other debris [21]. Our protocol adopted room temperature and H_2O_2 precisely to minimize organic changes in the synthesized material, according to the evidence cited.

In bone samples, the mineral fraction reaches 67% [17] and makes decalcification a key step for research aimed at access its organic portion. In the tested groups, the double treatment, started before and finished after decalcification, showed a greater degree of swelling. This could be explained by release of the phosphate ions, causing the remaining Ca^{2+} ions to attract hydroxide ions, leading to hydrogen bonding of the surrounding water and the increase of bound water, thus creating sample swelling [7]. Descaling can then create a highly hydrophilic environment. The effect of hydrochloric acid (HCl) on porcine cortical bone varies according to concentration and time, with immersion in 0.5M HCl for 2 hours or 2M HCl for 4 hours decreasing the thickness of the mineralized matrix from 5 to 35% and inducing an increase in precipitation of crystals in Haversian and Volkmann canals, suggesting that the decalcifier also acts in the gap-canalicular pathway [43]. Among the decalcifiers, 5% nitric acid or 10% HCl with 5% formic acid (CH_2O_2) solution were about 10 times faster than 7% ethylene-diamine-tetraacetic acid (EDTA) in rat bones, but the acids did not preserve cell morphology as well as the chelator [32]. Protocols in porcine cortical bone with 1M EDTA for 7 days, 0.5M CH_2O_2 for 2 days, 0.5M HCl for 7 hours and a mixture of 3.3M HCl and 2.4mM EDTA for 3 hours showed that HCl was the best bone demineralizer, leaving the collagen structure well preserved in a shorter time interval [33].

Lyophilization of bovine cortical bone, with freezing and drying for 48 hours, minimizes the water content and deformation of the material to less than 5%, promoting greater durability and the possibility of subsequent sterilization with gamma radiation [21]. Heating dehydrates, also causing the moist collagen to suffer a drastic reduction in its length, explained by the destabilization of the chemical bonds of the native collagen from 60 ^oC [44]. It should also be considered that UV light facilitates the formation of crosslinks within the chemical structure, with no strict mechanism due to the non-specificity of reactions provoked in radicals, from 30 min to 8 hours [45]. In this initial laboratory prototyping, a lyophilization step was not performed, but it will be important for manufacturing, industrial scalability and longer product storage. For the other parameters, lower conditions were used, both for the temperature of drying in a conventional oven for dehydration, and for the time of exposure to UV light for simple decontamination of the test sample.

Lyophilized demineralized bovine cortical bone treated extensively with 3% H_2O_2 for 5 to 6 days, followed by demineralization in 0.1% HCl, generated greater in vitro cytotoxicity, greater leukocyte infiltration up to 7

days post-implantation, but without producing in giant cells or degradation up to 4 weeks, when compared to commercial Jason bovine pericardium collagen membrane [21]. Processing with 30% H_2O_2, 4% NaOH and ethanol preserved collagen matrices after decellularization of a bovine bone scaffold, but despite good removal of residual proteins, which also microscopically promoted greater trabecular spacing, there was moderately reactive cytotoxicity [23]. These findings may be associated with H_2O_2 or HCl activity or both still retained in the material after the washing procedure, considering that the low porosity nature of cortical bone may require more cleaning time to completely remove the chemical agents absorbed during the manufacturing process [21]. A commercial demineralized bovine cortical bone membrane (GenDerm), processed with organic solvents, peroxides and acid treatment, maintained a type I collagen fibrillar mesh and a concentration of growth factors such as bone morphogenetic proteins and when implanted in the subcutaneous tissue of rats, it was well tolerated by the tissues and completely reabsorbed after 30–60 days by mononuclear cells and multinucleated giant cells [22], which suggests that the different processing can generate different immunoregulatory profiles.

Experiment with three commercial membranes implanted in rat cranial defects, two of demineralized bovine cortical bone, one thinner (GenDerm, 150–200μm) and the other thicker (GenDerm Flex, 200–250μm), in comparison with a porcine collagen pattern in pairs face (Bio-Gide), demonstrated a better osteopromotive performance up to 60 days associated with the thicker bovine cortical membrane, with greater bone gain and without resorption close to the porcine one, with a good amount of newly formed bone but already completely reabsorbed in 30 days and far superior to the thinner bovine cortical membrane [46]. There is the case of OsteoBiol Lamina Soft, a demineralized and collagenized porcine cortical lamina, with thickness variations from 3mm (standard) to 1mm (half curved), already used clinically for intraoral bone reconstruction prior to the placement of dental implants, with a high rate of success for 5 years [47]. This reinforces the potential of the porcine cortical source and the importance of final product design in influencing the results applied to GBR, which inspired the sample format of this pilot study.

Such evidence can contribute within the microstructure modeling strategies of a biomimetic cortical bone for additive manufacturing [48]. More studies are needed to idealize prototyping applied to the development of a product with an application profile as a membrane or carrier scaffold [2, 3, 31]. Still, within the limitations of this study, there is a need for future tests of biological parameters of porcine bone treatments, to ensure that biomaterials have biocompatibility, biodegradability and osteopromotion in vivo, for their future election as a material applicable to GBR [5, 29, 30, 49].

CONCLUSION

The treatment with hydrogen peroxide in porcine cortical matrix promoted changes in its composition, thermostability, bone porosity and swelling degree. Single exposure and at lower concentrations of the chemical agent suggest a better modulation of these determinants, for the development of biomimetic partially mineralized collagen biomaterial, with potential use as a membrane applied to bone regeneration.

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