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DIFFERENCES IN THE EXTRACELLULAR MATRIX OF BOVINE PERICARDIALS TREATED WITH GLUTARALDEHYDE RELATED TO THE STORAGE TIME IN FORMALDEHYDE

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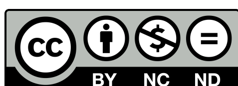

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Abstract: **INTRODUCTION:** Bovine pericardium is a xenogeneic membrane that requires pre-treatment to be used as an implant in humans. In order to do this, these membranes are treated with a warm solution of deglutaraldehyde, which cross-linking action reduces the immunogenicity of the xenograft and guarantees satisfactory biomechanical properties. These membranes are marketed under the name of "bovine pericardium patches", which are immersed in an aqueous formaldehyde solution as a preservative medium. OBJECTIVES: To evaluate the structure of the extracellular matrix and the microbiological properties of bovine pericardium previously treated with glutaraldehyde and stored in formaldehyde for more than 10 years. To analyze and quantify the presence of collagen fibers (I and IV), laminin and cementin in bovine pericardium from 2009 in comparison with the 2020 bovine pericardium.METHODS: Two patches of bovine pericardium were used. One, which consists of the control group, was produced in the year 2020 and the other, object of study, was produced in the year 2009. After treatment, the membranes were rolled, immersed in 10% formaldehyde, and embedded in paraffin to make the blocks. Cuts of 3um thick were obtained and subjected to HE staining and immunohistochemistry using the technique of streptovidin-perodixed for anti-patches I and IV, anti-vimentin and anti-laminin to ascertain these proteins in the patches. Digital images of the slides were taken, inserted into Photoshop for Windows, and through the image J program. RESULTS: No structural changes were detected when comparing the microscopic aspects between the membranes of 2009 and 2020. Collagen I occupied an area of 21.36% for the 2020 patch and 15.87% for the 2009 patch. Collagen IV occupied 24.67% and 12.02%, respectively, for the 2020 and 2009 patches. Vimentin was identified both in fusiform cells in the middle of the membrane and in areas compatible with vascular membranes.The percentage in cellular content was 54.34% for the 2020 patch and only 13% for the 2009 patch.Alaminin was not reacted among specimens and both membranes were negative for the presence of this protein. CONCLUSION: Although there are no important morphological alterations between the membranes, in this pilot study we can see that the 2009 membrane loses important proteins of sustentation and cellularity, suggesting loss of functional substrate that provides its use.

Keywords: BIOPROSTHESIS, GRAFTS STABILIZED BY GLUTARALDEHYDE, PERICARDIUM.

INTRODUCTION

Membranes of animal origin are widely used in medicine and their function is to replace an injured tissue and/or to serve as a base for the natural regeneration process, but tissues of animal origin are highly immunogenic for humans. To make them biologically compatible, it is necessary to treat these tissues through bioengineering methods.(ATHAR et al., 2014; CARPENTIER et al., 1969)

In 1969, Carpentier et al. introduced a method of treating animal membranes that is used to this day: cross-linking with glutaraldehyde. Glutaraldehyde in aqueous solution gives rise to several molecules, which react with the proteins of the extracellular matrix of the animal membranes (cross-linking reaction). It is undeniable that the cross-linking reaction with glutaraldehyde makes animal membranes safer for human implantation, but there is still a need to combine xenografts with increasingly modern bioengineering techniques in order to further assist in healing and regeneration procedures (ATHAR et al., 2014; IONESCU et al., 1977).

Among all the membranes of animal origin, one of the most practical and versatile is the bovine pericardium. In 1971, Marian Ionescu started research in order to use bovine pericardium, cross-linked with glutaraldehyde, as a method to manufacture valvular bioprostheses. At the end of the 1970s, the "Ionescu-Shiley" bovine pericardium graft was already considered as the standard method to manufacture these devices. The "Ionnescu-Shiley" pericardial graft obtained excellent results in the first 6 to 10 years, but after this period, serious problems of calcification and cytotoxicity of the implant emerged.(ATHAR et al., 2014; GOISSIS et al., 1998)

Taking as an example the problems of calcification and cytotoxicity of the "Ionescu-Shiley" graft after 10 years, several researchers started to develop alternative methods for the treatment of bovine pericardium. Among them, Domingo Marcolino Braile was a researcher physician who achieved international recognition due to the success of his research. In his studies, Dr. Braile continued using the aqueous deglutaraldehyde solution as the standard method to treat the bovine pericardium

However, with some differences; under different concentrations and even by transforming the aqueous derivatives of glutaraldehyde in glutaraldehyde acetals (protective chemical groups). (GOISSIS; YOSHIOKA; BRAILE; RAMIREZ, 1998; GOISSIS; BRAILE; GIGLIOTI, 1999)

Although the treatment with glutaraldehyde provides a biological tissue adequate for the confection of heart valves, by producing a relatively inert and biologically compatible material, it is not the ideal treatment because alterations may occur in the implanted tissue, asfibrotic infiltration, adgeneration, immunological rejection, sensitization, the toxicity of non-reactive dihydoglutaral, atrombosis, and in particular late post-implantation calcification, a predominant cause of heart valve failure. (BAUCIA, 2005, p.11)

The bovine pericardium patches produced and commercialized by Braile Biomédica® (São José do RioPreto-SP), after being treated withoglutaraldehyde, are stored in an aqueous solution of formaldehyde (preservative agent). All patches have a two-year shelf life. There is a lack of studies in the literature demonstrating the conservation, or degradation, of the microbiological properties of bovine pericardium patches many years after their manufacture. The present study intends, therefore, to provide data that will serve as a subsidy for future studies with animal membranes, and, above all, for the development of more efficient treatment methods.

OBJECTIVE

The present study aims to evaluate the extracellular matrix structure and microbiological properties of bovine pericardium previously treated with glutaraldehyde and stored in formaldehyde for more than 10 years. This will be a comparative evaluation with another patch of bovine pericardium, also treated with glutaraldehyde and stored in formaldehyde, but produced in the year 2020. In short, the final objective of the present study is to answer - or raise new hypotheses for future studies - the following questions: After a decade of its manufacture, will the bovine pericardium patch preserve the structure of its extracellular matrix? If not, what were the predominant changes?

SPECIFIC OBJECTIVES

To analyze and quantify the collagen fibers (I and IV) of the 2009 bovine pericardium, using the 2020 bovine pericardium as a control.

To analyze and quantify the vimentin

protein in the 2009 bovine pericardium, using the 2020 bovine pericardium as a control.

To analyze and quantify the laminin protein in the 2009 bovine pericardium, using the 2020 bovine pericardium as a control.

Evaluate the microscopic structure of the 2009 bovine pericardium, in the light of HE staining, using the 2020 bovine pericardium as a control.

LITERATURE REVISION

PERICARDIAL STRUCTURE

The pericardium is a serous membrane that surrounds the muscular body of the heart and the roots of the great vessels. It consists of two layers: fibrous pericardium and serous pericardium. The outer surface of the pericardial sac is formed by the fibrous pericardium, while the inner portion by the serous pericardium. The serous portion of the pericardium, in turn, gives rise to two layers, parietal and visceral. The visceral lamina (or epicardium) directly covers the cardiac surface. The parietal lamina covers the inner surface of the fibrous pericardium and originates in the reflection of the visceral lamina at the level of the basal vessels. (COELHO, 2011)

The two serous laminae delimit a cavity, the pericardial cavity. It is a virtual space containing a slightly alkaline viscous fluid, whose function is to decrease the friction between the epicardium and the parietal lamina during the periods of systole and diastole. (COELHO, 2011)

The pericardium, as a whole, is made up of collagen fibers of types I and III (predominantly) interspersed with short elastin fibers. The visceral layer of the serous pericardium, when analyzed by light microscopy, appears as a thin layer composed of a single row of mesothelial cells seated on a thin connective tissue formed by collagen and elastic fibers. fat cells. The parietal layer of the serous pericardium, unlike the visceral layer, is thick, rich in collagen and elastic fibers. Portion are concentrated in dense wavy bundles arranged three-dimensionally, being easily recognized during analysis by optical microscopy. (HOIT, 2017) "The abundance and orientation of collagen fibers are responsible for the viscoelastic mechanical properties characteristic of the pericardium..." (HOIT, 2017, p.483)

Tissues rich in collagen, such as bovine pericardium, undergo rapid degeneration and need to be stabilized in order to prolong the original structure and mechanical integrity. This stabilization consists in the creation of supplementary bonds in the collagen molecules, which reinforce the tissue, without altering its original characteristics. Furthermore, the supplementary bindings of the stabilization process aim to remove or neutralize antigenic properties inherent in the animal membrane. (BAUCIA, 2005)

COLLAGEN FIBERS

Collagens are the most abundant proteins in the mammalian body. It is a superfamily of proteins, containing 28 different types of collagen, which make up about 30% of the entire protein mass of the organism. (RICARD-BLUM, 2012)

Collagen fibers are formed by linearly polymerized tropocollagen subunits, with each tropocollagen molecule consisting of three polypeptide chains arranged in a triple helix, known as "alpha" chains. It is the sequence of amino acids in these chains that gives rise to the 28 different types of collagens. There are three different categories in the superfamily of collagens: fibrillar collagens, associated collagens, and network-forming collagens. (PORTO, 2007; RICARD-BLUM, 2012)

The category of fibrillar collagens is formed by collagen fibrils, extremely thin structures visible only to electron microscopy. Several

collagen fibrils together, arranged in parallel, give rise to collagen fibers (visible under light microscopy). The grouping of collagen fibers, in turn, gives rise to bundles, very abundant in tissues such as the pericardium. These bundles, in living tissue, have a whitish color, which is why collagen fibers are also known as "white fibers". When the beams are stained with hematoxylin and eosin under light microscopy, they appear as dense, wavy, serous structures. Most abundant in this category, giving the fabrics a very high resistance to tension forces. Another type of collagen that is very abundant in pericardial tissue is type III collagen. It is a fibrillar protein, synthesized by fibroblasts, which forms a structural fabric in the tissue. (PORTO, 2007)

"Fibrillar collagens are the most abundant types of collagen in vertebrates, playing a structural role by contributing to the molecular architecture, shape, and mechanical properties of tissues, such as tensile strength" (RICARD-BLUM, 2012, p.12)

ELASTIC FIBERS

The elasticity property of a cellular tissue is largely guaranteed by the elastic fibers present in the extracellular matrix. Such fibers, produced by connective tissue fibroblasts, can be stretched one and a half times their length without breaking, returning to their initial length when the force is stopped. (PORTO, 2007)

There are basically two main constituents of an elastic fiber: microfibrils and elastin. Microfibrils are made up of the glycoprotein fibrillin, while elastin is a protein rich in the amino acids glycine, lysine, alanine, valine, and proline. It is the deposition of elastin in the spaces between the microfibrils that gives rise to an elastic fiber. Chemically, what guarantees a high degree of elasticity to the components of the elastic system is the formation of desmosine cross-links (covalent bonds between lysine molecules). In the pericardium, under optical microscopy analysis, elastic fibers can be identified because they are located between the dense wavy bundles of collagen. (HOIT, 2017; PORTO, 2007)

USES OF BOVINONE PERICARDIOM MEDICINE

The body's natural response to any injury, acute or chronic, is to trigger a physiological repair of the affected tissue or organ. In this context, the regeneration process can be defined as a reconstruction of injured tissue in order to restore its original form and function. However, very extensive tissue damage and/or a limited ability to regenerate can inhibit or limit the tissue's ability to return to its original state (form and function). (ATHAR et al., 2014)

Therefore, tissue grafts produced by bioengineering methods are gaining ground. Basically, there are two major functions of tissue grafts: replacement of injured and/or auxiliary tissue and serving as a basis for the natural regeneration process. And there are some properties that must be inherent to the tissue graft in order to guarantee the success of the implant, they are: low immunogenicity and biodegradability, high resistance to mechanical forces, high ability to withstand high temperatures and resistance to pH variations. (ATHAR et al., 2014)

Xenografts, when compared to allografts, provide a greater "arsenal" of practical advantages. The supply of animal membranes is practically unlimited and, as it is possible to obtain them through a wide variety of species (pigs, sheep or goats), it is possible to have access to a wide variety of sizes needed for the most diverse surgeries. However, the biggest disadvantage of xenografts is the fact that these tissues are highly immunogenic. To circumvent this problem, several researchers

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use bioengineering to make animal membranes, biologically, more compatible. (ATHAR et al., 2014; CARPENTIER et al., 1969)

One of the most used methods for the treatment of biological membranes of animal origin is the reaction of these tissues with glutaraldehyde (GA). This treatment method was first described by Carpentier et al. in 1969. Glutaraldehyde reacts with proteins of the extracellular matrix of animal membranes forming cross-linking bonds. It is a complex chemical reaction, not yet fully elucidated, which guarantees the desired biomechanical properties for a xenograft. (ATHAR et al., 2014; CARPENTIER et al., 1969)

One of the most used animal membranes in medicine is bovine pericardium post-crosslinked with glutaraldehyde. It is a biological tissue widely used as a patch in cardiovascular surgery. Bovine pericardium treated with glutaraldehyde has been used for the correction of numerous heart diseases as a substitute for atrial, ventricular, arterial, and venous walls, and also for pericardial sac reconstruction. This material certifies good tensile strength due to the arrangement of collagen fibers in multiple directions. (PIRES et al., 1997; KAPAN et al., 2002)

The history of bovine pericardium treated with glutaraldehyde begins in 1971, introduced by Marian Ionescu in the construction of bioprostheses for heart valves.

In 1984, Yakirevich et al. reported excellent results in pericardial sac closure in 66 patients who underwent open heart surgery. All 66 pericardial sacs were closed with glutaraldehyde-treated bovine pericardium. Baharuddinetal., in 2002, used bovine pericardium membrane as a dural graft, obtaining positive results in 20 of the 22 treated patients. The other two patients experienced complications unrelated to the graft. In 2007, Lopes et al. reported the use of bovine pericardium in penile prosthesis implantation as a complement to the tunica albuginea, which, due to an infectious manifestation, suffered intense fibrosis and resulted in the destruction of the cavernous bodies. The author pointed out lower cost, low probability of retraction, good tensile strength and elasticity as reasons for choosing this material. (ATHAR et al., 2014)

The medical literature demonstrates that bovine pericardium treated with glutaraldehyde is a very practical and versatile material. However, there is still a need to combine this material with increasingly modern bioengineering techniques in order to further assist in healing and regeneration procedures. (ATHARetal., 2014)

ALDEHYDES AS CROSS-LINKING AGENTS

Fixation through crosslinking with aldehydes (especially glutaraldehyde and formaldehyde) is the most common method for preserving the biological architecture of a xenograft. (KIRKEBY; MOE, 1983)

GLUTARALDEHYDE

Glutaraldehyde (GA) is a linear di-aldehyde composed of five carbon atoms. Macroscopically, it appears as a clear, oily liquid and highly soluble in water, alcohol and organic solvents. It is marketed as an aqueous solution present in different concentrations (2% to 70%). In neutral pH solutions (such as those prepared during the treatment of bovine pericardium) it reacts rapidly with amino groups of proteins. (MIGNEAULT et al., 2004)

In January 1977, a study was published in "The Journal of Thoracic and Cardiovascular Surgery" that introduced the bovine pericardium graft, Ionescu-Shiley, as the standard method for the development of valve bioprostheses. The objectives of the

Ionescu-Shiley bovine pericardium graft were to extinguish thromboembolic events arising from valve transplantation, without the need for anticoagulation, to provide adequate hydrodynamic performance, and to ensure the structural and functional maintenance of the graft over a long period of time. (IONESCU et al., 1977)

In this study, pericardial tissue was obtained from calves that were 6 to 18 months of age and treated with glutaraldehyde (GA). Pericardial tissue was fixed with 0.5% purified GA aqueous solution in the presence of a phosphate buffer solution (pH 7.4) at 4 degrees Celsius for 2 weeks. After this period, the newly treated pericardium piece was stored in a 4% formaldehyde solution in the presence of a 0.2 M acetate buffer solution (pH 5.4). What occurs chemically during this method of fixation is the formation of crosslinks ("crosslinking") between the aldehyde functional group of the molecular derivatives of glutaraldehyde and the amino acid lysine and hydroxylysine groups present in collagen and elastin fibers, as well as in glycoproteins and proteoglycans. It is precisely this reaction with GA that makes the bovine pericardium non-immunogenic, less prone to thromboembolic events, more resistant to high temperatures and more resistant to mechanical forces. (IONESCU et al., 1977)

However, heart valves (bioprostheses) derived from bovine pericardium pieces treated by classical methods (such as the Ionescu-Shiley method) had a major problem: high levels of degenerative calcification after 7 years of implantation. (GOISSIS et al., 1998)

This occurs because the chemical reaction between the glutaraldehyde solution and the bovine pericardium is very complex, opening gaps for some undesirable results such as calcification. GA in aqueous solution gives rise to several molecular derivatives, each of which reacts with the s-amino groups of tissue proteins. This fact, added to the heterogeneity of the pericardial tissue, results in a non-homogeneous reaction. As a consequence, crosslinking is limited to surface protein fibers. Thus, internal regions of the tissue that were not crosslinked by the GA are obtained, resulting in a heterogeneous and waterproofed tissue; surface that has become biocompatible thanks to cross-linking, contrasting with the interior of the tissue that has not been cross-linked, therefore not biocompatible. (GOISSIS et al., 1998)

Valve bioprostheses, due to the mechanical forces to which they are subjected within the host organism, undergo micro-ruptures on their surface exposing the non-reticulated (non-biocompatible) internal regions to blood flow, thus serving as primary sites of calcification. (GOISSIS; BRAILE; GIGLIOTI, 1999)

In view of this and other problems, such as post-implantation cytotoxicity, alternative fixation processes with glutaraldehyde have been developed; among them, glutaraldehyde acetals. The method basically consists of using protective chemical groups that do not allow the reaction between the GA and the bovine pericardium to occur quickly (prevents waterproofing), allowing a more homogeneous distribution of the fixative agent throughout the thickness of the pericardial tissue. (GOISSIS et al., 1998)

This process is based on a double protection strategy, through protonation of the free end of pericardial tissue amino acid residues, and the formation of glutaraldehyde acetals from their molecular derivatives. A purified aqueous solution of 1% GA is reacted with absolute ethanol and 0.003 M hydrochloric acid. This reaction occurs over a period of 24 hours, resulting in the formation of glutaraldehyde acetals. The protonation of amino acid residues from bovine pericardium occurs by immersion of tissue fragments in

an initially aqueous ethanol solution which, by progressively increasing its concentration, culminates in an absolute ethanol solution. The final solution is acidified by the addition of 0.003M hydrochloric acid. After 24 hours reacting, the protonated tissue is transferred to the solution containing the glutaraldehyde acetals. Unlike traditional methods, the reaction does not occur immediately as both pivots of the cross-linking reaction (aldehydes and e-amino groups) are chemically "protected". The chemically protected bovine pericardium is immersed in the solution containing the glutaraldehyde acetals for a period of 96 hours, which allows a more homogeneous distribution of GA throughout the tissue. Crosslinking will only occur after exposure of this tissue to triethylamine (Et3N) vapors, which serves as an agent that removes chemical protective groups. (GOISSI etal., 1998) The chemically protected bovine pericardium is immersed in the solution containing the glutaraldehyde acetals for a period of 96 hours, which allows a more homogeneous distribution of GA throughout the tissue. Crosslinking will only occur after exposure of this tissue to triethylamine (Et3N) vapors, which serves as an agent that removes chemical protective groups. (GOISSI etal., 1998) The chemically protected bovine pericardium is immersed in the solution containing the glutaraldehyde acetals for a period of 96 hours, which allows a more homogeneous distribution of GA throughout the tissue. Crosslinking will only occur after exposure of this tissue to triethylamine (Et3N) vapors, which serves as an agent that removes chemical protective groups. (GOISSI etal., 1998)

FORMALDEHYDE

Formaldehyde is a monoaldehyde whose molecular formula is CH2O. In its pure form, it appears as a colorless gas with a high irritating potential. Its liquid form is basically obtained by the catalytic oxidation of methanol (process industrial) which, when reacting with water, similarly to glutaraldehyde, gives rise to several molecular derivatives. This complex aqueous solution is called formalin. Generally, the concentration of formaldehyde in the aqueous solution that is used for the preservation and microscopic study of animal tissues is 4%. (MUSIAL et al., 2016; SMITH, 1992)

Affixing tissues with formalin is the standard method for preserving tissues over a long period of time. Like glutaraldehyde, it interacts with tissue proteins through cross-linking, inactivating, stabilizing, or immobilizing the proteins. (HUTSONetal.,2018;METZetal.,2003)

Reticulation occurs when formaldehyde reacts as an N-terminal amino acid residue group with side chains of arginine, cysteine, histidine, and lysine residues. This way, three different types of interactions can arise depending on the sequence of amino acids in the peptides, they are: methylol groups, Schiff bases and methylene bridges. (METZ et al., 2003).

Hutson et al, in an article published in 2018 in the journal "Biotechnic & Histochemistry", reached the following conclusions when evaluating the modifications of the extracellular matrix induced by fixation with formaldehyde: "Although our findings indicate a very small impact of the preservation method on collagen fiber measurements, it is possible that the preservation method impacts the nano- or micro-architecture of other components of the extracellular matrix." (HUTSON et al., 2018, p.9)

GLUTARALDEHYDE X FORMALDEHYDE

The patches of bovine pericardium produced by the company Braile Biomédica®

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(São José do Rio Preto-SP) are crosslinked with glutaraldehyde and later stored in formaldehyde for conservation. There is a chemical explanation for the preference of glutaraldehyde as a crosslinking agent over formaldehyde.

In an article published in the journal BiochimicaetBiophysicaActa, in 1968, two researchers, Bowes and Cater, presented their results about the crosslinking of collagen fibers with different types of aldehydes. Monoaldehydes (such as formaldehyde) and di-aldehydes with chains ranging from 2 to 6 carbon atoms were used. for a given 5-carbon di-aldehyde, glutaraldehyde. (BOWES; CATER, 1968)

MATERIALS AND METHODS

DESCRIPTION OF THE BIOLOGICAL MATERIAL

The two patches of bovine pericardium used in the study were produced and marketed by the company BraileBiomédica® (SãoJosédoRioPreto-SP). Both were cross-linked in an aqueous solution of glutaraldehyde and, later, stored in an aqueous solution of formaldehyde at 4% for conservation (standard method of treatment used by the company). The main difference between the two patches is in the manufacturing date. The patch that was used as the object of study was manufactured on 01/13/2009, its validity expired on 01/13/2011. The 2009 patch had a thickness of 0.35mm and an area of 35cm2 (7x5cm). /10/2022. It had a thickness of 0.36 mm and an area of 11 x 9 cm. Both patches were acquired and made available for study, cordially,

STUDY DESIGN

This is a study based on qualitative and quantitative variables with the objective of analyzing the cellular components, and the extracellular matrix, of bovine pericardium patch stored in formaldehyde for a period exceeding 10 years. The research was carried out in vitro using a patch of bovine pericardium produced in 2020 as a control group. Data were obtained through a comparative analysis between the histological slides of the two pericardium samples (2009 and 2020).

BLADES PREPARATION

After treatment, the patch membranes were rolled up, each membrane was soaked in 10% formalin. Subsequently, each piece was semi-sectioned transversally to the patches, cleared, dehydrated and inserted into cassettes containing paraffin. From the paraffin blocks, serial longitudinal sections were obtained, measuring 5 mm in thickness. The slices were stained with hematoxylin and eosin according to the following specification:

1. Deparaffinization of sections, two xylene changes, 15 minutes each;
2. Hydration in two changes of absolute alcohol, 5 minutes each;
3. Immersion in 95% alcohol for 2 minutes, followed by 70% alcohol for 2 minutes;
4. Brief wash in distilled water;
5. Staining in Harris hematoxylin solution for 5 minutes;
6. Wash in running tap water for 5 minutes;
7. Differentiation in 1% alcohol-acid for 10 seconds;
8. Wash tap with running water for 1 minute;
9. Wash in running tap water for 5 minutes;
10. Rinse in 95% alcohol, 10 baths;
12. Counterstain in eosin solution for 20 seconds;
13. Dehydration using two changes of 75% alcohol, two changes of 95% alcohol and two changes of absolute alcohol, 5 minutes each;

14. Clearing (bleaching) in two xylene changes, 5 minutes each;
15. Mounting with xylene based on the mounting means Permount (Sigma-Aldrich, Darmstadt, Germany).

IMMUNOHISTOCHEMISTRY

Two μm thicknesses of each sample were deparaffinized in xylene and hydrated for 10 minutes in absolute alcohol, followed by 10 minutes each in 95% alcohol and 70% alcohol. The antigen retrieval process was carried out using 1% pepsin solution (pH 1.8) (Sigma-Aldrich, Darmstadt, Germany) for 1 hour at 37°C. They were left to cool down to room temperature for 20 minutes and washed in distilled water for 10 minutes. The activity of endogenous peroxidase was blocked in 0.1% hydrogen peroxide (Thermo Fisher Scientific, Waltham, MA, United States) for 15 minutes. The specimens were washed in running tap water for 5 minutes and then submerged in phosphate-buffered saline (Sigma-Aldrich, Darmstadt, Germany) for 5 minutes. The samples were then incubated overnight with the anti-anti-anti-Collages I primary antibody. and IV, subjected to immersion with diaminobenzidine chromogen (Universal HRP immunostaining kit) (Diagnostic BioSystems, Foster City, CA, United States) for 15 min. It produced a brownish precipitate at the antigen site. Samples were counterstained with Mayer's hematoxylin. A negative control was performed for all samples that omit the primary antibody. For each sample, three slides were used for incubation with each antibody.

SCANNING OF BLADES

Digital images of the slides were taken, inserted in a photoshop for Windows program, and through the image J program. All results were transformed into percentages.

RESULTS

MICROSCOPY

From a structural point of view, patches dated to 2009 and 2020 did not demonstrate autolysis or significant change differences. The fragments were composed of dense connective tissue that was not modeled and was poorly cellular. When present, cellularity was sparse and fusiform in appearance, similar to mature fibrocytes. Vascular areas represented by small capillaries defined the histological picture of both membranes.

COLLAGEN

In both specimens, collagen I was diffused through the connective tissue of the membranes. Although the structural difference was not evident, the protein percentages were different.

COLLAGENIV

Similar to what occurred for the immunoexpression of collagen I, the presence of collagen IV was also diffuse and irregularly arranged in the membrane for the different specimens. The percentage value for the 2020 specimen was 24.67%, and for the 2009 specimen, 12.02%.

LAMININ

Alaminin did not react between the specimens, and both were negative for this protein.

VIMENTINE

The pattern of immunopositivity for avimentin differed appreciably between membranes. In the 2009 membrane, only one face showed maintenance of protein expression, especially in fusiform cellularity spread by connective tissue. On the other hand, the entire membrane portion was positive for this protein in the 2020 specimen.

Antibody	Source	Dilution	Brand
Anti-Vimentine	Rabbit	1:500	Santa Cruz Biotechnology
Anti-Collagen I	Mouse	1:100	Sigma-Aldrich
Anti-Collagen IV	Mouse	1:300	Sigma-Aldrich
Anti-Laminin 5	Rabbit	1:100	Sigma-Aldrich

TABLE 1 - ANTIBODIES

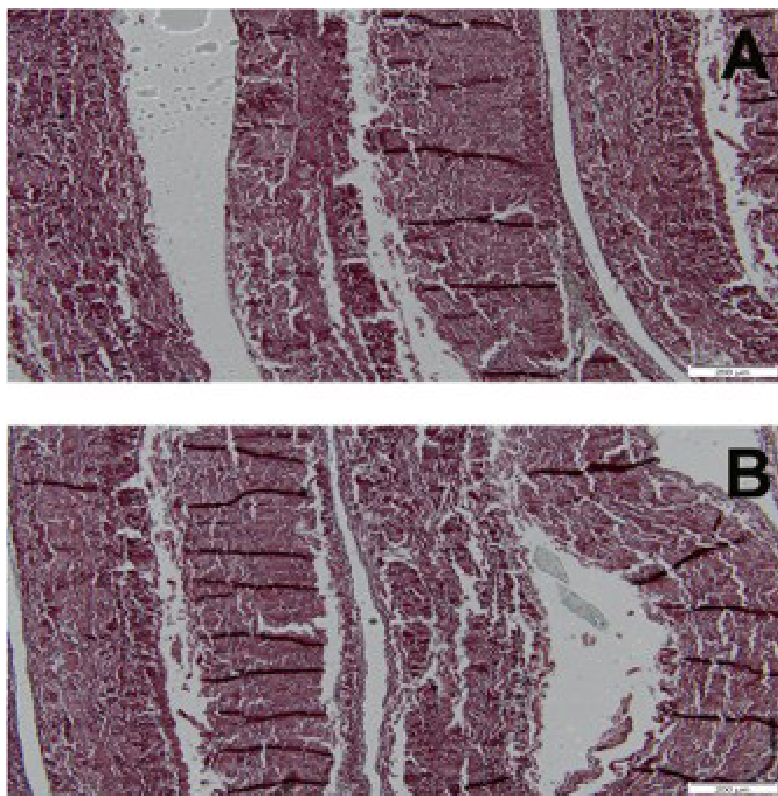

Figure 1.Histological micrograph demonstrating histological similarities between the pieces of 2009(A) and 2020(B).

Source: Courtesy of AllanFernandoGiovanini(2021)

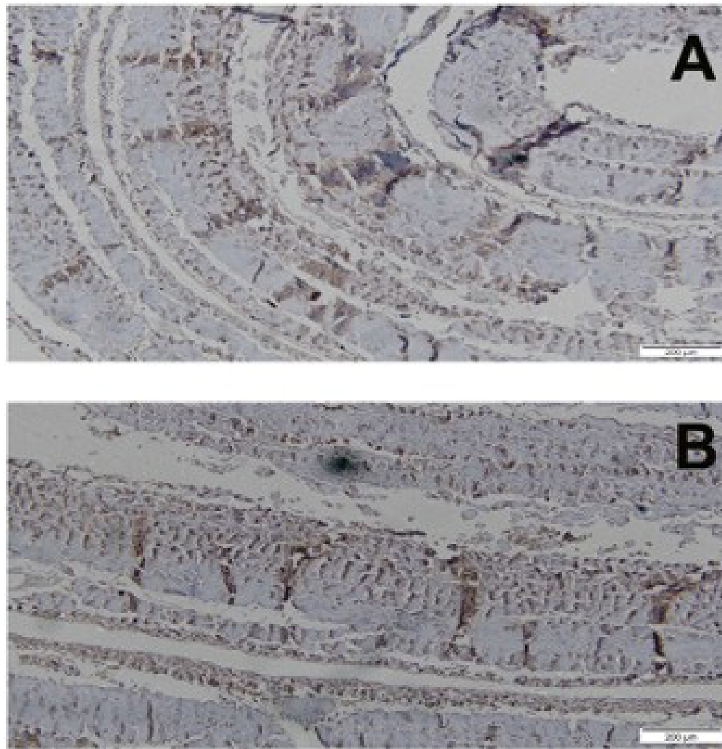

Figure2.Immunoexpression of collagen between the 2009(A) and 2020(B) specimens. Check biggest expression in B.

Source: Courtesy of AllanFernandoGiovanini(2021)

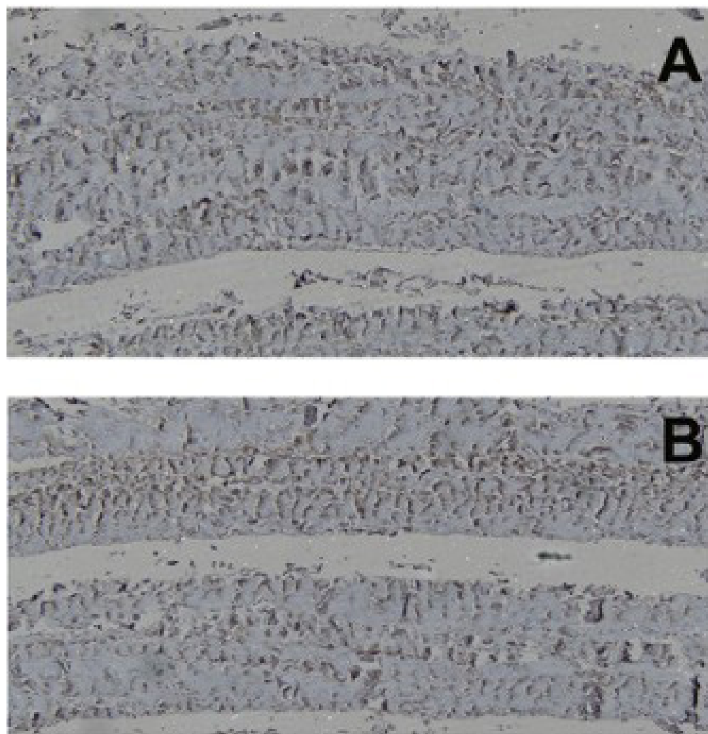

Figure 3. Immunoexpression of collagen IV among the 2009 (A) and 2020 (B) specimens. Check bigger expression in B.

Source: Courtesy of Allan Fernando Giovanini (2021).

As for the percentage occupied by vimentin, there was also a difference between the specimens. percentage found in cellular contingent was 54.34%, while a diffuse and little cellular finding (13%) was evidenced in the 2009 group.

DISCUSSION

Maintaining the integrity of membrane structural proteins is critical to the longevity of a xenograft. This applies not only to tissue implanted in a patient, which is subject to the shear forces caused by blood flow, but also to that membrane that has not yet been used, that is, that is stored in a preservation medium (eg, aqueous formaldehyde solution) for use in a future xenotransplantation. The present study brought data about the second situation mentioned above, which demonstrated an important process of degradation of structural proteins of membrane from a patch of bovine pericardium produced in 2009 (9 years after expiration). The proteins under study are: collagen I, collagen IV, laminin and vimentin.

When the microscopic appearance was observed, in the light of hematoxylin and eosin, it was not possible to observe differences or significant changes between the 2020 and 2009 patches (fig.1). Therefore, the microscopic structure of the 2009 pericardium was maintained, even after a decade of its manufacture. The 2009 patch, as well as the 2020 patch, showed a typical composition of membranes rich in collagen proteins, ie, rich in dense wavy bundles arranged three-dimensionally. Cellularity was sparse and fusiform in appearance, probably suggesting the presence of mature fibrocytes. According to HOIT, 2017, the quantity and orientation of collagen fibers are fundamental to guarantee the viscoelastic mechanical properties characteristic of a pericardium. However, only by microscopic analysis by HE it is not possible to say whether or not there was

Immunohistochemical analysis for type I collagen showed a significant percentage reduction of this protein in the 2009 patch when compared to the 2020 patch (15.87% versus 21.36%, respectively). According to PORTO, 2007, type I collagen is the most abundant representative of the fibrillar collagen category present in a collagen membrane, such as bovine pericardium, giving the tissue a very high resistance to tension forces. RICARD – BLUM, 2012, in a broader analysis of the fibrillar collagen category (which includes type III collagen), concluded that, in addition to tensile strength, fibrillar collagens play a very important structural role by contributing to the molecular architecture of the tissue.

Such loss of protein substrate also occurred when type IV collagen was analyzed by immunohistochemistry. Diffused and irregularly arranged in both membranes, however, showed a significant percentage difference between the 2020 patch and the 2009 patch (24.67% and 12.02%, respectively). Type IV collagens belong to the group of collagens that form a network of the extracellular matrix, participating especially as structural components of the basement membranes. These proteins bind to soluble glycoproteins, proteoglycans, and tissue growth factors to establish a fully functional basal membrane (WU;GE,2019). Such a property of collagen IV, according to the article, may be important for the in vivo remodeling of a xenograft, remodeling is understood to be the process of angiogenesis and repair of a previously injured endothelial tissue. Therefore, considering the studies mentioned above, it is hypothesized that the 2009 patch, when compared to the 2020 patch, has a considerably lower potential for remodeling in vivo.

Finally, immunohistochemical analysis regarding vimentin protein also showed a percentage reduction in the 2009 patch (13%, in contrast to 54.34% of the 2020 specimen).

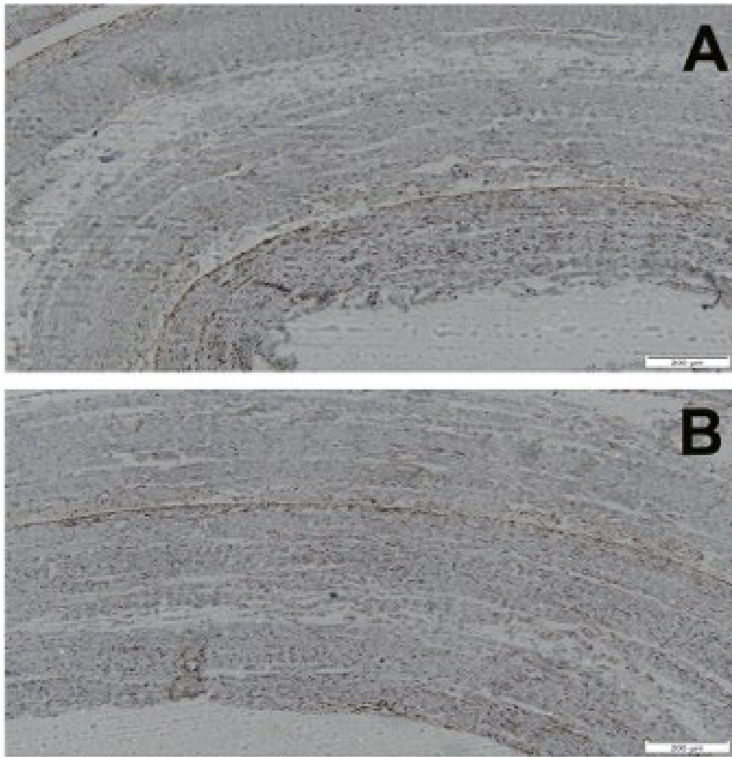

Figure4.Immunoexpression of vimentin between the 2009 (A) and 2020 (B) specimens. Check for greater expression in B, which shows positivity in the entire membrane area, while in A it reveals expression pattern restricted to a membrane face.

Source: Courtesy of Allan Fernando Giovanini (2021).

Unlike the 2020 patch, which showed positivity for this protein in the entire membrane area, the 2009 patch showed a diffuse, poorly cellularized patch concentrated in a single membrane surface. Vimentin is a marker protein of quiescent fibroblastic like cells (TEDDER et al., 2011). In an article published in the year 2019 in the journal Scientific Reports, Khorramirouz et al. demonstrated that the potential for tissue regeneration in a porcine pericardial membrane in vivo was directly related, among other factors, to the immunopositivity of the vimentin protein. Therefore, in light of the studies brought above, we can hypothesize that the 2009 bovine membrane has a considerably lower potential for tissue regeneration when compared to the 2020 membrane.

CONCLUSION

Although there are no important morphological changes between the membranes, in this pilot study we can see that the 2009 membrane loses important proteins of support and cellularity, suggesting a loss of functional substrate that provides its use for future valve implants.

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CERTIFICATE

We certify that the proposal entitled "DIFFERENCES IN THE EXTRACELLULAR MATRIX OF BOVINE PERICARDIES TREATED WITH GLUTARALDEHYDE RELATED TO THE STORAGE TIME IN

FORMALDEHYDE", protocol No. 0108/2021, under the responsibility of ALEXANDRE GELÁS HADDAD, DOUGLASMESADRIGEWEHR, FERNANDOBERMUDEZKUBRUSLYEVICTORDANIELFALKENBACH

TENIUSand participation/guidance of LUIZ FERNANDO KUBRUSLY - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except man), for scientific research (or teaching) purposes - is in accordance with the precepts of the Law No. 11,794 of October 8, 2008, of Decree No. 6,899, of July 15, 2009, and with the rules issued by the National Council for the Control of Animal Experimentation (CONCEA), and was approved by the Ethics Committee in the Use of Animals from Faculdade Evangélica Mackenzie do Paraná (CEUA/FEMPAR), at a meeting held on 04/22/2021.

Term of Authorization:	12 months (every 180 days Pa Reportfinal or final)
Purpose:	() Teaching (X) Scientific Research
Species / Lineage / Race:	-Number ofAnimals: -
Weight/Age:	-
Sex:	-
Origin:	Institute of the Heart of Curitiba-INCOR

Curitiba, April 22, 2021.

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