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A study on the biocompatibility and integration of acellular polyanionic collagen:elastin matrices by soft tissue

Um estudo sobre biocompatibilidade e integração de matrizes acelulares polianiônicas de colágeno:elastina com tecidos moles

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Abstract
One alternative for organ and tissue transplantation for the substitution or failure of a biological function is tissue engineering, based on the growth of isolated cells in three-dimensional biodegradable matrices. This work describes the preparation, characterization, and the behavior of polyanionic collagen:elastin matrices prepared from native bovine pericardium, and implanted in the subcutaneous of rats. The results showed these materials correspond to acellular polyanionic collagen:elastin matrices formed by preserved collagen fibers. In comparison to native bovine pericardium, the overall biological response was characterized by a progressive decrease of the inflammatory response, and practically non-existing for material with higher carboxyl group content, suggesting the high degree of biocompatibility of this acellular collagen:elastin matrices. Evidences for material incorporation by the surrounding tissue observed in this work indicate that polyanionic collagen:elastin matrices may be of potential use in tissue engineering.
Keywords: Acellular matrices, biocompatibility, elastin, polyanionic collagen.

Resumo
Uma alternativa para o transplante de tecido e órgãos ou para a substituição de funções biológicas é a engenharia de tecido, baseada no crescimento de células isoladas em matrizes tridimensionais biodegradáveis. Este trabalho descreve a preparação, a caracterização e o comportamento de matrizes polianiônicas de colágeno:elastina preparadas a partir de pericárdio bovino nativo e implantadas no subcutâneo de ratos. Os resultados mostraram que estes materiais correspondem a matrizes acelulares polianiônicas de colágeno:elastina formadas por fibras colágenas preservadas. Em comparação ao pericárdio nativo, a resposta biológica foi caracterizada pelo decréscimo progressivo da resposta inflamatória, e, praticamente, não existindo para o material com elevados grupos de carboxila, sugerindo alto grau de biocompatibilidade destas matrizes acelulares de colágeno:elastina. Os resultados mostram também evidências da incorporação do material pelo tecido adjacente, sugerindo que as matrizes polianiônicas de colágeno:elastina podem ser de uso potencial na engenharia de tecido.
Palavras-chave: *Biocompatibilidade, colágeno polianiônico, elastina, matrizes acelulares.*

Introduction

One alternative for organ and tissue transplantation for the substitution or failure of a biological function is tissue engineering (Langer *et al.*, 1993; Deuel e Zhang, 1997; Kim *et al.*,1998) based on the growth of isolated cells in three-dimensional biodegradable synthetic matrices (ECM), to produce in vitro or in vivo functional structure similar to the original tissue (Langer *et al.*, 1993; Deuel e Zhang, 1997; Kim *et al.*,1998). These matrices are usually prepared from synthetic or natural materials and the following characteristics are desirable: a) highly biocompatible; b) appropriate mechanical properties adapted to tissue-specific function and cellular growth; c) controlled rates of biodegradation or resorption in order to allow for host cells to spread and increase in quantity for efficiently tissue reconstruction; and d) it should allow for interaction with specific cellular growth factors to induce rapid and more specific response. In this respect synthetic polyesters, among other polymers, derived from hydroxy acids such as polyglycolic, polylactic and their copolymers have been widely used (Hutmacher *et al.*, 1998). Nevertheless, they are not exempt from undesirable side reactions due to dynamic changes in biodegradation kinetics, to local release of by-products (Bergsman *et al.*, 1993), and frequently are associated to an undesirable and constantly active inflammatory process (Bergsman *et al.*, 1993). Natural polymers for ECM manufacture include among other products, proteoglycans, collagen and elastin (Kim *et al.*,1998), and are widely used not only due to their high biocompatibility, but also because they actively interact with specific cell receptor, participate in cellular phenotypic expression, and therefore in the maintenance of tissue morphology, function and remodeling (Alberts *et al.*, 1994). Based on this, the use of collagen:elastin ECM produced from homologous or heterologous sources by processes capable to remove cells and other tissue components have been described (Probst *et al.*, 1997; Delustro *et al.*, 1990; Goissis *et al.*, 2000).

In most studies with collagen intended for biomedical applications, type I collagen have been the material of choice without any chemical or structural modifications. In this work we describe the biocompatibility behavior of polyanionic collagen: elastin matrices prepared from bovine pericardium (BP) and treated with an alkaline solution for the removal of cellular and other tissue components in a similar way as described for the preparation of matrices from segments of the thoracic and abdominal aorta (Goissis *et al.*, 2000). Under these conditions carboxyamide groups from asparagine and glutamine are selectively hydrolyzed, resulting in a collagen matrix with an excess of carboxyl groups (Bet *et al.*, 2001). Membranes and composites with hydroxyapatite prepared with polyanionic collagen gel were highly biocompatible (Goissis *et al.*, 1999) and efficient in the repair of periodontal ligament (Cirelli *et al.*, 1997; Rosetti *et al.*, 2000). Besides this properties, polyanionic collagen materials have been characterized by increased dielectric properties (Plepis *et al.*, 1996) particularly in the form of composites with poly(vinylidene fluoride):trifluorethylene (Goissis *et al.*, 1999).

Material And Methods

Material used in this work was bovine pericardium (BP) furnished by Braile Biomédica S.A., São José do Rio Preto, SP, Brazil, from animals between 30 and 60 month of age, selected, cleaned and manipulated (except for glutaraldehyde treatment) as described for the manufacture of cardiac valves.

Polyanionic collagen:elastin matrices (PACE) (Bet *et al.*, 2001): To hydrolyze amide groups selectively in collagen rich tissues (MB), 50 g of BP, and in the wet state, were treated at 20°C from periods of 24, 36 and 48 hours, with an alkaline solution (3 mL of solution/g of tissue) containing 6% by volume of dimethylsulfoxide, salts (chlorides and sulfate), bases of alkaline (K^+, 1.19 M and Na^+, 1.74 M), and alkaline earth metals (Ca^{++}, 0.86 M). The resulting materials were equilibrated with a solution containing Na_2SO_4, NaCl, KCl, and $CaSO_4$ (6 mL of solution/g of tissue) for a period of 12 hours and after removal of residual salt, materials were equilibrated in 0.14 M phosphate buffer pH 7.4, washed once with water. After lyophilization in EDWADS Mod. FREEZE DRYER Modulyo (Edwards High Vacuum International, Manor Royal, Crawly, West Sussex, RH11 2LW, England), and characterization (potentiometric titration, elastin content, DSC, light microscopy, SEM, TEM), PACE with 46 ± 12 (PACE46), 66 ± 12 (PACE66) and 87 ± 17 (PACE87) extra carboxyl groups materials were stored at 5ºC in aqueous 70% ethanol.

Subcutaneous Experiments with Polyanionic collagen:elastin matrices: After equilibration in physiological sterile saline solution, native BP, PACE46, PACE66 and PACE87 were implanted subcutaneously in 30 male rats, Wistar strain, with an average weight of 220 g. During the experiment they were fed with a semi-solid diet and water at will. The animals were

submitted to anesthesia with ethyl ether and after antiseptic cleaning done with an iodine solution, tricotomy was performed on the dorsal scapular and pelvic regions. Incisions of 0.7 mm in length were done in the regions above (right and left) and PACE pieces of 1.0 cm2, were introduced at 3.0 cm distance from the incision. The animals were killed after 5, 14, 28, 74, 102, 120 and 180 days from implantation, the implants removed and immediately immersed in phosphate buffered 10% formalin and fixed for 24 hours. The specimen after embedding in paraffin using standard procedures, 5.0 mm section were stained by routine procedures, with Hematoxylin/Eosin, Gomori's trichrome stain, Weighert's resorcin:fuchsin and Von Kossa's stain, and examined under light microscope.

Results

Figure 1 showed that pore size of lyophilized PACE materials increased with the time of alkaline treatment and in the range from 5.9 to 10.3 m for PACE46 (Figure 1a) and PACE87 (Figure 1b) respectively. Although not shown, swelling results indicate pore may be significantly higher in implanted materials.

Major components in PACE46 correspond to well preserved collagen fibrils associated to elastin in decreasing amount with increasing time of tissue processing. While in native BP the determined elastin content was 4.8 ±0.8 % w/w, it reduced to 3.2 ±1.0 % w/w after 72 hours processing. Morphologically, PACE66 and PACE87 were similar to PACE46, except with increases in space between collagen fibrils.

The biocompatibility studies of polyanionic collagen:elastin matrices were performed with lyophilized PACE with 46, 66 and 87 extra-carboxyl groups and the extension of tissue response evaluated after days 5, 14, 28, 74, 102, 120 and 180 from implantation.

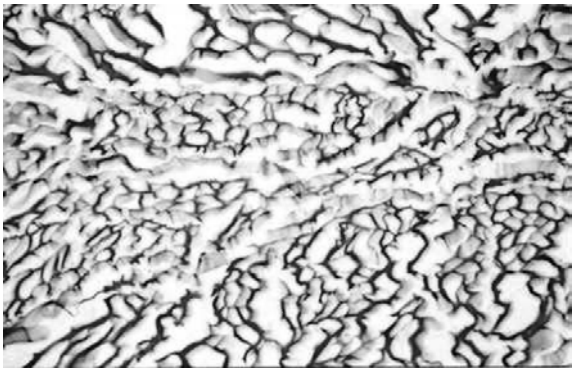
Day 5

Native BP: Implants were characterized by the appearance of fibrous connective tissue associated to pseudocapsule formation, with an increased cell population and peri-implant vascularization. The inflammatory infiltrate adjacent to the implant was moderate to intense with predominance of polymorphonuclear neutrophils, lymphocytes and histiocytes. In the internal region of BP implants, the inflammatory infiltrate was discreet with the prevalence of mononuclear cells. Gigantocytes were not observed. In comparison to native BP before implantation, collagenic and elastic fibers were still well preserved.

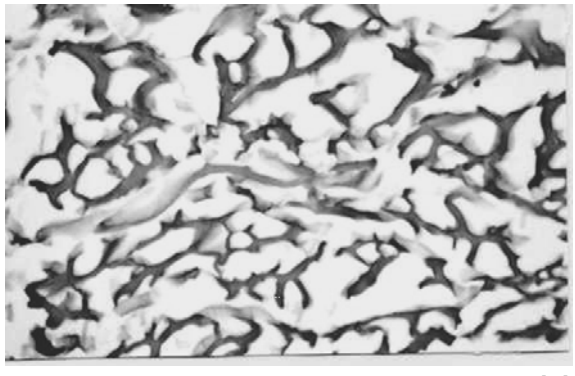
PACE46, 66 and 87: Although the overall characteristics adjacent to the implant were the same as those described for native BP, as exemplified by PACE87, the inflammatory infiltrate was less intense, with prevalence of polymorphonuclear neutrophils, lymphocytes and histiocytes. Other major difference in respect to native BP implants was the presence of only a few inflammatory cells in PACE46 and 66, and the complete absence in PACE87. In comparison to native BP, collagenic and elastic fibers were less preserved, but much more as a result of the alkaline conditions used to hydrolyze carboxyamide side chain of Asn and Gln side chains, than as a result of the inflammatory process over the implants.

Day 14

Native BP: Compared to day 5 the inflammatory infiltrate adjacent to the implant was of higher intensity, with the prevalence of lymphocytes, histiocytes,



(a)



(b)

Figure 1. Photomicrographs of polyanionic collagen:elastin matrices prepared after selective hydrolysis of asparagine and glutamine amide side chains, after Hematoxylin/Eosin staining. **(a)** Net increase of 46 ± 12; and **(b)** 87 ± 17 extra carboxyl groups HE, 350x.

associated with multinucleated giant cells, characterizing a foreign body type of reaction. A similar behavior was observed in the internal region of the implant. Except in one of the explants, collagenic and elastic fibers were still well preserved.

PACE46, 66 and 87: Inflammatory infiltrate adjacent to all PACE implants were similar to that described after day 5, except less intense, and with practically no inflammatory cells detected in the internal region of all PACE the explants. Collagen and elastic fibers still showed a high degree of preservation.

Day 28

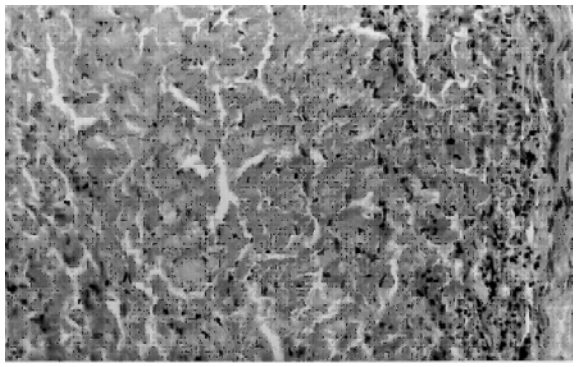
Native BP: After this period implants were characterized by an increase in fibrous connective tissue, cell population and peri-implant vascularization (Figure 2a). In comparison to day 14 from implantation the inflammatory infiltrate was more intense adjacent to the implant (Figure 2a), with alterations in the morphology of collagenic and elastic fibers already being detected.

PACE46, 66 and 87: No increases in fibrous connective tissue or vascularization were observed. As after day 14, no inflammatory cell was observed adjacent or in the internal region of PACE87 (Figure 2b) and only a few were observed in PACE46 and 66. Collagen and elastic fibers showed the same degree of preservation compared to day 14, suggesting the low activity of the inflammatory process over PACE implants. One interesting observation was the presence of fibroblasts-like cells in all implanted PACE, as exemplified PACE87 which became more evident after 74 days of implantation. Although not shown, Von Kossa's stain did not detect calcification in any of the implants, with minimum cell alterations and no observed evidences of contact necrosis.

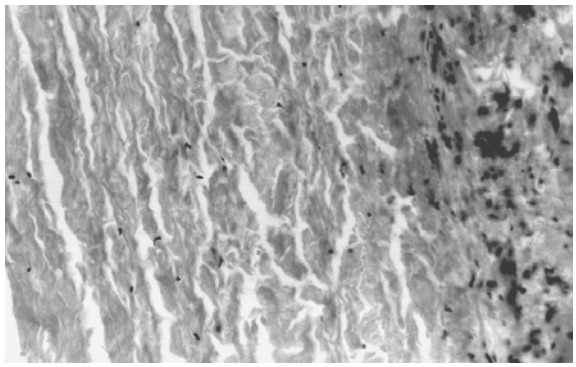
Day 74

Native BP: In comparison to day 28, increase in fibrous connective tissue was accompanied with a decrease in vascularization, and changes in collagen and elastic fibers being more intense. The inflammatory infiltrate adjacent to the implants was intense, and characterized by the presence of mononuclear cells, with prevalence of limphocytes and plasmocytes (Figure 3a).

PACE46, 66 and 87: Although fibrous connective tissue increased in all PACE implants, they were less intense in comparison to BP. Contrary to native BP neovascularization was increased in peri-implants region of PACE. The cell pattern of the inflammatory



(a)



(b)

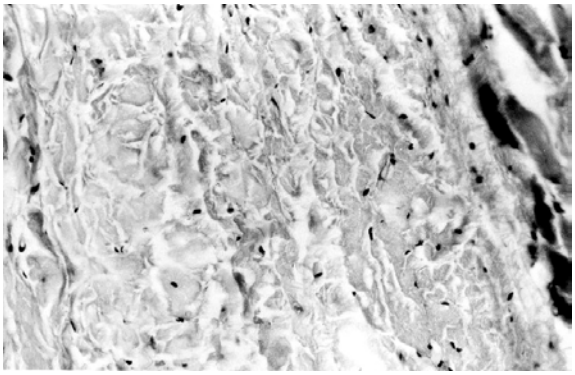
Figure 2. Micrographs from rat subcutaneous implants of bovine pericardium derived materials after 28 days from the implant. **(a)** Native: intense inflammatory infiltrate adjacent to the implants, and discreet in the internal region with prevalence of neutrophils, mononuclear lymphocytes and histiocytes; **(b)** Polyanionic collagen:elastin matrix with an excess of 87 ± 17 extra carboxyl groups: inflammatory infiltrate adjacent to the implants almost non existent and absent in the internal region. HE, 20x.

infiltrate adjacent to the implants was similar to that described for native BP, but the occurrence was very discreet or absent (Figure 3b). In the internal region, the inflammatory infiltrate was only discreet in one of the PACE46 explants and absent in PACE66 and 87. As in native BP, alterations were observed in collagen and elastic fiber morphology, except less intense compared to native BP. After this period fibroblast like cells were observed in all PACE implants.

Day 102

Native BP: After 102 days tissue response was similar to that observed after 74 days from implantation with the inflammatory infiltrate adjacent to BP implants very intense with prevalence of lymphocytes,

(a)



(b)

Figure 3. Micrographs from rat subcutaneous implants of bovine pericardium derived materials after 74 days from the implant. **(a)** Native: intense inflammatory infiltrate adjacent to the implants, and discreet in the internal region with prevalence of mononuclear limphocytes and plasmocytes, Verhoeff, 10x. **(b)** Polyanionic collagen:elastin matrix with an excess of 87 ± 17 extra carboxyl groups: inflammatory infiltrate adjacent to the implants almost non existent and absent in the internal region. HE, 20x.

neutrophiles and mastocytes. In the internal regions of the implant it was only discreet.

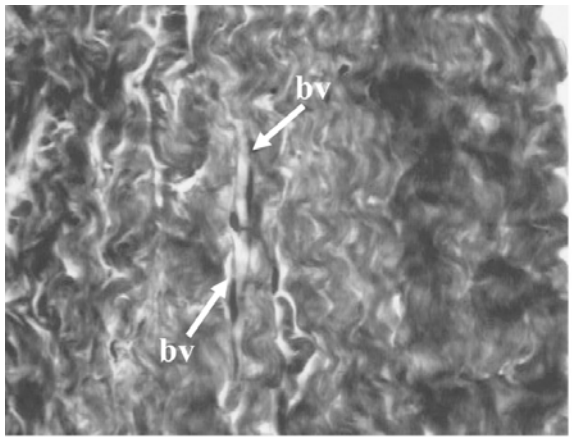
PACE46, 66 and 87: In PACE46 and PACE66 the inflammatory infiltrate adjacent to the implants was discreet or absent as in PACE87. It was absent in most of the internal regions of PACE implants. Collagenic and elastic fibers were altered in all implants, except more intense on native BP.

Day 120

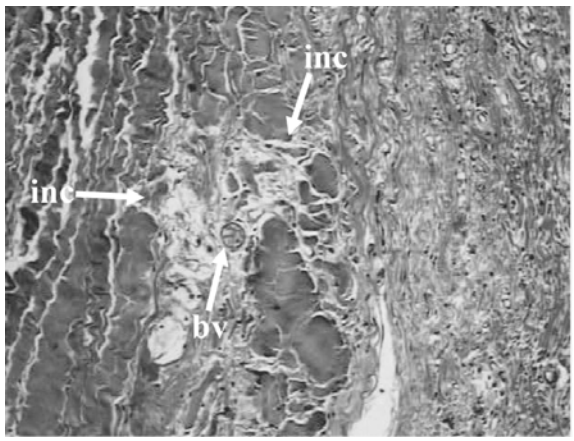
PACE46 and 87: The inflammatory infiltrate adjacent to implants was discreet and it was not detected on surface or internal region of these materials. Collagen and elastic fibers still showed a high degree of preservation, especially in PACE87, associated with neo-vascularization, now observed within the internal region of the implants (*Figure 4a*). Fibroblast like cells were observed throughout the implants. Fibrosis process, with neovascularization (*Figure 4b*), was dissociating collagenic fibers showing the incorporation of PACE implants (*Figure 4b – delimited area with arrows*).

Day 180

PACE46 and 87: Rare mononuclear inflammatory cells could be found in areas adjacent to all PACE implants.



(a)



(b)

Figure 4. Micrographs from rat subcutaneous implants of bovine pericardium derived materials after 120 days from the implant. **(a)** (PACE with 46 ± 12) transition among surface and internal region of the implanted tissue with blood vessel (bv), and preservation of collagenic fibers, Masson Trichromic 20x; **(b)** (PACE with 87 ± 17) collagen fibers with discreet alteration (on the left); fibrosis process dissociating collagenic fibers (on the right) with vascularization to the internal region of the implant and incorporation process (inc). Masson Trichromic, 20x.

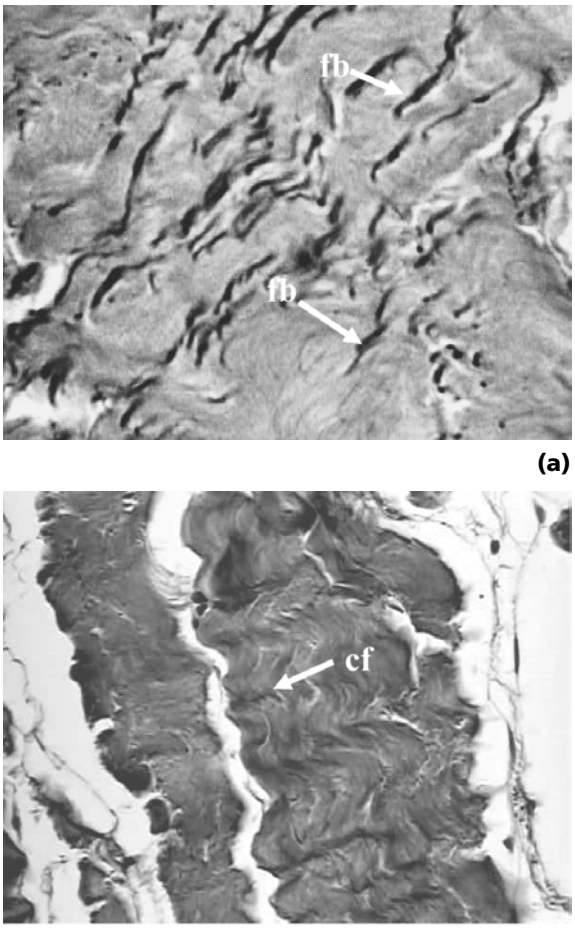


Figure 5. Micrographs from rat subcutaneous implants of bovine pericardium derived materials after 180 days from the implant. **(a)** Internal region of PACE with 46 ± 12, with preserved collagenic fibers and presence of fibroblast-like cells (fb), H.E., 20x**. (b)** Preservation of collagenic fibers (cf), Masson Trichromic, 20x.

Inflammatory cells were not detected on the surface in the internal region of the implants (*Figure 5a*). Collagen and elastic fibers organization of the implants were still observed even after this long period from implantation (*Figure 5b*). At this stage, the population of fibroblast like cells was quite significant, and similar to that found in native BP (*Figure 5a*). In theses materials, vascularization accompained the fibrosis process and was intense both on the surface and in the internal region.

Discussion

The results above showed that independently from the number of extra-carboxyl group present in PACE, the inflammatory process was discreet and decreasing (Figures 3-5), and apparently related to the inverse of carboxyl group content, as suggested by PACE87 implants. This results associated with the absence of calcification, cell alterations or contact necrosis, points to the high biocompatibility of PACE matrices, an important feature for materials intended for tissue engineering. On the contrary, native BP implants were always associated intense tissular response that increased with the time of the implants. The higher biocompatibility of PACE compared to native BP is probably associated with the removal of cellular and other tissue components since failures associated to bovine pericardium-based biomedical devices are attributed to the presence of cells, particularly phospholipids responsible for the observed dystrophic calcification (Rossi *et al.*, 1990) described for native BP implants, which is associated with an intense inflammatory foreign body type of reaction (Rossi *et al.*, 1990), in agreement with the cellular response described in this work.

Although the interconnected porous structure of PACE (*Figure 1*) may explain the migration of fibroblast to the internal region of the implants, the low or the absence of an inflammatory process associated to tissue incorporation, as shown by PACE87 after 120 days (*Figure 4*) are not easily explained, unless PACE implants are in close interaction with cell and the surrounding tissue, as suggested by the primary ossification detected around polypropylene mesh coated with polyanionic collagen prepared from materials corresponding to PACE46 (Goissis *et al.*, 2001) which are characterized by improved dielectric properties (Plepis *et al.*, 1996). With respect to incorporation of the material by the surrounding tissue, similar results were also observed for PACE46 explants after 90 days in the rabbit sclera (Rocha *et al.*, 2001) and for PACE46, 66 and 87 in the tibia of rats (Rocha *et al.*, 2001).

These results on the incorporation of PACE implants are uncommon for other biomaterials from synthetic or natural origin due to the installation of chronic inflammatory reaction as a result of a clear interface between implant and surrounding tissue (Kao *et al.*, 1999).

Conclusions

The results show that materials prepared by the selective hydrolysis of carboxyamide side chains of Asn and Gln present in type I collagen of native BP correspond to acellular polyanionic collagen:elastin materials formed by preserved collagen fibers, except with an increasing carboxyl group content. In comparison to native bovine pericardium, the overall biological

response was characterized by a progressive decrease of the inflammatory process, that was practically non-existing for material with higher carboxyl content, suggesting the high degree of biocompatibility of this acellular polyanionic collagen:elastin matrices. Evidences for material incorporation by the surrounding tissue observed in this work, and already detected for the same materials implanted in the sclera of rabbits and in the tibia of rats indicate that polyanionic collagen:elastin matrices may be of potential use in tissue engineering.

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