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Biocompatibility *in vivo* of elastic cartilage treated in alkaline solutions

[*Biocompatibilidade* in vivo *de cartilagem elástica tratada em soluções alcalinas*]

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ABSTRACT

This study verified the in vivo biocompatibility of bovine elastic cartilage decellularized with alkaline solution in relation to the non-decellularized cartilage implanted in rats. Fifty Wistar rats were divided into two groups, with the experimental group (EG) receiving subcutaneous implants of cartilage treated in alkaline solution and the control group (CG) receiving subcutaneous implants of untreated cartilage. In both groups, the implants were removed on days 3, 7, 14, 21 and 42 with the preparation of histological slides stained with Hematoxylin and Eosin for the quantification of inflammatory cells, fibroblasts, angiogenesis, and cartilage degradation. The results showed that EG presented a less intense inflammatory infiltrate and better organization of collagen fibers compared to CG. It was concluded that the alkaline treatment provided better biocompatibility for elastic cartilage when implanted subcutaneously in rats.

Keywords: alkaline solution, biomaterial, cartilage, and collagen

RESUMO

Este estudo verificou a biocompatibilidade in vivo *de cartilagem elástica bovina descelularizada com solução alcalina em relação à cartilagem não descelularizada implantada em ratos. Cinquenta ratos Wistar foram divididos em dois grupos; o grupo experimental (GE) recebeu implantes subcutâneos de cartilagem tratada em solução alcalina, e o grupo controle (GC) recebeu implantes subcutâneos de cartilagem não tratada. Em ambos os grupos, os implantes foram retirados nos dias três, sete, 14, 21 e 42, com a preparação de lâminas histológicas coradas com hematoxilina e eosina para a quantificação de células inflamatórias, de fibroblastos, de angiogênese e para a degradação da cartilagem. Os resultados mostraram que o GE apresentou infiltrado inflamatório menos intenso e melhor organização das fibras de colágeno em relação ao GC. Concluiu-se que o tratamento alcalino proporcionou melhor biocompatibilidade para a cartilagem elástica quando implantado por via subcutânea em ratos.*

Palavras-chave: solução alcalina, biomaterial, cartilagem e colágeno

INTRODUCTION

The use of animal cartilage as a source of collagen and elastin has attracted the attention of researchers due to its availability, particularly in its bovine and swine forms, and because the cartilaginous tissue is very resistant to autolysis (Shin *et al.*, 2018).

The direct use of cartilage without previous decellularizing treatment provokes an immediate inflammatory response to the host tissue against the implant, regardless of whether this material belongs to animals of the same species or if it is xenogeneic (Reis Filho *et al.*, 2017). To avoid these intense reactions, the cartilage has to undergo a decellularization treatment to reduce the antigenicity and slow down the inflammatory process (Gilpin and Yang, 2017).

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The decellularized matrix of the cartilage which is implanted in animals can guarantee a better biocompatibility and generate new medical devices, such as pharmacological vehicles, or serve as a matrix in which the cells inserted in the host — or those that belong to the host — can adhere to, proliferate, differentiate, and reoccupy the lost tissue space (Nürnberger *et al*., 2019).

Amongst the chemical methods for decellularization, the use of alkaline solutions can be highlighted. These solutions are generally composed of bases which contain different concentration of various alkaline elements, such as ammonium, sodium sulfide and sodium hydroxide. The high pH resulting from this solution can solubilize cell components like the plasma membranes, organelles, and nucleic acids (Keane *et al*., 2015).

The decellularization method by alkaline solution can assist in the production of biomaterials which have better biocompatibility. This method can also further improve the biocompatibility of biomaterials already known in the medical routine, such as collagen of animal origin. This study aims to compare the biocompatibility of bovine elastic cartilage treated in alkaline solutions with those that are not treated in rats of the Wistar breed.

MATERIAL AND METHODS

This research and the use of rats were authorized by the Ethics Committee of the UFG-Jataí regional, protocol Nº. 019/2017. The cartilaginous matrix used to produce the biomaterial that was collected from auricular cartilage of bovine ears and cut in squared fragments of 1.5cm each.

Half of the cartilage fragments (N=25) were taken to the Biochemistry and Biomaterials Laboratory (Chemistry Institute – Universidade de São Paulo – São Carlos, São Paulo, Brazil). In the laboratory, the material was defrosted and immersed in a 0.9% sodium chloride solution for four hours, and the solution was changed every hour. Subsequently, the cartilage was treated in an alkaline solution containing salts (chlorides and sulfates) of alkaline (K+ and Na+) and alkaline earth metals (Ca+2) as described earlier (Horn *et al*., 2009). The other half of the cartilage fragments (N=25) did not undergo the alkaline treatment. The treated and untreated cartilages were sterilized in ethylene oxide.

After the preparation of the two types of cartilage (treated and untreated in alkaline solution), these were implanted in animals. Fifty male rats of the Wistar breed were utilized. They were around ten weeks old and weighted approximately 400g.

The group of fifty rats were randomly distributed between two experimental subgroups (N=25) according to the biomaterial to be implanted. Those allocated to the Experimental Group (EG) received the implants with cartilage treated in alkaline solution, whereas those allocated to the Control Group (CG) received the untreated cartilage.

During the implantation process, the rats were anesthetized with Xylazine and Ketamine (8mg/kg and 60mg/kg respectively, IP), with the surgical region being shaved and with local antisepsis performed with an alcoholic solution of chlorhexidine (0.5%). The surgical incision was made in the dorsal region between the scapulae with the cartilage tissue placed in the subcutaneous space. The surgeon was careful to place the implant as far as possible from the surgical incision (International…, 2007). The incision was sutured with nylon (4-0) thread in single stitches and the post-surgical anesthesia was performed with tramadol (1 ampoule with 100mg diluted in 500ml of water).

After the cartilage implementation, the CG and EG groups were divided in five subgroups each (N=5) according to the period of cartilage removal, which was performed on the third, seventh, fourteenth, twenty-first, and forty-second day post implantation. The same unaesthetic and surgical procedures were performed for the removal of the implants, considering that there were higher levels of anesthesia and a euthanasia due to cervical dislocation.

The analysis and the macroscopic scoring were performed during the removal of the implants following a modified model of the ISO 10993-6, (International…, 2007) standards, in which the characteristics of the neovascularization, the adherence of the cartilage to deeper planes, their encapsulation and their change in coloration were evaluated. These changes were graded according to the intensity of the lesions in grade I, II, III or

absent. Other characteristics that eventually occurred were clots, exudate, transudate, fibrosis, and other types of alterations.

The morphometric analysis of the tissues colored in H&E and Picro-Sirus Red was performed under light microscope with the polarizing lens being used for the Picro-Sirus Red.

The analysis in H&E of the mononuclear (MN), polymorphonuclear (PN) and the adjacent connective tissue (ACT) populations resulted in five random fields observed with 400x magnification in the interface region between the cartilage and the subcutaneous tissue in each of the fifty samples collected. In order to rank the quantity of cellular types, a score of 0, 1, 2 and 3 was established in which "0" represented the absence of inflammatory cells and connective tissue; score "1" possessed a discreet quantity of cells without forming large clusters; score "2" contained a moderate quantity of cells forming clusters which were not very concentrated; and score "3" presented an accentuated quantity of cells forming clusters which were very concentrated and coalescent in the most part of the tissue evaluated (Garros *et al*., 2006 and Branco Neto *et al*., 2006 modified). Both the evaluations in H&E and Picrosirus Red were performed blindly and by a single evaluator.

In the tissues colored with Picro-Sirus Red, five random fields were analyzed in high magnification (400x) following the same principle as to the tissue in H&E. The images were captured by a Sony® NEX-3 camera and analyzed with the "Imagem J®" software, version 1.3.1 with the "*Threshold Color*" plugin to characterize the types I and II collagen fibers.

The data was analyzed by the GraphPad Prism 7.04 (San Diego, CA, USA) software. For PMN and MN cells, Mann-Whitney test was implemented, $p<0.05$. The concentration of red collagen (type I) or green/yellow (type III) for CG and EG, Holm-Sidak test was used, $p < 0.05$.

RESULTS AND DISCUSSION

During the experiment, no animals died or had complications related to the surgery or the biomaterial implantation, like edema, formation of exudate, transudate, or suture dehiscence, which is an important macroscopic indication for the com patibility of both materials (International…, 2007; Modulevsky and Cuerrier, 2016) - (Fig.1).

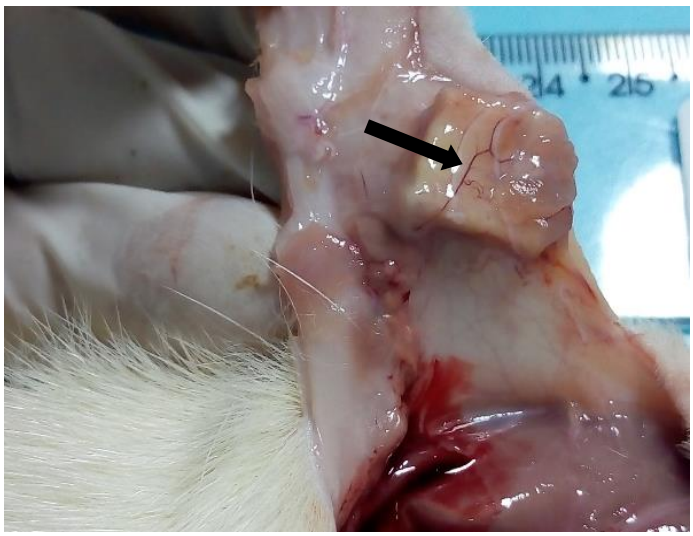

Figure 1. Rat from 3 Day experimental group (EG). Macroscopic aspect of the implantation area of the elastic cartilage treated in alkaline solution. The formation of a thin and translucent connective tissue capsule is noted around the implant. The arrow indicates a blood vessel formed on the connective tissue. neovascularization

The macroscopic results (Table 1) obtained during the removal of the implants did not demonstrate a significant difference either between EG and CG or amongst the days of observation in terms of the encapsulation of the implant which was involved by a thin capsule of connective tissue. In terms of the neovascularisation and adherence of the material, there were no differences between the groups but there were discrepancies amongst the days of observation from the fourteenth day onwards (Table 1).

Table 1. Gross Evaluation

	3 Day		7 Day		14 Day		21 Day		42 Day	
	EG	CG	EG	CG	EG	CG	EG	CG	EG	CG
Neovascularization	+	+	+	+	++	++	++	++	++	++
Adhesion "cartilage-subcutaneous"	+	+	+	+	++	++	++	++	++	++
Change in cartilage color	-	-	-	-	-	-	+	-	+	-
Encapsulation	+	+	+	+	+	+	+	+	+	+

As for microscopic results, it was noted that both types of cartilage had a degree of inflammation. However, they had significantly different concentrations. As the cartilage has a xenogeneic aspect, it is possible that it could still contain protein antigens of animal origin and chondrocytes which are responsible for generating an immunological response on the host (Almine *et al*., 2013). These events could also be related to immunological factors related to the adsorption of the biomaterial and not depend solely on the presence or absence of the chondrocytes, but also on common factors shared by both tissues, like the proteins present in the perichondrium (Thevenot *et al*., 2008).

The difference between the concentrations of inflammatory cells between the EG and the CG is related to the smaller concentration of xenogen antigen in the EG which was decellularized with alkaline solution. This made the EG lose most of the antigens which were derived from the chondrocytes and, consequently, end up with less antigen components present (Wong and Griffths, 2014). It is known that the chondrocytes antigens can trigger immunological and histocompatibility response types I and II which attracts several polymorphonuclear cells (PMN) and mononuclear (MN) cells (Osiecka-Iwan *et al*., 2018). The low concentration of inflammatory cells and its reduction over time can be considered a good indicator of biocompatibility in the EG (Silveira *et al*., 2011).

On the third day of observation, it was observed a high concentration of (PMN) cells in both the EG and the CG, with an accentuated decrease in the following days. Even though the number of PMN was high in both groups, in the CG this number was significantly higher (Fig.2A). The neutrophils are the main PMN observed on the third day, and they practically disappeared in the following days (Fig.3).

For mononuclear cells, it was noted a difference between the groups and over the treatment duration from the seventh day onwards which maintains itself until the forty-second day (Fig.2B). It is mainly observed the macrophage population standing out on the seventh day and being replaced by lymphocytes and plasma cells from the fourteenth day onwards. The giant cells are in discreet quantities from the seventh day onwards and do not change in concentration during the following days of observation. All cell groups had larger populations in the CG (Fig.3).

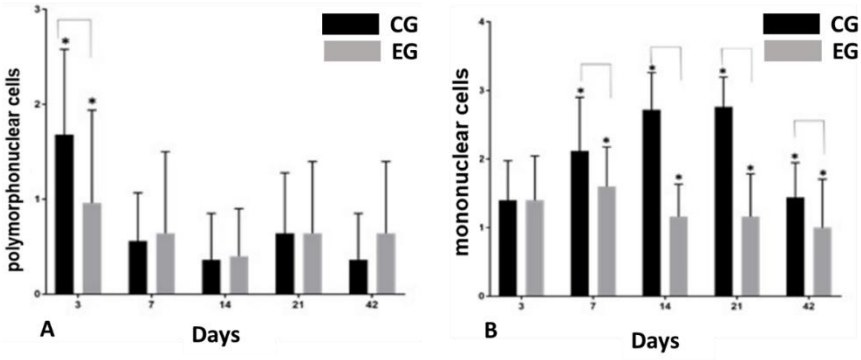

Figure 2. Ranking of the population of polymorphonuclear (PMN) cells (A) and mononuclear (MN) cells (B) for Experimental Group (EG) and Control Group (CG). In A, on day three, both groups presented an increase in PMN cells, particularly the CG. Over the 42 days, there is a decrease in the cell quantity. (*) - Statistical difference compared to the Control Group within the same period, $p > 0.05$. In B, MN increases after the seventh day and maintains itself until the forty-second day. (*) — statistical difference compared to the Control Group (CG) in the same period, $p > 0.05$.

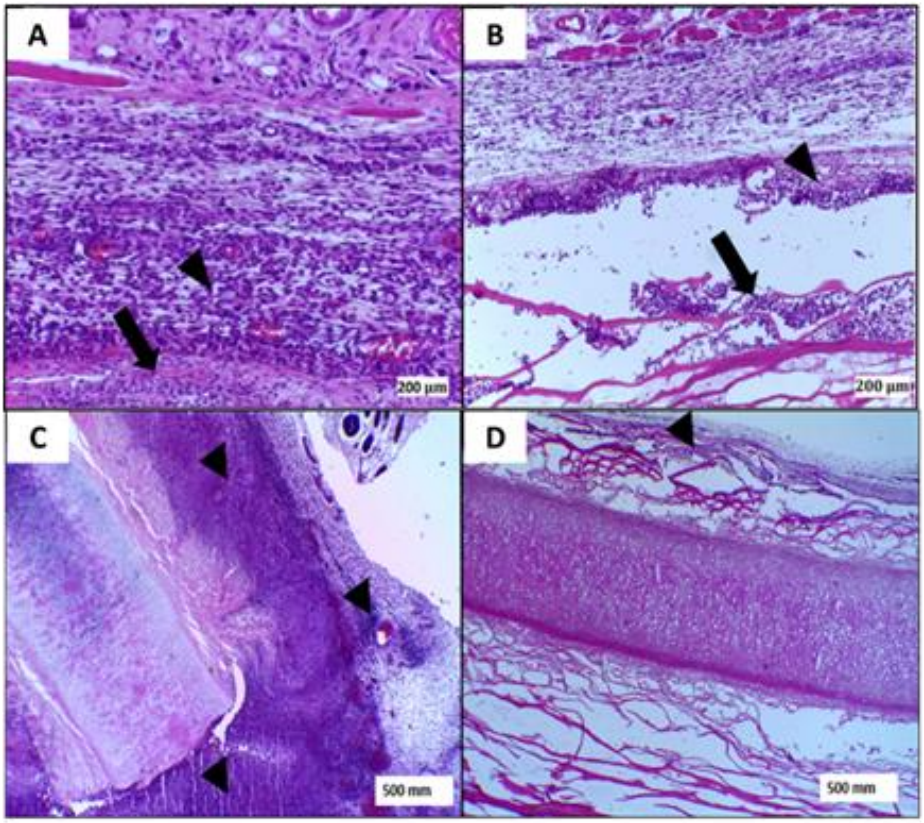

Figure 3. Polymorphonuclear cells in the Control Group – CG (A) and the Experimental Group - EG (B) after three days of implant removal. Arrows = neutrophils; Arrow tips = Macrophages and Monocytes. It is noted that the neutrophils are interspersed in the outer perichondria layers of the cartilage. Coloration H&E, 400x.

The inflammation and replacement of the population of inflammatory cells over the course of the experiment is commonly associated to the innate immune system's reaction to the presence of a foreign body, which is common in material implantation (Trindade *et al*., 2016; Kar *et al*., 2016).

The giant cells are present in similar quantities between the two groups which is in line with what is reported in other research work involving polymeric biomaterials (Subramanian *et al.*, 2013). This feature can be considered a natural reaction to implantation and not a pathological factor. (Al-Maawi *et al*., 2018).

Regarding the disposition of inflammatory cells, it was observed that the surface on the superior and inferior edges of the cartilage, which would correspond to its perichondrium, attracted a large concentration of inflammatory infiltrate (PMN or MN). However, in both groups, these did not deeply penetrate the perichondria layers and therefore did not reach the cartilaginous matrix during the observation (Figure 3). On the side edges, where the cartilaginous matrix is exposed to the animal's organism, there is also a strong inflammatory reaction, particularly in the CG where the most observed cells were the lymphocytes and plasma cells. It was observed that the inflammation is generally less intense and multifocal in the EG for both PN and PMN; as opposed to the CG in which the inflammation is more intense and diffuse.

The strong inflammatory reaction seen on the side borders of the cartilage, which is more evident in the CG, is related mainly to the exposition of the biomaterial's chondrocytes to the host organism which stimulates its immune system. This factor is observed even if the tissue is allogeneic (Osiecka-Iwan *et al*., 2018). In the EG, this response is still present, however it is less intense because the cartilage may contain some cell remnants derived from the chondrocytes which could have been stuck to the cartilage even after successive washes in H3BO3 3% and EDTA 3%.

The remaining small quantity of cells over the course of time — seen in both the EG and the CG — is common to the process of continuous degradation of the biocompatible biomaterials (Gillitzer and Goebeler, 2001). The decellularized materials, as much as they are biocompatible, can still trigger an inflammatory response due to the characteristics of the implant itself, the environment of the implantation, the duration of the implantation and the epitopes which are not correlated to the cells, but to the extracellular matrix (Wong and Griffths, 2014).

Regarding the cartilaginous matrix, it is possible to notice that in both the EG and CG it loses intensity in its basophilic coloration from the edges to the center from the seventh day. It intensifies on the twenty-first day and by the forty-second day, part of the cartilage is 100% without basophilia in the matrix area, possessing only collagen and elastin eosinophilic fibers that are thin and disconnected (Fig. 4). In the CG, it can be observed that the chondrocytes in the cartilaginous matrix gradually disappear, leaving only gaps from the edges to the center. This starts on the fourteenth day and by the forty-second day, there are no longer chondrocytes in the cartilage.

In relation to the loss of basophilia in the cartilaginous matrix and in the CG's chondrocytes from the edges to the center, it can be linked with possible antigenic factors present in the matrix, like the proteoglycans, glycosaminoglycans and the chondrocytes themselves which were exposed to the immune system of the host animal at the interface area of the cartilaginous border. This could have induced the activation of the histocompatibility system types I and II (Osiecka-Iwan *et al*., 2018).

Regarding the connective tissue adjacent to the implant, it was noted a significant difference between the groups (days three and twenty-one) ($p>0.05$), especially in the quantity of fibroblasts being larger in the CG. Qualitatively, the fibroblasts in the EG were better organized from the third day onwards, as they were located parallel to the edges of the cartilage. They were also more concentrated and possessed a low quantity of inflammatory cells interspersed in a pattern that maintained itself until the end of the observation, on the forty-second day. In the CG, it can be observed that the fibroblasts, although surrounding the cartilage, were not parallel and often were distributed randomly or attempting to form multiple non-caseous granulomas over the length of the cartilage. Its fibers were not compact, but more distant from one another with inflammatory cells interspersed between them.

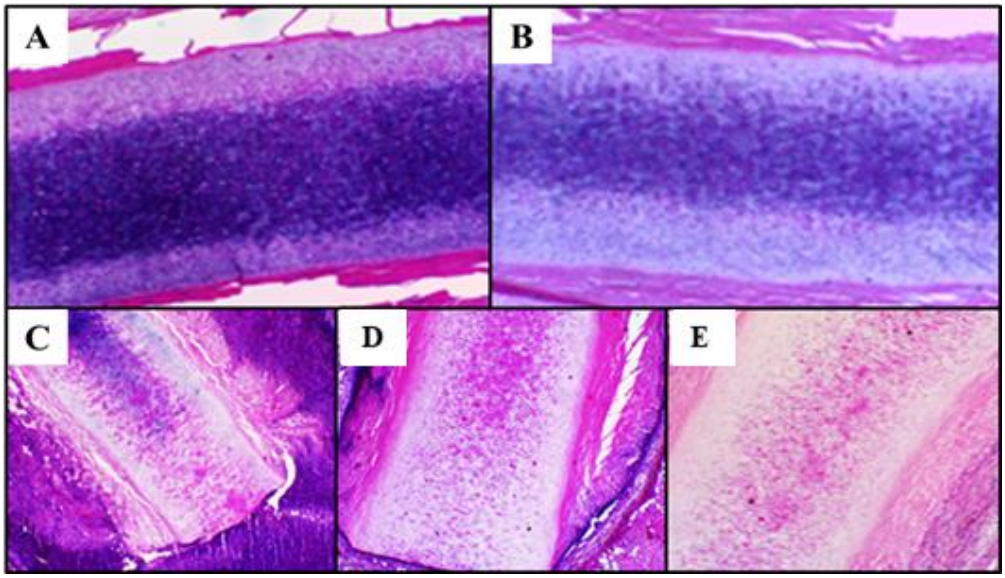

Figure 4. Decrease of the cartilaginous matrix basophilia (dark bluish coloration inside the cartilage) in the Control Group **(CG)** over the course of the experiment. A = 3rd day, B = 7th day, C = 14th day, D = 21st day, E = 41st day. Both the thickness and the intensity of coloring decrease with the advancement of days. Coloration H&E, 40x

On the twenty-first day, there is an attempt in the CG to encapsulate the entire cartilage and the various granulomas in a single, large fibrous capsule with fibroblasts oriented in random directions (Fig. 5). However, on the forty-second day, it can be observed that there is a remodeling of the fibroblasts with less granulomas and these are located more in parallel with the cartilage.

As for the fibroblasts, although there is significant difference between the groups and days 3 and 21. This is since the cartilage possesses some pro-fibroblasts factors which are not associated to the chondrocytes but to the actual cartilaginous material (Meneghin and Hogaboam, 2007), or to factors associated to the surgical implantation like the transforming and growing factors of the platelets (Sinno and Prakash, 2013) and the active presence of the elastin (Almine *et al*., 2013) and fibronectin (Zhu, 2010). However, more studies are needed to know which of these factors influence the most.

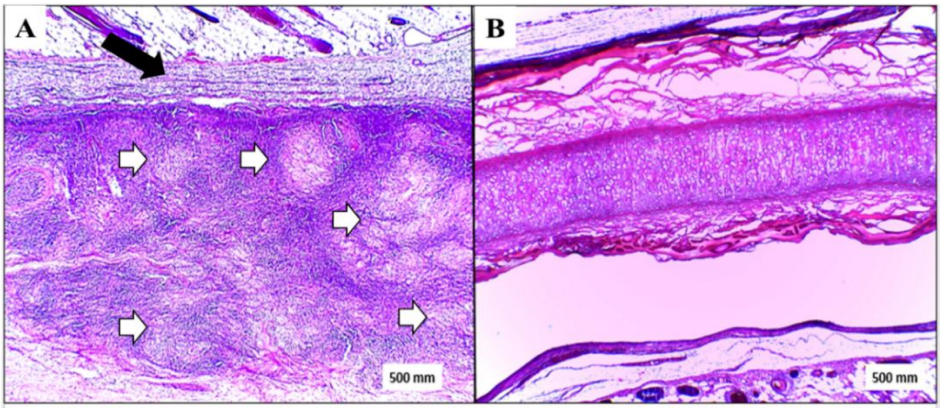

Figure 5. Difference in the arrangement of the fibrous capsule between the Control Group (Figure A) and the Experimental Group (Figure B) in implants removed after 21 days. In the CG, there is an attempt to form multiple granulomas (white arrows) which are then all englobed by a single granuloma (black arrow). Coloration H&E, 40x.

The disorganized arrangement of the fibrosis seen in the CG with the formation of multiple non-caseous granulomas and the attempt to encapsulate all these granulomas and the cartilage could be due to the intense immune stimulation provoked by the chondrocyte's xenogeneic antigens (Ma *et al.*, 2013). The fibroblastic remodeling mainly seen in the CG can be linked primarily to factors induced by the macrophages which can release factors of fibroblastic degradation and remodeling (Brown *et al*., 2012). The good orientation of the collagen fibers is relevant even if compared to other polymers which are considered a positive control in biocompatibility, like the empty polyethylene tubes (Mori *et al*., 2014).

Regarding the formation of new blood vessels, it was observed, microscopically, that there was a difference between the groups on the fourteenth day with the highest mean for CG.

The neovascularization observed in both groups can be linked to the components of the cartilaginous matrix themselves, particularly the elastin whose protein sequences can help in the adherence and the angiogenic proliferation (Staubli *et al*., 2017).

In relation to the quantity of collagen fibers, there was a significant difference in the typification in the areas of collagen type I and also differences between the groups and days 3, 7, 14 and 21 (Fig.6 A), with a higher concentration in the CG. In relation to the collagen type III, there was also a difference between the groups and days 3, 7 and 21, again with the CG presenting higher concentration (Fig.6 B). The fibers of the collagen type I were more concentrated in the form of cords surrounding the cartilage that were more disorganized and fragmented in the CG (Fig.6 C). The fibers of collagen type III were more commonly found next to the fibers type I and normally had various sizes and shapes.

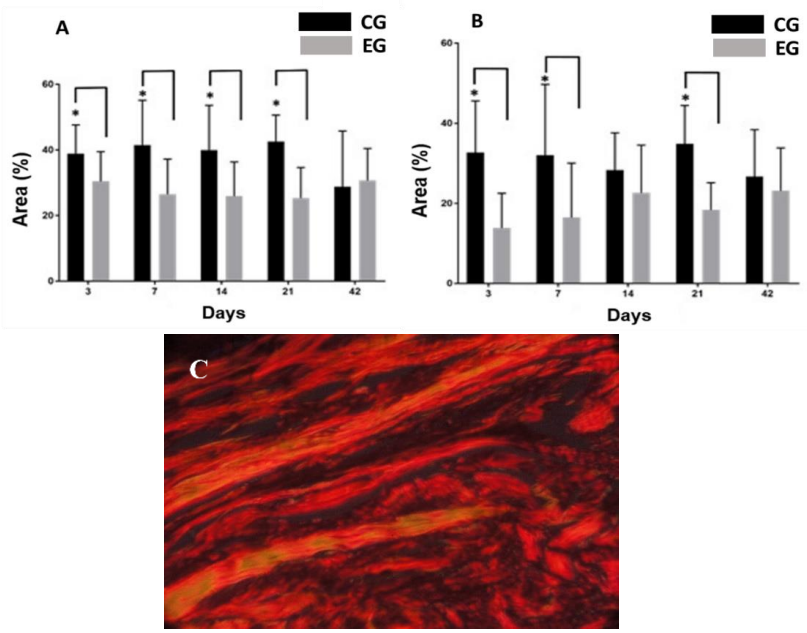

Figure 6. In "A", chart of the percentage quantification of the area in red (Collagen type-I). It can be noted the difference between the Experimental Group (EG) and the Control Group (CG) shown by the "bar" and amongst the days, shown by the star "*". Statistical difference in relation to the control group in the same period, $p > 0.05$. In "B", ranking of the percentage of area in green and yellow (collagen type III) There is a difference between the EG and CG demonstrated by the "bar" and amongst the days indicated by the "*". Statistical difference in relation to the Control Group in the same period, $p>0.05$. In "C", example image of the arrangement of fibers found in the fibrous capsule formed around the biomaterial. The fibers marked in red are type-I collagen and the fibers marked in green and yellow are type-III collagen. Rat of the control group (CG) aged 21 days. Picrosirius red stain observed under polarized light microscope. 400x.

The concentration of collagen types I and III in the CG and EG, which was observed in the first few days of the implant removal (third day), is high when compared to a normal adult rat (Cheung *et al*., 1990). This high concentration of collagen types I and III in both groups indicate that there are common pro-fibroblast factors which can be linked not only to the antigenicity of the chondrocytes but also to other components of the extracellular matrix in the cartilage and its surface. These factors may not be correlated to the composition of the biomaterial as factors released during the surgical act (Creag and O´Neil, 2006; Meneghin and Hogaboam, 2007; Zhu, 2010).

In the CG, the strong presence of antigens that derive from chondrocytes may have indirectly contributed to the large concentration of collagen observed, since these antigens can induce acute and chronic inflammatory responses which can consequently induce the secretion of collagen, particularly in its type III (Akilbekova and Bratlie, 2015).

The smaller concentration of collagen and associated connective tissue can be a good biocompatibility factor in the EG, since the excess of fibrous capsule could remove or reduce the future functions of this material, such as drug release.

CONCLUSION

The decellularized bovine elastic cartilage in alkaline solution, when compared to those that were not treated in alkaline solution, demonstrated a low ability to attract inflammatory cells and showed the formation of fibrous capsules with collagen fibers, besides not inducing necrosis, edema, and seroma. Thus, the alkaline treatment was effective in the decellularization and decrease of the antigenicity, demonstrating an acceptable biocompatibility in relation to the non-treated material.

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