An Acad Bras Cienc (2023) 95(Suppl. 1): e20220288 DOI 10.1590/0001-3765202320220288
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

HEALTH SCIENCES

Tracheal regeneration with acellular human amniotic membrane and 15-deoxy-Δ12,14prostaglandinj2 nanoparticles in a rabbit model

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Abstract: The treatment of tracheal pathologies remains challenging.Nanotechnology allows adding substances to decellularized human amniotic membrane (DHAM), such as 15-Deoxy-Δ12,14ProstaglandinJ2 nanoparticles (15D-PGJ2-NC).This study performed a tracheotomy in rabbits randomized into three groups.The tissue repair process was evaluated when treated with DHAM associated or not with 15D-PGJ2-NC.The average of the area in the control group was 54.76% smaller than DHAM group and 41.98% smaller than DHAM + 15D-PGJ2-NC group (p=0.004 for both).The DHAM + 15D-PGJ2-NC group had significantly more immature cartilage (p=0.015).DHAM impregnated with 15D-PGJ2-NC could provide support for the healing of the tracheal defect and may prevent reduction of its lumen.

Key words: 15D-PGJ2, amniotic membrane, healing, tracheal regeneration, tracheotomy.

INTRODUCTION

The trachea has a complex anatomical and histological structure, and the treatment of its defects requires improved medical techniques (Grillo 2003). The main causes of tracheal injuries are complications of tracheal intubation or tracheostomy, inflammatory diseases, infections, neoplasms, and idiopathic pathologies (Ha et al. 2017). The treatment of tracheal defects depends largely on the extent of the tracheal injuries. Although there are several studies with prostheses, grafts and materials for tracheal replacement, surgical treatment with end-to-end anastomosis remains the gold standard (Grillo 2002). When defects exceed the maximum limits, it is necessary to search for alternative therapies (Bergonse Neto et al. 2018).

The amniotic membrane (AM) is suitable for the development of biomaterials because it has factors that allow adequate regeneration and healing of lesions (Lacorzana 2020, Takejima et al. 2021). The application of AM has been studied in the regeneration of various systems including ocular, nervous and pericardial tissues. Importantly, AM is known to allow the incorporation of substances through nanotechnology (Francisco et al. 2016a, Francisco et al. 2020). In the trachea, although recent studies have reported promising results, repairing the injured trachea has remained a great challenge (Jorge et al. 2018).

The metabolite 15-Deoxy-Δ12,14ProstaglandinJ2 (15d-PGJ2) has been described as an anti-inflammatory molecule

due to its modulating activity in diseases such as rheumatoid arthritis, neural lesions, and myocardial ischemia (Francisco et al. 2016a). Current studies are focusing on understanding its applications in biological tissues, interactions with proteins, modulation of enzymes, and its response to diverse stimuli (Hironaka et al. 2009). Because various obstacles can interrupt proper tracheal reconstruction, the decellularized human amniotic membrane (DHAM) can be a biomaterial with regenerative potential. Besides, 15d-PGJ2 nanoparticles have been demonstrated to possess anti-inflammatory properties. Therefore, we used a rabbit model of tracheal injury to assess tissue regeneration after implantation of these materials. The evaluated parameters were tissue regeneration, cartilaginous neoformation, inflammation, and the capacity to maintain proper tracheal architecture compared to the control.

MATERIALS AND METHODS

In this experimental study, we used fifteen New Zealand white rabbits, with an average weight of 3.00 ± 0.45 kg. The animals were randomized into three groups (five each). The experiments were performed according to the institutional ethical guidelines of the Brazilian College of Animal Experimentation and approved by the Committee of Ethics in Research in Animal at Pontifícia Universidade Católica do Paraná (CEUA PUC-PR, Brazil), under the approval number 01025 and Research Ethics Committee (CEP) of the Hospital Pequeno Principe, under the approval number 0948-11.

Preparation and Decellularization of Amniotic Membrane

The amniotic membrane was obtained from one parturient, gestacional age 40 weeks and negative results for HIV-1 and 2, hepatitis B and C, and syphilis, who underwent elective cesarean section and signed the Informed Consent Form. The membrane was collected in sterile conditions, separated from the chorion and placed in a phosphate buffer solution (PBS) containing 10-mM hypotonic Tris–HCl buffer at pH 8.0 for 24h. The AM was then treated with 100 U/mL of penicillin and 100 µg/mL of streptomycin (Thermo Fisher Scientific, USA) for 30 min and rinsed with sterile saline. Thereafter, it was treated with 0.01% SDS solution (sodium dodecyl sulfate) and SD (sodium deoxycholate) at 0.01% for 24 hours at 37° C, with the aid of a stirrer mechanic (Shaking table 109 M, Nova ÉticaLtda, Brazil.) (Riau et al. 2010).

The AM was then preserved in PBS at 4 °C (Riau et al. 2010) and sectioned into appropriately sized pieces (3 x 5 mm), which was similar to the tracheal lesion induced in the rabbits.

Nanoparticles of 15D-PGJ2

Nanoencapsulation of 15D-PGJ2 in poly D, L-lactide-co-glycolide (PLGA) nanocapsules was provided by the Laboratory of Immunology and Molecular Biology, São Leopoldo Mandic Institute and Research Center, Campinas - São Paulo, Brazil.

The nanoprecipitation method used, described by Fessi, is comprised of two phases (Fessi et al. 1989). The organic phase is composed of PLGA polymer (100 mg), acetone (30 mL), 15D-PGJ2 (100 µg) - Sigma-Aldrich, St. Louis, MO, sorbitan monostearate (40 mg), and caprylic acid triglycerides (200mg). The aqueous phase is composed of polysorbate 80 (60 mg) and deionized water (30 mL). After dissolving the components of both phases, the organic phase is gradually added to the aqueous phase, and the suspension is kept under stirring for ten minutes, to obtain a colloidal suspension of 15D-PGJ2 with a final concentration of 10 µmol/mL. A control formulation (without 15D-PGJ2) was

also prepared, following the same methodology (Fessi et al. 1989).

The incorporation of 15-Deoxy-Δ12,14ProstaglandinJ2 nanoparticles (15D-PGJ2-NC) into the amniotic membranes was performed by immersing the fragments of the DHAM inside a colloidal solution of 15d-PGJ-NC at a concentration of 10 µmol / mL, for 60 minutes, at room temperature. In parallel, surgical procedures were performed and the membranes were implanted in the tracheal defect.

The morphology and structure of the DHAM added to the 15D-PGJ2-NC were examined in a JEOL 1200EX II microscope (Jeol Ltda, Akishima) operating at 80 kV.

Surgical procedure and euthanasia

The animals received intramuscular xylazine (2.0 mg/kg) as a pre-anesthetic medication, and the anesthetic induction was performed with intramuscular ketamine (20 mg/kg) and intravenous fentanyl (8.3 mcg/kg). The procedure started with antisepsis of the anterior cervical region using topical povidone-iodine, subcutaneous infiltration of the anesthetic lidocaine 2% without vasoconstrictor (10 to 20 mg/kg), and median longitudinal incision of 3 to 4 cm, followed by dissection, preserving the lateral portion of the trachea. Tracheotomy was performed by resecting a tracheal rectangle, in the anterior face, measuring 3 x 5 mm (15mm^2) (Jorge et al. 2018). In the control group, the tracheotomy orifice was kept open for secondary intention healing. In the membrane group, at the time of the surgery, the animals received an DHAM implant over the tracheal defect and in the DHAM + 15D-PGJ2-NC, the DHAM implant was added with 15D-PGJ2-NC. The amniotic membrane was sutured in the trachea with four points of prolene 7-0 thread at the ends.

Then, the sternohyoid muscle and homohyoid muscle were sutured by continuous stitches. As postoperative analgesia, meloxicam veterinary 0.2% (0.2 mg/kg/day) was administered in the first 72 hours after surgery. All animals were euthanized on the 35th postoperative day, using a lethal dose of thiopental (180 mg/kg) and 19.1% potassium chloride (1.74 mEq/kg), both intravenously. For the histological study, the laryngotracheal block was resected up to 1 cm above the main carina, including the fully injured trachea.

Figure 1 demonstrates these stages.

Histological examination and statistical analysis

Samples were fixed in 10% paraformaldehyde and remained for 48 hours. Then they were dehydrated by successive alcohol baths, soaked in liquid paraffin using two baths at 65 °C, and cut into 4-µm-thick sections. The samples were stained with hematoxylin and eosin stain (HE), Sirius Red stain (SR), and immunohistochemistry was using Anti-Aggrecan primary monoclonal antibody (clone BC-3; ABCAM; Cambridge, UK, 1: 200 concentration). The sections were then incubated with biotinylated goat antirabbit antibodies (HRP-Conjugate, Abcam, USA) and mouse specifying reagent (Abcam, USA). After incubation, the polymer amplification system was applied. In order to reveal the reactions was used diaminobenzidine (DAB; Sigma Chemical Co®, St. Louis, MO, USA) and counterstaining with Harris hematoxylin.

HE stained sections containing the defects were examined by optical microscopy to analyze the histological findings of inflammation according described by Vizzotto Junior. The parameters evaluated were presence and graduation of polymorphonuclear cells, vascular congestion and edema (acute and subacute inflammation), and monomorphonuclear cells,

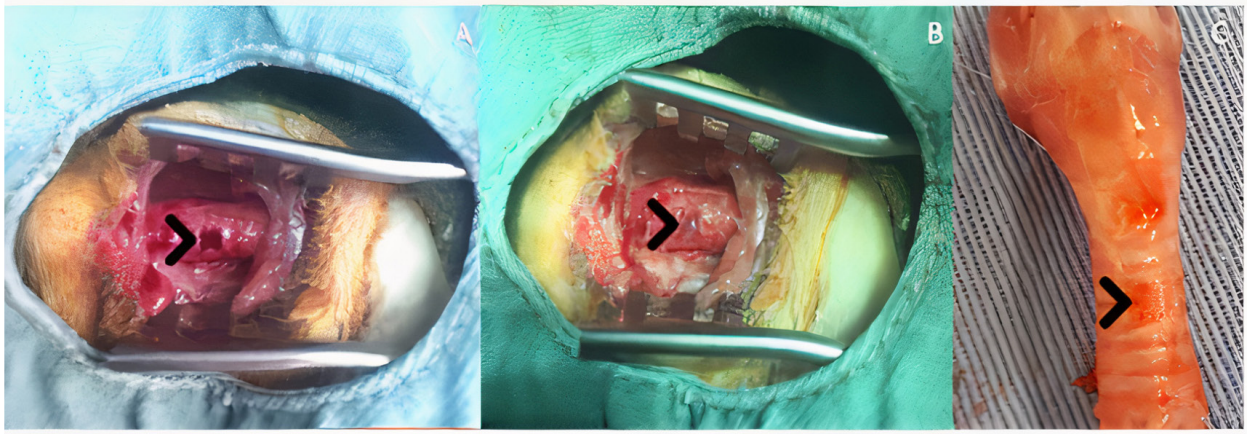

Figure 1. The tracheal defect. a. Image of the surgical site, in which the arrowhead shows the tracheal defect by the tracheotomy in an animal in the control group. b. Image of the surgical site showing the tracheal defect covered by the amniotic membrane (arrowhead), in an animal in the DHAM group. c. Image of the trachea after resection of an animal in the DHAM + 15D-PGJ2-NC group. The arrowhead points to the area of the previous tracheotomy.

fibrosis, neovascularization, and granulation tissue (chronic inflammation). From this rule, an inflammation score (-9 to +9) was determined for each animal (Vizzotto Junior et al. 2003).

The analyses of collagens and cartilage were performed at the area of 1000 μm² which occupied exactly the central region of the tracheal defect. It is important to note that this specific region was standardized for the analysis of the identical place in all animals. We chose the middle part of the lesions because the tissue repair starts from the ends of the defect and progresses towards the lesion center.

To identify types I and III collagens, we used Sirius Red F 3B solution (direct red), diluted in picric acid at a concentration of 0,3%. The image analysis software used was Image-Pro Plus version 4.5, in which the masks identified type III were green and type I were red. After overlaying the mask and identifying collagens, the software provided the area in square micrometers, occupied by these two types of collagen in each image of the defect. These data were used for later calculation of the area/percentage of the two collagens in the defect of each animal.

To analyze the cartilaginous tissue, we used anti-aggrecan immunohistochemistry. The software used to superimpose a mask on the area of the defect was Image-Pro Plus version 4.5, in which masks identified the ocher color in the positive areas for the anti-aggrecan antibody. After overlaying the mask and identifying the immunopositive regions, the software provided the area, in square micrometers, occupied by these images. These data were used for later calculation of the area/percentage of aggrecan in the defect of each animal. It is important to note that the two animals were excluded during the analyzes. One from the DHAM group, and another from the DHAM + 15D-PGJ2-NC group. The exclusions were necessary because the slides of the tracheal lesions of these animals did not show minimum characteristics for appropriate analysis by immunohistochemistry.

To calculate the tracheal lumen area, the slides containing the defect were selected and scanned, and measurements were made using the ZEN 2.6 software (blue edition) © Carl Zeiss Microscopy GmbH, 2018.

Statistical Analysis

To compare various parameters in the studied groups, the Kruskal-Wallis non-parametric test was used and p <0.05 indicated the statistical

significance. The data were analyzed with the computer program Stata / SE v.14.1. Stata Corp LP, USA.

RESULTS

Evaluation of the tracheal lumen area (defect region)

After the recovery period (35 days), the average area (cm²) of the tracheal lumen at the defect site was blindly quantified and compared. In the control group, this average area was 54.7% smaller than the DHAM group and 41.9% smaller than that of the DHAM + 15D-PGJ2-NC group, both statistically significant (p=0.004). Considering that a significant difference was found, the groups were compared two by two (Table I).

Histological Findings

Statistical analysis of the inflammatory activity demonstrated that there was no significant difference between groups (p=0.289). Table II shows the inflammation profile of each group (Vizzotto Junior et al. 2003).

There was also no significance between the groups regarding the quantification types I and III of collagen (p=0.281).

However, a significant amount of immature cartilaginous tissue was found in animals of the DHAM + 15D-PGJ2-NC group compared to the other groups (p=0.015). Table III and Figure 2 demonstrates the amniotic membrane impregnated with 15D-PGJ2 nanoparticles implantation at the site of the tracheal defect causing significant tracheal cartilaginous regeneration and the comparison between the groups.

Figure 3 shows samples of all performed stains, immunohistochemistry, and reporting of the analysis.

DISCUSSION

Surgical procedures for tracheal reconstruction are limited by the difficulty in removing the extension of the trachea, leading to the need for new efficient strategies (Grillo 2003, Tsukada et al. 2010, Wurtz et al. 2013). Cartilage regeneration is considered an important aspect for successful tracheal reconstruction. Cartilaginous tissue generally has a low capacity for spontaneous regeneration, maybe because of its discreet vascularization and low cellularity. Therefore, few researchers have induced cartilage repair in tracheal defects successfully (Bergonse Neto et al. 2018, Kim et al. 2020). Kim et al. 2020 developed an artificial trachea with mechanical properties similar to the native trachea that can enhance the regeneration of tracheal mucosa and cartilage through the optimal combination of a tubular scaffold and human pluripotent stem cells (Kim et al. 2020).

Biological membranes have been shown as good choices for supporting tracheal

Table I. Comparison of the area at the site of the tracheal defect between groups, two by two.

Compared groups	*p*
Control x DHAM	<0.001
Control x DHAM+15D-PGJ2-NC	0.005
DHAM x DHAM+15D-PGJ2-NC	0.111

DHAM, Decellularized Human Amniotic Membrane; 15D-PGJ2-NC, 15-Deoxy-Δ12,14ProstaglandinJ2 nanoparticles.

Table II. Comparison of averages indices of histological findings and characterization of the inflammatory profile.

Histological Indexes	Average Index Inflammation	
Control	+3.6	Chronic
DHAM	+2.8	Chronic
DHAM+15D-PGJ2-NC	+2.0	Subacute

DHAM, Decellularized Human Amniotic Membrane; 15D-PGJ2-NC, 15-Deoxy-Δ12,14ProstaglandinJ2 nanoparticles.

Table III. Comparison of the cartilaginous tissue (aggrecan) between groups, two by two.

Compared Groups	*p*
Control x DHAM	1
Control x DHAM+15D-PGJ2-NC	0.048
DHAM x DHAM+15D-PGJ2-NC	0.007

DHAM, Decellularized Human Amniotic Membrane; 15D-PGJ2-NC, 15-Deoxy-Δ12,14ProstaglandinJ2 nanoparticles.

reconstruction because they promote a scaffolding effect for re-epithelialization of the defect and cartilage tissue (Bergonse Neto et al. 2018, Jorge et al. 2018, Wurtz et al. 2013). The AM is considered very useful in regenerative medicine due to its high structural support, resistance, and immunological properties exemplified by its application in ophthalmology and skin reconstruction (Lacorzana 2020, Kim & Tseng 1995a, Hopper et al. 2003).

The use of DHAM as a biomaterial has been proved to be advantageous because it is easily obtained and processed. In addition, it has been shown to allow the incorporation of various products, for example, through nanotechnology (Francisco et al. 2016a). The association of 15D-PGJ2-NC, a metabolite of prostaglandin D2 (PGD2) with known anti-inflammatory properties could represent a biomaterial with immunoregulatory activity in the trachea as observed in other tissues (Francisco et al. 2020, Francisco et al. 2016b).

The cyclooxygenases (COXs), especially COX-2, are upregulated in acute inflammation. On a carrageenan-induced pleurisy model, it is suggested that COX-2 late-phase induction may aid in the resolution of inflammation by the production of prostaglandins from cyclopentenone, including 15d-PGJ2 (Gilroy et al. 1999). It has been suggested that 15d-PGJ2 exerts its anti-inflammatory activity through the activation of the peroxisome proliferator-activated receptor gamma (Jiang et al. 1998, Ricote et al. 1998), and also through the inactivation of the nuclear factor-kappaB directly inhibiting the effect of kappaB kinase or the p50 subunit (Rossi et al. 2000, Cernuda-Morollon et al. 2001).

This study provided significant data about the area of the tracheal lumen at the injury site, in which the AM implant (associated or not with 15D-PGJ2-NC) played a scaffolding role, making it difficult to retract the lesion. Therefore, our findings offer promising information that may

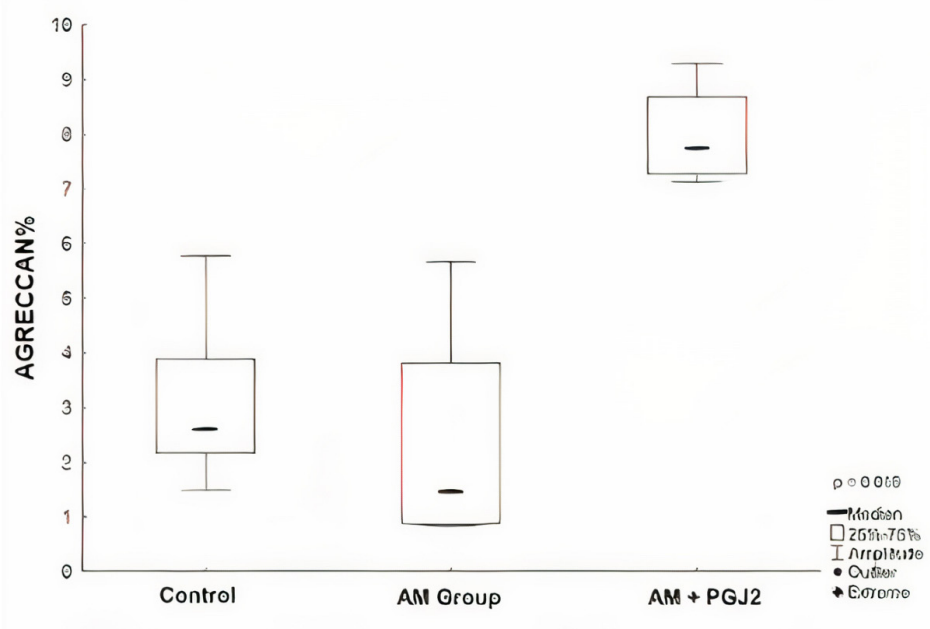

Figure 2. Comparison of the cartilaginous tissue (aggrecan) between groups. AM, Amniotic Membrane; PGJ2, ProstaglandinJ2.

help in the treatment of tracheal pathologies as well as in the maintenance of its lumen. Thus, it is suggested here that AM implantation in the lesion can prevent tracheal lumen reduction commonly caused by healing. This result is in agreement with a study carried out by Jorge et al. 2018 which used a similar methodology and concluded that the tracheal lumen area was significantly larger in the group that received AM implant. These results indicate that AM could facilitate neovascularization and regeneration of immature cartilage in a model of tracheal injury and its use may lower the risk of stenosis of trachea (Jorge et al. 2018).

Another study used porcine small intestine submucous (SIS) and showed a reduction in the area of the tracheal lumen at the lesion site, demonstrating that the SIS does not have the same scaffolding function as AM. Although facilitating neovascularization, reepithelialization, and neoformation of immature cartilage (Bergonse Neto et al. 2018).

The supportive role of AM is due to its unique structural features, and even when decellularized it preserves its basement membrane together with the innermost layer of the stroma including types I, III, IV, V, and VII collagens, elastin, fibronectin, and several integrins (Kim & Tseng 1995b). In this study, there was no difference between the two AM groups, concluding that the presence of nanoparticles does not seem to interfere with the scaffolding function of this membrane.

In this study, there was no significant difference regarding the inflammatory activity, considering the predicted anti-inflammatory role of 15D-PGJ2 in tracheal tissue. However, previous reports showed that the anti-inflammatory activity was observed in several other tissues (Gilroy et al. 1999, Scher & Pillinger 2005, Napimoga et al. 2012). Francisco et al. 2020 used DHAM associated with 15D-PGJ2-NC and applied the same impregnation technique in a post-ischemia model of the rat myocardium. They observed inflammatory changes with improvement of ventricular function (Francisco et al. 2020) and suggested certain inflammation profiles in the studied groups. The control and DHAM groups data indicated the occurrence of chronic inflammation, while the group that received DHAM impregnated with 15D-PGJ2-NC showed features of subacute inflammation, suggesting a slower resolution or an exacerbated inflammatory reaction in this latter group. That is relevant because it indicates the anti-inflammatory potential of this metabolite when applied to respiratory tissue. Experiments using models of bronchial asthma and lung infections raised the hypothesis that PGD2 and its metabolite 15D-PGJ2 may have a pro-inflammatory effect on respiratory tissue. These substances have also been shown to be mediators in the onset of allergic reactions and were suggested to be have a causative role in increased macrophage activity in models of pulmonary fungal infection (Matsuoka et al. 2000, Pereira 2013). Previous works uncovered a pulmonary pro-inflammatory role when the prostaglandin D2 receptor 2 (DP2) is activated, while the activation of prostaglandin D1 receptor 1 (DP1) antagonized the process of inflammation. They concluded that the degree of inflammation is a result of the action of prostaglandin DP2 and is dependent on the quantity and activation of these receptors (Spik et al. 2005, Rajakariar et al. 2007).

The role of collagens in tracheal regeneration was previously analyzed, using the quantitative methodology, and there were no statistically significant differences between the groups. There were predominant mature collagen fibers (type I) and immature fibers (type III), demonstrating that within 35 days of healing the remodeling

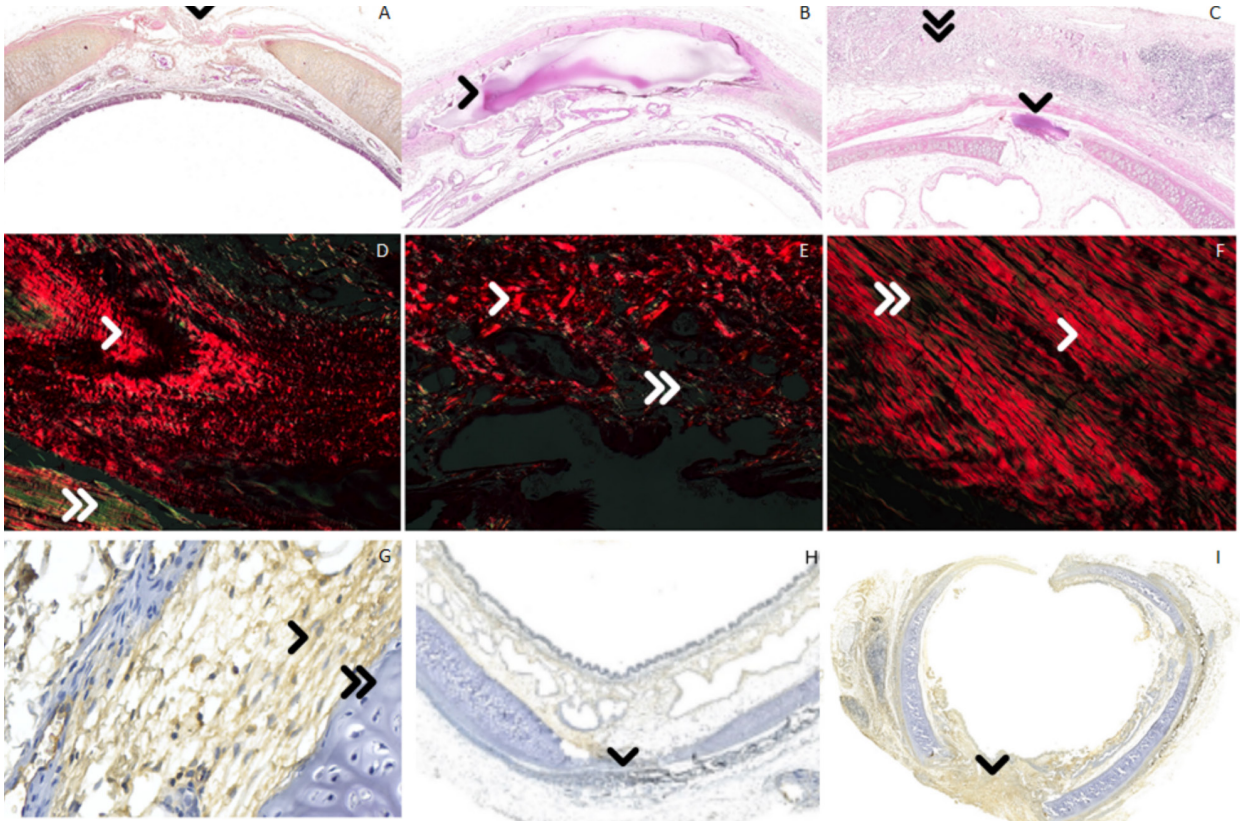

Figure 3. Photographs of the performed stains and immunohistochemistry. a. Hematoxylin-Eosin (HE) staining of the lesion area (arrowhead) in a control group. b. HE Stain of the lesion in the DHAM group in which the arrowhead points to the remaining tissue of the amniotic membrane. c. HE Stain of the lesion in the DHAM + 15D-PGJ2-NC group in which the arrowhead points to the remaining tissue from the amniotic membrane added to 15D-PGJ2-NC, and the double arrowhead to the inflammatory infiltrate. d. Image of Sirius Red stain, at the region of the lesion, in the control group. The red images represent type I collagen (arrowhead), and the green images show type III collagen (double arrowhead). e. Collagen type III (arrowhead) and type I (double arrowhead) of the DHAM group. f. Collagen type III (arrowhead) and type I (double arrowhead) of the DHAM + 15D-PGJ2-NC group. g. Anti-aggrecan immunohistochemistry (40x magnification) in a control group animal. The arrowhead points to the aggrecan (ocher color) and the double arrowhead points to mature cartilage. h. Anti-aggrecan immunohistochemistry (10x magnification) in an DHAM group animal. The arrowhead points to the injury site. i. Anti-aggrecan immunohistochemistry in DHAM + 15D-PGJ2-NC group animal. The arrowhead points to the injury site.

phase was established in all groups (Campos et al. 2007).

In the present study, the association of 15D-PGJ2 nanoparticles with DHAM promoted greater formation of immature cartilage tissue compared to the control group and the group that received only DHAM. This finding indicates that 15D-PGJ2-NC can contribute to cartilaginous regeneration, as well as to maintenance of the tracheal structure. Overall, there are now numerous efforts to achieve cartilage repair by using cellular biotechnologies, chemical complexes and biomaterials (Dashmana et al. 2020, Jeong et al. 2020, Giraldo-Goez et al. 2019, O'Leary et al. 2020).

In conclusion, the implantation of acellular amniotic membrane impregnated with 15D-PGJ2 nanoparticles in tracheal lesions in rabbits contributes to tissue regeneration suggested by the development of immature cartilage and collagen formation, facilitation of the healing process and prevention of the tracheal lumen

reduction. Despite these results, the presence of the 15D-PGJ2 nanoparticles caused a more intense inflammatory reaction and slower resolution when compared to the other groups.

Acknowledgments

We are grateful to CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), foundation of the Ministry of Education, in Brazil. We are grateful to Maternity Victor Ferreira do Amaral and the donor for enabling us to collect the placenta and amniotic membrane.

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How to cite

MIGGIOLARO AFRS ET AL. 2023. Tracheal regeneration with acellular human amniotic membrane and 15-deoxy-Δ12,14prostaglandinj2 nanoparticles in a rabbit model. An Acad Bras Cienc 95: e20220288. DOI 10.1590/0001-3765202320220288.

Manuscript received on April 2, 2022;
accepted for publication on September 5, 2022

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