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EVALUATION OF BIOLOGICAL MEMBRANE AND STEM CELLS AS POTENTIAL REPAIRS OF TENORRAPHY IN WISTAR RATS

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Abstract: Tendon rupture and lacerations are orthopedic problems of great importance in veterinary medicine. Many times, animals are discarded or removed from athletic life due to ruptured tendons. Therefore, new therapeutic possibilities have been used to improve and accelerate tendon healing. The objective of the work was to evaluate the use of a biological membrane derived from bovine tunica albuginea and stem cells in tenorrhaphies in veterinary medicine. Thirty-one adult Wistar rats were used, subdivided into 4 experimental groups, the control group (CG) with 4 animals, the tenotomy group (GT), the biological membrane group (GMB) and the stem cell group (GCT), each with 9 animals. The albugineous tunics were collected and underwent cleaning, conservation and quality analysis, making them ready for use. Three young Wistar rats were used to extract subcutaneous adipose tissue for stem cell cultivation. After the surgeries, the animals were euthanized in 3 moments; 7, 14 and 21 days post-operatively, where they were evaluated macroscopically and microscopically. The GT and GMB demonstrated low quality of healing and inflammation at almost all evaluated moments. The GCT presented a much better result, in relation to all the parameters evaluated between the groups, low tendon inflammation, no limb edema, much higher quantity and quality of collagen and demonstrating rapid healing and high tissue quality. We conclude that stem cell therapy in cases of tendon ruptures is very promising.
Keywords: Tendon rupture, stem cells, histology.

INTRODUCTION

Muscles and tendons are of great importance and play a role in the orthopedic problems of domestic animals. Tendon lacerations range from milder forms, with minimal signs of pain and without sharp injuries, to more severe forms, generating intense pain and often associated with piercing-cutting injuries. In the search to improve and accelerate regenerative processes, research has been carried out in different tissues such as skin (KOTTON et al., 2001), bone (KOSACHENCO et al., 1998), nerves (STAINKI et al., 1995) and muscles and tendons (REDDY et al., 1998; BOTTAGISIO and LOVATI, 2017). In particular, the study of tendon repair has been growing, since tissue recovery after injury is still a major challenge in clinical practice (LUCKE, 2018).

The tendon is a structure made up of connective tissue, giving it a fibrous, thick, strong and flexible, whitish color and is present in several places on the animal's body, at the ends of the muscles and inserting into the periosteum. Therefore, it has the function of maintaining the static and dynamic balance of the body through the transmission of muscular force to the bones and joints, thus allowing locomotion. (CORRÊA, 2012).

The tendon repair process occurs in three overlapping phases: Inflammatory, proliferative and remodeling (ALMEIDA, 2015). Soon after the injury, the inflammatory sequence lasts for three to seven days, characterized by changes in endothelial cells resulting from blood vessel injury, and activation of platelets that undergo degranulation and secrete multiple mediators, forming clots. At the end of this phase, the beginning of angiogenesis, stimulation of tenocyte proliferation, the beginning of collagen III production and also the expression of cytokines responsible for activating anti-inflammatory pathways such as the deactivation of macrophages and natural

killer cells (LUCKE, 2018; CLUTTERBUCK et al., 2010; MORITA et al., 2017).

During the second week (8th day to 14th day), the so-called proliferative phase, dramatic fibroblastic proliferation and production of collagen (extracellular matrix) and angiogenesis (granulation tissue) continues. This phase is responsible for closing the injury itself. The peak of collagen III production still occurs, starting the organization of the bundles of this fiber. (SHARMA and MAFFULLI, 2005). From the 14th day to the 21st day, the third and final phase of the healing process begins, remodeling, which can last up to 1 year after the injury (ABATE et al, 2009). At this stage, there is a marked change in the proportion of collagen type, where collagen III is degraded while a large amount of collagen I is degraded (LUCKE, 2018; RAISER, 2001).

The testicles, in general, have a thin capsule of connective tissue called the tunica albuginea, which does not present much difference in its histological architecture and anatomy among domestic animal species. It is composed of dense connective tissue, characterized by a greater quantity of collagen fibers and a smaller number of cells, compared to loose connective tissue (QUEIROZ et al., 2012).

Glycerin 98% has been widely used as a means of preserving biological membranes, due to its advantages such as the material's ability to dehydrate, antiseptic power, little toxicity at low concentrations and low cost (RODASKI et al., 2000; MARQUES et al., 2015).

Stem cells constitute a population of undifferentiated cells with unique characteristics of self-renewal and plasticity. These cells are present in all tissues throughout life and are involved in the processes of embryogenesis and regeneration (LUCKE, 2018). Fundamentally, there are two types of stem cells: the embryonic stem cell (ESC), which comes from the inner mass of the blastocyst, and the adult stem cell, which is divided into a mesenchymal stem cell (MSC) and a hematopoietic stem cell (HSC).

MSCs have the ability to differentiate into mesodermal tissue lineages, such as skeletal muscle, bones, tendons, cartilage and fat, when under appropriate culture conditions (GONDA et al., 2008). MSCs have several properties that suggest high capacity for cell therapy in numerous pathologies. Modulation of the recipient animal's immune system, differentiating into osteoblasts, fibroblasts, myocytes, tenocytes, osteocytes and adipocytes, in addition to the ability to promote neovascularization and decrease the production of pro-inflammatory cells and cytokines, promoting the process of tissue repair and remodeling (SANTOS, 2017; PERONI et al., 2011; MAXSON et al., 2012; SCHIPPER et al. 2008). In addition to these characteristics, MSCs have a migratory behavior, being the basis for stem cells to reach diseased sites after intravenous, intralesional or perilesional administration. (MAIA, 2012).

Regarding tissue sources in veterinary medicine, adipose tissue, the umbilical cord as well as its blood are the most frequently used sources (SANTOS, 2017). However, in view of the simple surgical procedure, easy access and simple isolation procedures based on tissue digestion enzymes have made subcutaneous adipose tissue an attractive source for obtaining MSC for researchers and clinicians in almost all areas of medicine. and veterinary medicine (MARTINS, 2019; SANTOS, 2017). In addition to these characteristics, adipose tissue can yield 500 times more cells from the same amount of tissue compared to bone marrow, and are genetically more stable in long-term cultures (MIZUNO, 2009).

MATERIAL AND METHODS

Thirty-one Wistar rats (Rattus norvegicus albinus) were used, from 3 months of age, reaching adulthood, weighing between 350 and 450g. All were weighed, identified and remained individually in standard polypropylene cages in the vivarium of the Anatomy Sector. Animal (LMPA-HVET-UENF), kept under controlled temperature (22ºC ±2ºC) and relative humidity (55% ±5%) and a 12/12-hour photoperiod, receiving balanced food and water ad libitum. In addition to the 31 adult animals, 3 young rats, approximately 1 month old, were euthanized by infusion of anesthetics (2% xylazine and 10% ketamine), according to one of the indications for small laboratory rodents (LUCKE, 2018). An incision of the cutaneous and subcutaneous tissue was then made in the inguinal region, gaining access to the adipose tissue. The tissue was extracted, cut into small pieces and samples of the tissue with blood vessels were discarded to avoid contamination. The samples were placed in 3 sterile falcon tubes and added with 4% penicillin and streptomycin in PBS solution (phosphate buffered saline). Immediately after the procedure, the tubes were placed in a thermal box with ice and transported to CELLTROVET, a stem cell preparation and culture laboratory, located in the city of São Paulo – SP. The laboratory sent the stem cells frozen in cryovials, where each cryovial contained 3 million cells. At the Reconhecimento Biology Laboratory (LBR) of the Center for Biological Sciences (CBB) at UENF, the process of thawing and preparing the cells for use was carried out. First, after removing the cryotube from the nitrogen cylinder, it was placed in distilled water in a "water bath" until the entire contents of the cryotube were liquid, with an estimated time of 5 minutes on average. After removing the "Bath Maria", 70º alcohol was sprayed on the cryotube and it was taken to the flow hood, where, with the aid of a Pauster pipette, all the liquid was removed from the cryogenic tube and transferred to a sterile conical tube, which was then also physiological solution was slowly added in a 1:1 ratio, homogenizing with caution. After closing, the sterile conical tube was taken to the centrifuge for 5 minutes at 1500 rpm, so that the cells were slowly added. Then 3 ml of physiological solution was slowly added, resuspending the cells, and centrifuged again for 5 minutes at 1500 rpm. This procedure was performed 3 times in total. Finally, the cells were resuspended in the desired final volume, ensuring that each 0.1 ml of solution contained at least 500,000 viable stem cells. To confirm the success of the technique after all preparation, 1 aliquot of the prepared solution was stained with 1% Trypan Blue for quantification and cell viability testing. Where this aliquot stained in the Neubauer chamber was observed under a microscope to perform viable cell counts.

The 31 adult rats were randomly separated into 4 groups, the first being the control group (CG)(n=4), which did not undergo any type of procedure, but underwent all examinations and analyses. The second group (GT) (n=9) underwent the simple tenorrhaphy procedure, starting with manual trichotomy of the skin over the caudal region of the tibia of the right pelvic limb (MPD), in the location corresponding to the Achilles tendon. After local asepsis (iodinated alcohol), a longitudinal incision was made in the skin over the site corresponding to the tenotomy, between the calcaneal insertion and the myotendinous junction, where total tenotomy was performed by transverse shear, using a number 12 scalpel blade. procedure in the animals in the GT group served as control, with a complete tenotomy followed by tenorrhaphy with 5.0 polyglactin 910 monofilament thread allowing the approximation of the tendon stumps. In

the third group, the tenorrhaphy procedure involved the association of the biological membrane (GMB)(n=9) and the fourth group (GCT)(n=9) involved the application of stem cells to the tenorrhaphy focus. The animals in the treatment groups (GT, GMB and GCT) were initially weighed and anesthetized with 10% Ketamine Hydrochloride (75 mg/kg) and 2% Xylazine Hydrochloride (4 mg/kg) with intraperitoneal application. And for the analgesic association, Morphine 1% (3 mg/kg) was also used intraperitoneally. The latter, in turn, continued to be performed every 4 hours postoperatively subcutaneously during the first 24 hours after surgery. In the GMB group, tenorrhaphy was associated with the use of a fragment of biological membrane, measuring 3 mm x 5 mm, and in the GCT group, 500,000 stem cells were applied to the tenorrhaphy site. Next, the skin was sutured with 5.0 non-absorbable nylon thread and subjected to local cleaning (with iodinated alcohol), followed by immobilization of the limb with orthopedic cotton and an elastic bandage. In the immediate postoperative period, the animals received 10% ketoprofen (3 mg/kg-^{1} SC, SID) for three consecutive days. The evaluation of each individual was monitored in the first 12 hours after surgery and then daily.

After carrying out the procedure, the animals were randomly selected to be euthanized and undergo macro and microscopic evaluations. These were carried out on three occasions (7, 14- and 21-days post-surgery), each time with one animal from the GC group and three animals from each treatment group, namely GT, GMB and GCT. These euthanasia times chosen correspond to the tissue regeneration times of the tendon, in their respective inflammatory, proliferative and remodeling phases (CLUTTERBUCK et al., 2010; MORITA et al., 2017; LUCKE, 2018; RAISER, 2001). Macroscopic evaluations followed the following parameters: presence and color of the biological membrane, consistency, edema, presence of vascularization, surgical wound infection, stitch dehiscence, tendon inflammation and adhesion formation. In addition to these parameters, tendon thickness was also measured in each operated and non-operated tendon. Microscopic evaluations were carried out after harvesting the surgical wound, where the Achilles tendon was dissected, from its myotendinous origin to its insertion in the calcaneal tuberosity and then fixed in a 10% aqueous solution of neutral buffered formalin. After all, histotechnical preparation, which was carried out in the Animal Pathology sector (LMPA-HVET-UENF), the slides were stained with Hematoxylin Eosin (HE), Picrosirius-Red (PS) and Masson's Trichrome (TM) and examined by microscopy. light. The HE technique aimed to evaluate the inflammatory reaction, such as the presence of macrophages, fibrin, neutrophils, granulation tissue, fibrosis and the presence of the biological membrane. The PS technique using polarized light microscopy aimed at a qualitative analysis of the collagen fibers of the connective tissue, through the different interference of colors, intensity and birefringence of the stained tissues, mainly differentiating type I and type III fibers. Type I fibers appear as thick fibers, highly birefringent and red in color, while type III fibers appear in thin bundles, with weak birefringence and greenish-yellow in color. The TM technique aimed to quantitatively analyze the collagen fibers of connective tissue, giving a blue color to all these fibers, but not allowing differentiation between types I and III.

RESULTS

In macroscopic analyses, it was possible to observe a significant difference in the healing pattern between the groups, where the GCT group demonstrated much more efficient and organized healing. This conclusion can be answered through some parameters that made it possible to make a comparison between the experimental groups. The GT and GMB groups presented edema in the region of the operated limb until the 21st day after surgery, whereas in the GCT, since the 7th day after surgery, no signs of edema were observed in the operated limb. This parameter could also be evaluated and compared by measuring the tendons after necropsy with the aid of a digital caliper. In the microscopic analyzes it was also possible to visualize striking differences between the experimental groups, initially during the analysis of the HE-stained slides, the GMB on the 7th, 14th and 21st post-operative day, showed the presence of macrophages and neutrophils, indicating a very active inflammatory process. throughout this healing process, in addition to an active immune response signaling as well. The GT on the 7th postoperative day confirmed the presence of macrophages and neutrophils, but on the 14th day only the presence of macrophages and on the 21st day there was no presence of these cells, indicating that after the 14th postoperative day, the animals in this group passed the inflammatory wound. As for the GCT from the 7th day onwards, all animals in the group did not present macrophages and neutrophils in the scar region, thus indicating a very rapid and efficient inflammatory phase in the GT on the 7th day, they showed fibrin and the beginning of granulation tissue, on the 14th day no longer presented fibrin and only granulation tissue with some points of fibrosis and on the 21st day only fibrosis. The GMB on the 7th day showed the beginning of granulation tissue, along with some points of initial fibrosis and from the 14th day onwards, only fibrosis was identified, as well as on the 21st day. In the GCT on the 7th day, there was already very well-established fibrosis throughout the injured area. When evaluating the slides stained with TM staining, where the collagen tissue is stained blue, different characteristics were also observed between the groups, mainly highlighting the GCT group from the others. Since the GCT slides showed an intense collagen density from the 7th day after surgery, while the other groups (GMB and GT) showed a light collagen density on the 7th day and moderate on the 14th day. Once again, demonstrating that the use of biological membrane in GMB did not cause improvement or acceleration in the healing effect, compared to GT. On the other hand, GCT showed a large amount of collagen production in a shorter time and analyzing the slides from the 14th and 21st day, in addition to collagen intensity, GCT demonstrated an accelerated progression in tissue modeling (Figure 2). In PS staining, in the GT and GMB groups we observed a prevalence of type III collagen until the 14th day and from the 21st day, equality between collagen types. This demonstrates that only from this moment onwards is a transition from type III collagen, which is a collagen in the initial healing phase, with good resistance but little elastic capacity, to type I collagen, which is the predominant collagen in tissues. in the modeling phase, having a wide elasticity capacity. The GCT showed a significant difference from the other groups, where a large amount of type I collagen and a smaller amount of type III collagen were observed from the 7th postoperative day (Figure 3).

DISCUSSION

In the literature, some anesthetic protocols are found for performing surgical procedures on Wistar rats intraperitoneally, however, when carrying out a pilot project, it was observed that the protocol suggested by Lucke et al. (2018) was not giving satisfactory results, where the recommended dose was 80 mg/kg of ketamine and 10 mg/kg of xylazine. The animals went very deep during surgery, took a long time to regain consciousness post-surgery and subsequently died. As a result, we adjusted the doses, in addition to associating morphine with the anesthetic protocol, as well as with postoperative therapy to control pain, as mentioned in this study. Martins (2019) also used an analgesic protocol to control pain, using tramadol (20 mg/kg) intraperitoneally during the surgical procedure and afterwards. After these adjustments, in the experimental groups we managed to have an excellent anesthetic induction, with faster recovery of consciousness and efficient pain control, avoiding the death of the animals and guaranteeing their well-being during the research. Costa (2011) used anesthesia in his experiment through vaporization in an inhalation apparatus with halothane (induction 4%, maintenance 1.5 to 2%, diluted in 70% N2O and 30% O2). Like Felix (2013), we used MSCs extracted from adipose tissue due to their good performance, easy cell reproduction in culture, high anti-inflammatory capacity, low expression of antigens and their immunomodulatory capacity. The adipose tissue collection site chosen was the subcutaneous access to the inguinal region of the rats, as in the study by Lucke (2018). Felix (2013) and Martins (2019) describe that they collect tissue from the abdominal region of animals. In general, there will be subcutaneous adipose tissue in both the inguinal and abdominal regions. Santos (2017) describes that in domestic animals the most used source for isolation and culture of stem cells is adipose tissue, as does Oliveira (2019), who published a work collecting adipose tissue in horses, in the region parallel to the base of the tail, through a 4 to 5 cm skin incision reaching the adipose tissue between the skin and the muscles. However, Bernardo et al. (2017) carried out a study in horses, describing a technique for obtaining stem cells from the bloodstream, called peripheral progenitor cells. Where he reported that it is an easy technique to perform, being possible in an outpatient environment, as it is only necessary to access the equine vein without the need for anesthesia or sedation and with a lower risk of contamination. However, compared to other sources of stem cells, the effectiveness of this technique is slightly lower, requiring further studies to improve and reduce the collection procedure time, which is considered high, averaging 2 hours and 30 minutes. In the present work, in relation to the experimental groups, it was possible to obtain a very significant result in relation to the animals treated with stem cells, due to the lesions without an inflammatory or infectious aspect, no type of immunological reaction to the use of the cells, high healing capacity and modeling in a short space of time, ensuring high-quality and agile tendon healing, which is extremely sought after in cases of tendon ruptures in veterinary routine. In the study carried out by Lucke (2018), after partial transection of the Achilles tendons of Wistar rats, 4.5 x 105 intralesional CTM was used and then skin suture. By polarization microscopy evaluating birefringence, comparing tendons treated with CTM and those treated with low-power laser, they obtained a better collagen organization in tendons treated with laser alone than with CTM alone. However, the group treated with CTM obtained better results compared to the control group, where it was only transected. In HE staining, the group

treated with laser showed more cellularity compared to the group treated with MSC. In the present work, we used 5 x 10^5 Intralesional CTM and in slides stained in PS and TM, to evaluate the quantity and quality of collagen, there was a significant difference in relation to the other groups, as well as in the analyzes of slides stained in HE, demonstrating a much higher efficacy than conventional treatments performed in comparison. That is why more studies are needed on treatments using stem cells, to determine criteria and standards, from the choice of the supplier animal, the source that will be used, through the collection and culture method to the application with doses, so that there is better understanding of technique, safety and therapeutic efficacy (Lucke 2018). In work on humans related to the central nervous system, MSCs have shown efficacy, with the ability to differentiate into neurons and glial cells, migrate to the injured site and promote functional recovery (Costa, 2011). Oliveira (2019), describes the use of autologous MSCs derived from adipose tissue, by regional perfusion in horses with chronic laminitis, where the dose of 2 x 10^7 cells in physiological solution. Obtaining a satisfactory result compared to placebo treatment, in relation to hoof growth rate, spatial relationship between the third phalanx and the corneal case, vascular perfusion and lamellar healing process. Biological membranes derived from animal tissues have been studied and used mainly in surgical repairs of hernias in veterinary medicine (Vidor et al. 2013; Marques et al. 2015) and as dressings for external wounds and grafts (Aceto et al. 2007; Queiroz et al. 2012). In the present work, biological membranes derived from bovine tunica albuginea were used in conjunction with tenorrhaphy, with the aim of evaluating their ability to aid healing. However, we observed that there was no healing improvement in the tissues and also an exacerbated inflammatory reaction. Demonstrating that its use must be more applied to skin dressings and places where there are tissue defects, as already successfully described (Queiroz et al. 2012; Aceto et al. 2007; Salbego, 2010; Vidor et al. 2013; Marques et al. 2015).

CONCLUSION

The tunica albuginea, despite its high resistance and healing capacity, as an aid in tenorrhaphies does not prove to be a viable technique, as it has a negative influence through intense inflammatory processes, causing a delay in the healing of tendons and poor quality. Stem cells prove to be an excellent tool for accelerating the healing process of tendons, with quantitative and qualitative growth of connective tissue at the tenorrhaphy site. Being of great importance for clinicians and veterinary surgeons in severe tendon injuries. To achieve this, it is important to know how to collect cells, doses, and evaluate cells before carrying out each procedure, to achieve success.

GOVERNO DO ESTADO
RIO DE JANEIRO

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RECTORY
Ethics Committee on the Use of Animals-CEUA

CERTIFICATE

We certify that Protocol No. 492, entitled "MORPHOLOGICAL AND MICROBIOLOGICAL EVALUATION OF THE BOVINE TESTICULAR TUNIA ALBUGINEA AND STEM CELLS AS POTENTIAL REPAIRS OF THE TENORRAPHY TECHNIQUE IN WISTAR RATS" under the responsibility of Dr. Eulógio Carlos Queiroz de Carvalho, ``Centro de Ciências Tecnologicas e Tecnologias Agrícolas`` of the Universidade Estadual do Norte Fluminense, is in accordance with the Ethical Principles in Animal Experimentation adopted by the Brazilian Society of Laboratory Animal Science/Colegio Brasileiro de Experimentação Animal(SBCAL/COBEA) as well as federal law 11,794 and was approved by the COMMISSION OF ETHICS FOR THE USE OF ANIMAL(CEUA-UENF) in a meeting held on 10/14/2021. This program is licensed in the current format and is valid until 10/14/2025

Campos dos Goytacazes, October 14, 2021

Clóvis de Paula Santos

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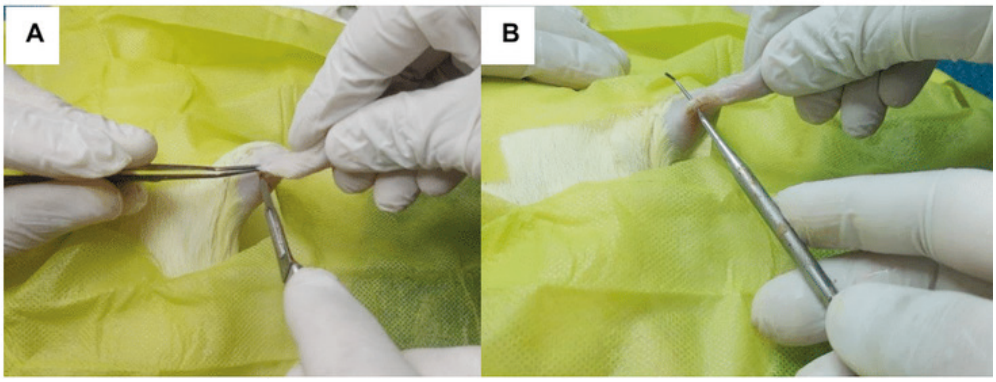

Figure 1: (A) Skin incision in the caudal region of the tibia. (B) Exposure of the common Achilles tendon for subsequent transverse shear tenotomy.

Source: Personal archive.

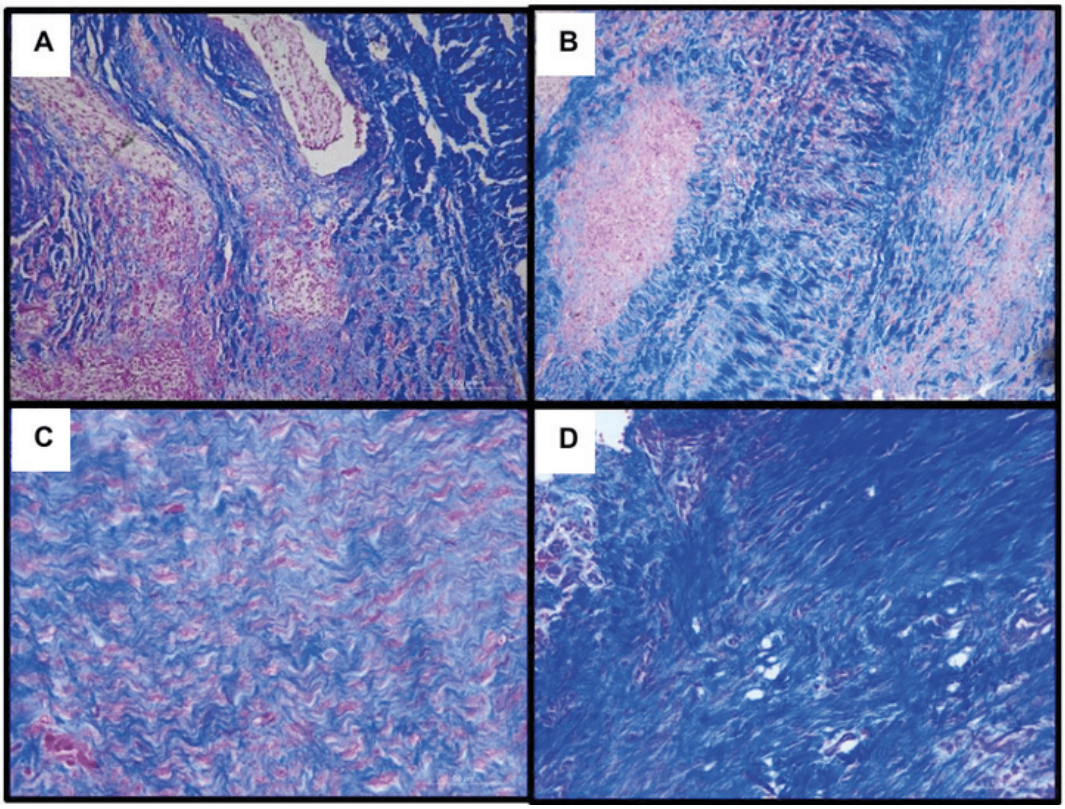

Figure 2: Photomicrographs of slides stained in TM with objective magnification 10X (A) and (D) 40X (B) and (C). (A) GMB on the 7th day showing mild collagen intensity. (B) GMB on the 14th day with moderate presence of collagen. (C) GT demonstrating intense presence of collagen on the 21st day. (D) GCT on the 7th day showing the intense presence of collagen and in an organized manner.

Source: Personal archive

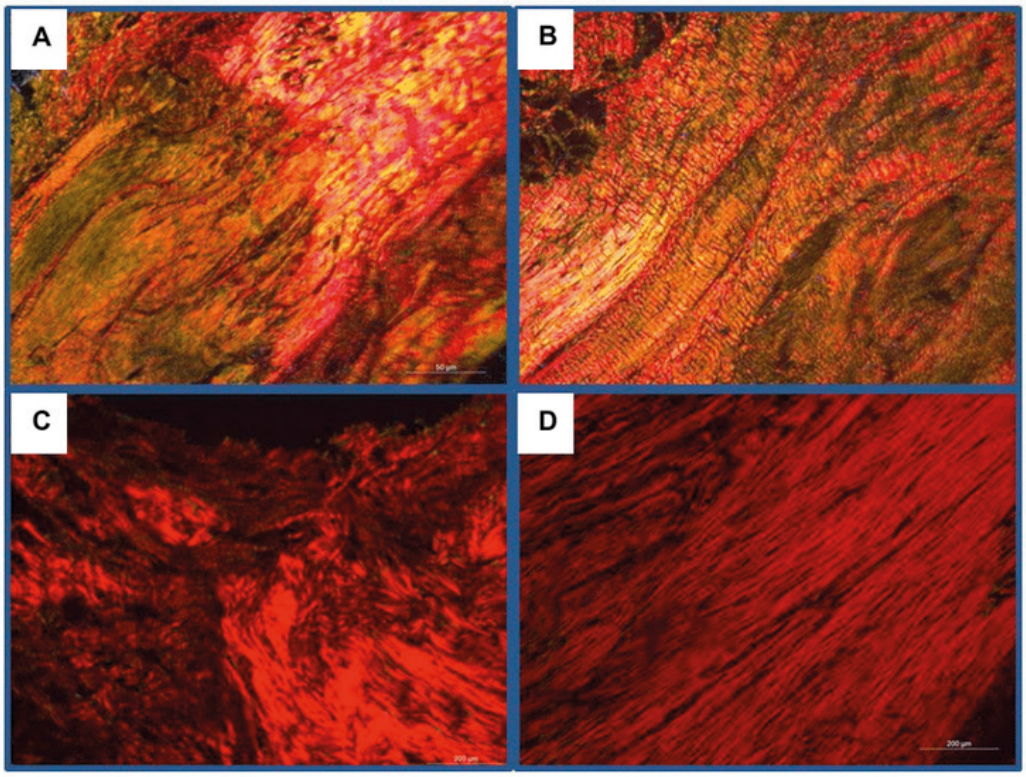

Figure 3: Photomicrograph of slides stained in Pricosírus-Red with objective magnification 40X (A) and (B) 10X (C) and (D). Thick, red fibers: Type I collagen. Thin, greenish-yellow fibers: Type III collagen. (A) GT slide on the 21st day, demonstrating equality of collagen types. (B) GMB slide on the 21st day, with equal types of collagen. (C) GCT lamina on the 7th day, showing a considerable predominance of type I collagen. (D) GCT lamina on the 21st day, also with a greater amount of type I collagen in a well-modeled form.

Source: Personal archive