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Heterologous Bovine Tunica Albuginea Graft Conserved in Honey as Abdominal Wall Reinforcement in Rats

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Abstract

Complex abdominal wall defects might be challenging for human and veterinary surgeons worldwide. Defects from trauma or congenital causes may lead to hernias development. The introduction of meshes to reinforce hernia repairs has improved surgical outcomes and several synthetic and biologic materials have been used. In this context, biomaterial prosthesis seems to be a satisfactory solution when managing great abdominal wall defects. The aim of the current study is to evaluate the bovine tunica albuginea (BTA) preserved in honey as graft material for rats' abdominal wall reinforcement in incisional herniorrhaphy surgery as well as its viability, cicatrization and integration into the host tissue. Wistar rats were assigned to two main groups: 1) animals (n = 20) underwent median longitudinal laparotomy followed by laparorraphy with suture of the bovine tunica albuginea (BTA) graft as abdominal wall reinforcement; and 2) animals (n = 20) underwent only laparotomy and subsequent laparorrhaphy. Rats were clinically evaluated until euthanasia at post-surgical day 7, 14, 21 and 28. Necropsy and histopathological analysis of abdominal wall fragments were performed to compare groups and subgroups findings. BTA promoted abundant fibrosis, providing resistance and low postoperative complication rates. Besides, animals did not show rejection signs to the implant. In conclusion, BTA preserved in honey is an affordable, easy collection and handling biomaterial for graft, demanding simple surgical implantation technique for abdominal wall repair in rats.

Keywords

Biomaterial, Hernia, Reconstructive Surgery, Xenograft

1. Introduction

Complex abdominal wall defects might be a real challenge for human and veterinary surgeons worldwide. The abdominal wall is a muscle layer which protects organs. Defects in the form of rupture or tear occur either due to trauma or congenital causes and may lead to hernias development [1]. The incisional hernias are commons complications of abdominal surgeries [2] [3] [4] and its primary repair can be performed in small lesion (<4 cm) when surrounding tissues are viable. Given large lesions (>4 cm) have a higher recurrence rate when primarily closed, it must be repaired with prosthesis support [5]. In this regard, the introduction of meshes to reinforce hernia repairs has improved surgical outcomes [6] and several materials have been identified, such as sorts of grafts and synthetic meshes. However, some disadvantages include 1) implants low elasticity do not keep up with animal individual grow and 2) exacerbated inflammatory reaction leading to prothesis removal necessity [7] [8]. In this context, biological prosthesis as a bridge to close the abdomen seems to be the best obvious solution when managing great abdominal wall defects [9].

A wide variety of biomaterials such as bovine pericardium [10] [11], bovine peritoneum [12], bubaline aortic matrix [13], bubaline diaphragm [14], porcine small intestinal submucosa [15], caprine rumen [14], ovine tunica albuginea [16] [17] and dog tunica albuginea [18] have been reported as biologic scaffolds in abdominal wall repair surgery. In this regard, various studies have shown tunica albuginea as a useful biologic mesh due to its low cost, easy collection, conservation, processing and application technique [16]-[22].

The purpose of this work is to evaluate the bovine tunica albuginea (BTA) fragment preserved in honey as graft material for rats abdominal wall reinforcement in incisional herniorrhaphy surgery as well as its viability, cicatrization and integration into the host tissue.

2. Materials and Methods

2.1. Ethical Approval

The study was approved by the Ethics Committee on the Use of Animals of Universidade Federal Fluminense with the verdict number 532/2014.

2.2. Graft Collection and Conservation

The BTA fragment was obtained by open orchiectomy technique of a healthy six-month-old crossbred calf from Teaching Farm of Universidade Federal Fluminense. The biomaterial was extracted sterilely and preserved in organic raw honey (orange tree flower origin) in such a way that entire fragment got in con-

tact with solution. Then, it was stored at room temperature (not above 35˚C) for at least 60 days.

2.3. Animals Selection

A total of 40 four-month-old female Wistar rats (*Rattus novergicus albinus*) from Animal Center Laboratory of Universidade Federal Fluminense were selected for this study. The animals were maintained in environmental, nutritional, and health-controlled conditions in Morphology Department Vivarium of same intitution. They were housed in polycarbonate individual boxes with saw dust bed, kept under temperature controlled (22˚C ± 2˚C), 12 hours light-dark cycles, food, and water *ad libitum*. All animals were in anestrus during the whole experimentation period.

2.4. Animals Groups Division

Wistar rats were assigned to two groups: 1) a test group (T), whose animals (n = 20) underwent median longitudinal laparotomy followed by laparorraphy with suture of the tunica graft as abdominal wall reinforcement; and 2) a simulation group (S), in which animals (n = 20) underwent only laparotomy and subsequent laparorrhaphy. Besides, each of these groups was further divided into four subgroups (n = 5) according to euthanasia period at post-surgery day 7, 14, 21 and 28.

2.5. Graft Prepare

The honey preserved BTA fragment was considered contamination free after morphological and microbiological (culture) evaluations. Before T group surgical procedure, the bioprosthesis was rehydrated in sterile saline 0.9% for about 30 minutes in order to remove all honey solution and to return its physical properties.

2.6. Anesthesia and Surgical Technique

Anesthetic induction of T and S groups animals was performed with ketamine (75 mg/kg) and midazolam (10 mg/kg) combined by intraperitoneal injection. Then, a broad abdominal trichotomy and antissepsia (1% chlorhexidine digluconate and 70% alcohol) of the operative field were executed.

The rats were placed in dorsal decubitus position and underwent median longitudinal laparotomy with an approximately 1.5-centimeter incision, followed by laparorrhaphy with simple continous pattern (braided synthetic absorbable multifilament made of Polyglycolic acid 4-0 size thread). Subsequently, T group animals had BTA grafted over abdominal wall laparorraphy with simple interrupted pattern (absorbable multifilament Polyglycolic acid 4-0 size thread) as Figure 1 shows. Dermorraphy was performed with simple interrupted pattern (inabsorbable monofilament Nylon 4-0 size thread). Surgical time lasted about seven minutes.

Animals postoperative clinical evaluation took into consideration behavior and

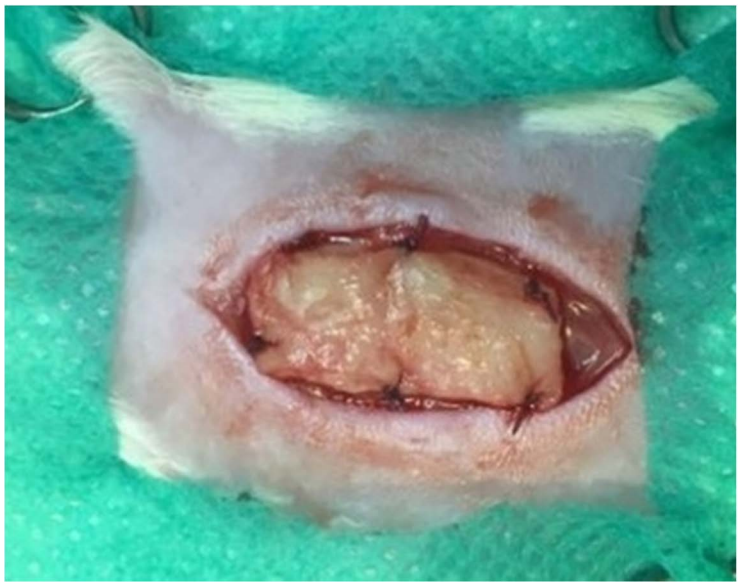

Figure 1. Bovine tunica albuginea grafted in a rat abdominal wall.

clinical aspects such as: general condition, appetite, activity, and surgical wound specially regards to dehiscence, infection, and seroma formation.

Chlorhexidine spray on surgical wound (every 24 hours), enrofloxacin (5 mg/kg SC every 24 hours), ketoprofen (10 mg/kg SC every 24 hours) and dipyrone (100 mg/kg SC every 24 hours) were given for five days after surgery.

2.7. Euthanasia, Necropsy, and Samples Collection

Animals were euthanized by inhalation of isoflurane overdose at post surgical day 7 (subgroups T7 and S7), 14 (subgroups T14 and S14), 21 (subgroups T21 and S21) and 28 (subgroups T28 and S28).

During necropsy, signs of extra and intra-abdominal inflammation, suture dehiscence, efusion and adhesions were assessed. An abdominal wall fragment of approximately 4 cm, including whole surgical site with (T group) or without (S group) the BTA xenograft, was excised and fixed in 10% buffered formalin solution for 48 hours until histological processing.

2.8. Histopathological Processing and Analysis

Abdominal wall fragments excised and fixed in 10% buffered formalin were embedded in paraffin tissue blocks, cut in 5 μm sections and stained with Haemotoxylin and Eosin (HE) and PicroSirius Red. For histopathological analysis, stained sections were evaluated with a Leica DM500 microscope. Images captured by Leica icc50 HD camera were processed by LAS CORE™ software in 40×, 100× and 400×. The microscope slides were assessed by the same observer considering the following criteria: 1) inflammation, 2) neovascularization, 3) fibrosis and 4) graft integration into the recipient tissue. These parameters were graded by comparing samples in absent (−), rare (+), low (++), moderate (+++) or high (++++) levels.

3. Results

After microbiological analysis, the BTA converved in honey did not have bacterial nor fungal growth, being free from contamination.

All animals from both S and T groups had complete recovery until two hours after surgery, showing activity and appetite. No deaths or clinical changes—such as apathy, hyporexia, isolation or pain signs—were reported during the entire experiment period. Two T group animals (10%, n = 2/20) had skin suture dehiscence due to pulling threads out by biting. Nevertheless, it happened after injury healing with no wounds edges spacing.

Immediately after euthanasia, the post-mortem macroscopic evaluation showed one animal from subgroup T14 (5%; n = 1/20) had omentum adherence to abdominal wall muscle suture area.

At histopathological analyzes, all T7 animals (100%, n = 5/5) had inflammatory infiltrate around the xenograft and a little inside it. The infiltrade was mainly composed by polymorphonuclear specialy at suture region (**Figure 2(A)**). Picrosirius Red stained sections evaluated under a polarized light microscope reveled a remarcable presence of strongly birrefringent red fibers, that denotes thick collagen fibers arrangement (type I), near muscle and along graft extension. A rare presence of slightly yellowish green birrefringent fibers that hints thin collagen fibers (type III) was also noted near scar and musculature region (**Figure 2(B)**). S7 rats subgroup differed from T7 one by presenting fewer polymorphonuclear cells infiltrate (**Figure 2(C)**).

Compared to T7 previous subgroup, all T14 animals (100%, n = 5/5) showed neovascularization and inflammatory infiltrate increases with greater polymorphonuclear cells foci inside the graft, leading to a granuloma aspect. Near muscle, surrounding the tunic implant and suture area, inflammation was even more evident. The entire graft was still viable by post-surgery day 14 (**Figure 2(D)**). Under polarized light microscope we observed high levels of thin yellowish green fibers compatible with type III collagenous fibers, but no significant difference in quantity nor location of red fibers (**Figure 2(E)**) compared to T7 subgroup. S14 subgroup had a lower inflammatory infiltrate than T14 and greater fibrosis formation (**Figure 2(F)**) than S7.

All T21 subgroup animals (100%, n = 5/5) had moderate inflammatory infiltrate dispersed throughout graft extension and an initial loss of implant integrity with its incorporation into abdominal wall host. An intense neovascularization was noted with vessels calibres variation (**Figure 2(G)**). Picrosirius Red staining revealed an increase of weakly birefringent thin yellowish green fibers (type III collagen) below the xenograft area (**Figure 2(H)**) compared to T14 rats. On the other hand, S21 subgroup showed mild inflammatory infiltrate (**Figure 2(I)**), fibrosis and consolidated cicatrization features suggesting complete healing.

T28 subgroup animals (100%, n = 5/5) showed complete implant invasion by inflammatory infiltrate with loss of integrity and no viability anymore. Besides, intense neovascularization, fibrosis and complete healing were observed (**Figure 2(J)**).

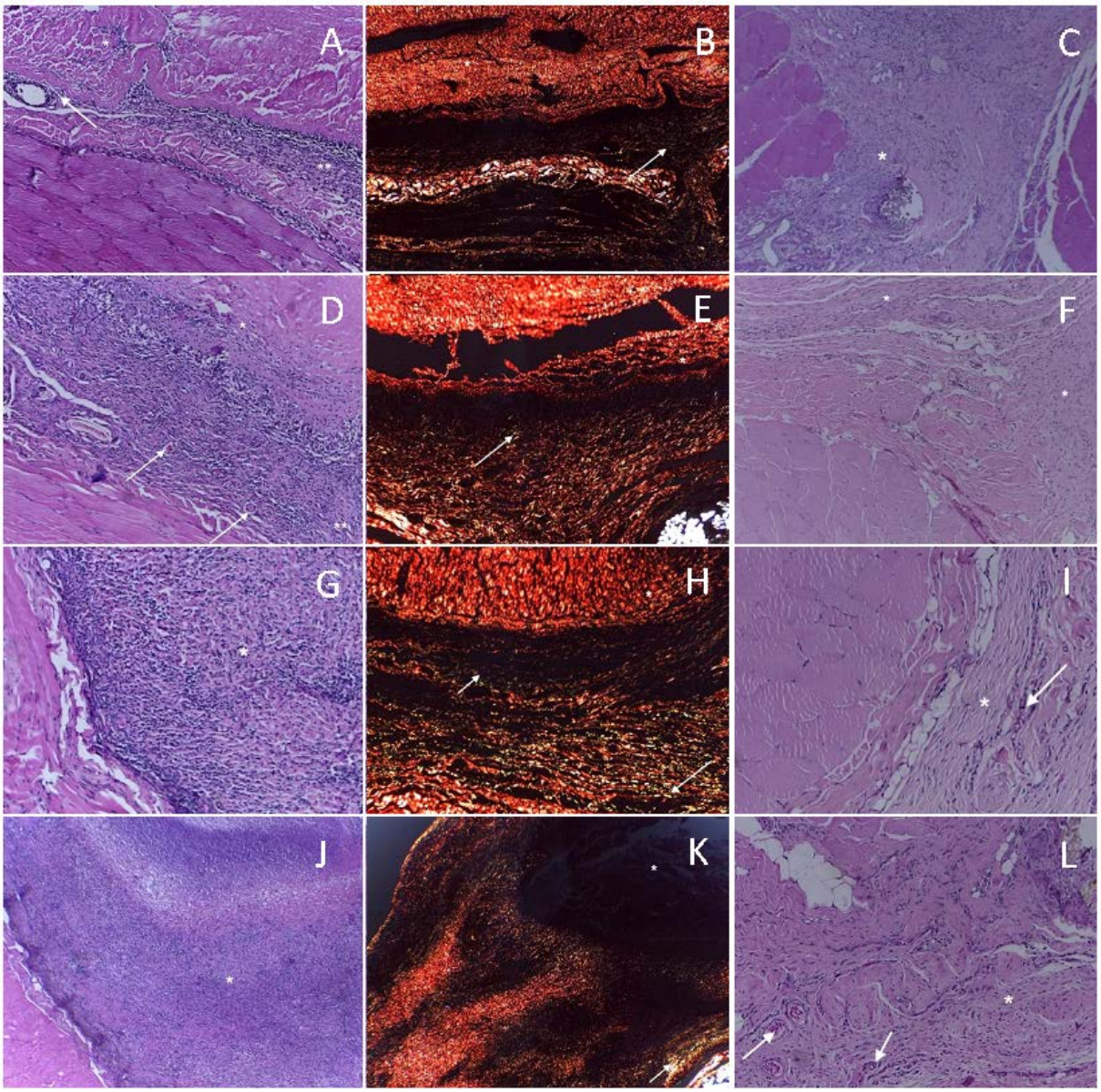

Figure 2. Microscopic abdominal wall samples evaluations from different rats' subgroups. (A) T7 subgroup section showing little inflammatory infiltrate inside the graft (*), higher infiltrate levels around the implant (**) and near suture area (arrow). HE, 10×. (B) T7 subgroup section evidencing elevated amount of red collagen fibers (*) and rare presence of thin yellowish green collagen near scar region (arrow). Picrosirius Red, 4×. (C) S7 subgroup slide showing polymorphonuclear cells dispersed throughout healing area (*). HE, 10×. (D) T14 subgroup section demonstrating moderate inflammatory infiltrate inside the graft (*) and around it (**) with moderate neovascularization (arrow). HE, 10×. (E) T14 subgroup section evidencing high red collagen (*) and thin yellowish green collagen fibers deposition near musculature (arrow). Picrosirius Red, 10×. (F) S14 subgroup slide showing polymorphonuclear cells dispersed throughout healing area and also moderate fibrosis (*). HE, 10×. (G) T21 subgroup section demonstrating intense inflammatory infiltrate inside the graft (*). HE, 10×. (H) T21 subgroup section evidencing red collagen fibers (*) and higher thin yellowish green collagen deposition near scar region (arrow). Picrosirius Red, 10×. (I) S21 subgroup slide showing polymorphonuclear cells dispersed throughout healing area near musculature (*) and neovascularization (arrow). HE, 20×. (J) T28 subgroup section demonstrating intense inflammatory infiltrate in entire graft extension (*). HE, 4×. (K) T28 subgroup section evidencing no refringence in graft area (*) and higher thin yellowish green collagen deposition near suture region (arrow). Picrosirius Red, 4×. (L) S28 subgroup slide showing polymorphonuclear cells dispersed throughout healing area (*) and neovascularization with different calibres vessels (arrow). HE, 10×.

Under polarized light microscope we did not note birefringence at graft area, although higher levels of thin yellowish green fibers (type III collagen) compared to previous T21 subgroup were reported mainly near suture region (Figure 2(K)). Meanwhile in S28 subgroup, fibrosis and complete healing were remarked (Figure 2(L)).

Picrosirius Red stained sections evaluated under a polarized light microscope reveled collagen arrangement in T group animals abdominal wall samples. On early postoperative days (T7 subgroup), a remarcable presence of strongly birrefringent red fibers (type I collagen) was noted specially along bioprosthesis extension, while no significant weakly birefringent yellowish green fibers (type III collagen) deposition was observed. However, the last fiber type became more frequent by late post-surgery days (T14 and T21 subgroups), mainly muscle wounds area where inflammation and neovascularization were intense. In addition, BTA xenograft no longer showed refringence at postoperative day 28 (T28 subgroup), suggesting collagen absence and therefore loss of function as support matrix since it seemed to be completely degraded. Nevertheless, S group animals samples had fibrosis and granuloma with considerable type III collagen fiber amount on early post-surgery days. However, type I collagen became more prevalent from the 21st postoperative day onwards (S21 and S28 subgroups). Therefore, group T had a greater type III collagen deposition than S group.

Table 1 and Table 2 demonstrate some microscopic parameters analyzed in T

Table 1. Evaluation of inflammation, neovascularization, fibrosis formation, xenograft integration into the host tissue and collagen fibers deposion from test (T) group animals at postoperative days 7, 14, 21 and 28.

T Group	Inflammation	Neovascularization	Fibrosis	Graft integration	Collagen Fiber	
					Type I	Type III
T7	+	+	+	−	++++	+
T14	++	++	+	+	++	++
T21	+++	+++	+++	++	+	+++
T28	++++	++++	++++	++++	−	+++

Absent (−); Rare (+); Low (++); Moderate (+++); High (++++) levels.

Table 2. Evaluation of inflammation, neovascularization, fibrosis formation and collagen fibers deposition from simulation (S) group animals at postoperative days 7, 14, 21 and 28.

S Group	Inflammation	Neovascularization	Fibrosis	Collagen Fiber	
				Type I	Type III
S7	+	+	+	+	++++
S14	++	++	++	++	+++
S21	++	++	+++	+++	++
S28	+	+++	+++	+++	+

Rare (+); Low (++); Moderate (+++); High (++++) levels.

and S group, respectively.

4. Discussion

The absence of contamination in tunica albuginea fragment after microbiological cultures can be attributed to honey physicochemical and phytotherapeutic properties [23] [24] [25]. The antimicrobial property is related to honey's low pH [26], hyperosmolarity, and non-peroxide antibacterial factors from some phytochemical substances [27]. Preservation in honey has also been applied to bone [28] [29] [30] [31] [32], cornea [33] and skin [23] grafs conservation. There are many graft processing and storing methods available, but honey has been chosen for the current research due to its advantages over other techniques such as its cost and handling. Studies compared honey and 98% glycerin for dog bones conservation methods and while some authors found glycerin superior in bone withstand compressive strength [34], other reported honey as a better preservation techinique to keep bone resistance [35].

The satisfactory animals' postoperative recovery resembled Kapan, *et al.* [10] and Canellas [36] findings. They used metedology similar to our and same experimental model, but Kapan, *et al.* [10] compared three different types of graft in incisional hernias repair while Canellas [36], tunica albuginea and polypropylene mesh as abdominal wall reinforce. In addition, the recovery quality may also be related to adequate animal management, drug protocols and postoperative care. The absence of deaths or even postoperative complications was compatible with appropriate surgical field antisepsis, operative technique, and drug protocol. Likewise, wound complications such as seroma and infections were not reported by Aramayo, *et al.* [37] and Mohsina, *et al.* [38]. In this context, biological prosthesis seems to be a valid option for abdominal wall repair sice minimize mesh-related complications [9].

The suture dehiscence in two T group rats resulted from animals pulling out stitches and did not cause any negative postoperative interference. This ocurrence was also observed by Brun and colleagues [39]. The fact might be due to biomaterial presence below the skin suture leading to animal stress and discomfort, despite of no behavior changes being noticed. This hypothesis can be corroborated by S group animals—in which no heterologous implant was placed—not having skin suture dehiscence. Still, in Nunes [18] study, where an autologous tunica albuginea was used in dogs abdominal wall reinforce, there was no suture dehiscence. These results demonstrate that autologous biomaterial has sactisfatory acceptance and does not promote tissue irritation like the heterologous graft we use in current experiment.

Inflammation at injury site can inhibit or delay the normal fibrinolytic activity, leading to persistent fibrin deposits that become an insoluble network and then postoperative adhesions [40] [41] [42]. Greca, *et al.* [15] observed omentum adhesion to abdominal wall in 90% of rats that had intestinal submucosa applied as reinforcement, while we reported same issue in 5% (n = 1/20) of animals that

had BTA grafted. This low adherence rate might be related to bioprosthesis placement site being over abdominal musculature and not at visceral peritoneum interface and even to implant inducing a minimal inflammatory response in adjacent tissues.

Other kinds of reinforcement material are more likely to adhesion formation. Isa [43] and Aramayo, *et al.* [37] used different kinds of surgical meshes and recorded adhesions as well as Greca, *et al.* [15] and Canellas [36] that reported, respectively, intestinal adhesion in 4% and omentum adhesion in 5% of rats underwent polypropylene mesh implant in abdominal wall.

According to Evans and colleagues [44], the healing first phase is caracterized by fluids and plasma proteins exudation. Besides, leukocytes, specially neutrophils, migrate to interstitium by diapedesis and transmigration. In our experiment, initial neovascularization, little collagen deposition and full implant integrity were observed on cicatrization first phase. Our findings of inflammatory infiltrate around the graft and little reaction inside it (T7 subgroup) are similar to those found by Nunes [18] who studied autologous tunica albuginea in dogs abdominal wall and by Silva, *et al.* [17] that analyzed ovine tunica albuginea xenograft in rats abdominal wall.

In compliance with Nunes [18], we found same evolutionary healing changes in T14 subgroup animals, demonstrating inflammatory response induction and fast graft incorporation into recipient's abdominal wall tissue. Equal changes were reported by Canellas, *et al.* [16] with ovine tunica albuginea as xenograft in rats abdominal wall.

Silva, *et al.* [17] studied heterologous ovine tunica albuginea graft in rats abdominal wall and observed moderate inflammatory infiltrate, moderate neovascularization and intense graft absorption with little viability, showing similarities with our experiment, particularly regards to T21 subgroup.

T28 animals had tunica albuginea absorption findings comparable to Canellas, *et al.* [16] assay, but not so advanced xenograft incorporation to recipient's abdominal wall. This result might be related to bovine tunica thicker than ovine's one leading to a longer absorption time in our study.

Regards to S group, fibroplasia phase in S7 subgroup matched with Andrade [45] and Cotran, *et al.* [46] descriptions. Moreover, Cotran, *et al.* [46] and Pereira [47] described healing process phases as seen in S14 rats. Also, the complete cicatrization observed in S21 and S28 subgroups corroborates with Cotran, *et al.* [46], Andrade [45] and Silva [48] notes.

Considering Picrosirius Red stained sections evaluated under a polarized light microscope, type I collagen was more present in T group healing initial stages, that is animals' subgroups euthanized before the others. This finding is due to tunica albuginea composition which is mostly type I collagen. Collagen type III increasing levels are expected with regular healing evolution [49]. Note that type III collagen was already abundant since cicatrization initial stages in S group rats as the patterns of scar evolution described by Robson and colleagues [50]. How-

ever, we had a greater type III collagen deposition in group T rats abdominal wall than S group what led to profuse fibrosis process and consequential xenograft reinforcement.

Experimental animal models have become vital in the evaluation of abdominal meshes for hernia repair since they allow comparison between different implants placed with same surgical technique. This fact provides information about abdominal mesh performance parameters as integration into the recipient tissue, encapsulation, infection susceptibility, remesothelialization capacity and adhesiogenic potential [51].

Biological prosthesis for reconstruction of the abdominal wall in humans seems to be associated with a high rate of hernia recurrence in long-term follow-up [9], however our study lasted a short period (euthanized rats at most 28 days after surgery). Long-term follow-up studies are essential to evaluate BTA graft rejection and incisional hernia recurrence.

The primary purposes for utilizing biomaterials in hernia repair are mechanical support and native tissue integration [6] and both were achieved in our experiment. Thus, the BTA proved to be efficient as abdominal wall reinforcement since it promoted abundant fibrosis, providing resistance and consequently decreasing postoperative complication rates. Besides, rats did not show rejection signs to bovine tunica implant and it fit biocompatibility criteria for a graft, even though being a heterologous biomaterial. Honey was pointed out as a competent low-cost and available preservative solution.

5. Conclusion

To summarize, these findings support that BTA preserved in honey is an affordable alternative and an easy collection and handling biomaterial for graft, demanding simple surgical implantation technique. The tunica was an excellent substrate in rats abdominal wall repair, promoting ideal reinforcement and early healing. Furthermore, rats represent a valid animal model to comparative future studies that might be undertaken with BTA xenograft in human beings and even domestic animal species in experimental or clinical trials.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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