



## Evaluation of the efficacy of freeze-dried bovine pericardium and acellular bovine skin in the treatment of diaphragmatic hernia in dogs

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### Abstract

The article was designed to evaluate the efficiency of lyophilized bovine pericardium and acellular bovine skin for repairing diaphragmatic hernia in dogs. Twelve local breed adult dogs were used. In all animals, the diaphragmatic hernia was induced by removing 5cm diameter circular piece the diaphragm. The animals were divided into two equal groups (n=6). In the first group, the diaphragmatic hernia was repaired using lyophilized bovine pericardium, and in the second group, acellular bovine skin was applied. The results were evaluated studying the gross and histopathological changes on the 15<sup>th</sup> and 30<sup>th</sup> postoperative days. The gross results showed a different degree of adhesion at the site of hernial repair. The adhesion rate was developed more severely in the second group animals compared to the first group. The histopathological manifestation of the first group showed excessive and more mature granulation tissue formation with few inflammatory cell infiltrations with good angiogenesis comparable to the second group, which revealed moderate granulation tissue formation and blood vessel formation with sever infiltration of inflammatory cells. In conclusion, we can use lyophilized bovine pericardium and acellular bovine skin to repair diaphragmatic hernias in dogs, although lyophilized bovine pericardium is regarded as the best option despite its high cost.

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### Introduction

The diaphragm is the musculoaponeurotic dome-shaped physical wall that separates the abdominal and thoracic cavities and arches over the abdomen, allowing the liver and the spleen to be located underneath it and to be protected by the lower ribs and the chest wall. It consists of a noncontractile central tendon part and two other muscular portions, the crural and costal, and a minor sternal muscular portion (1). Diaphragmatic hernia (DH) is the most common disorder occurring in dogs and cats due to trauma or congenital causes. Rupture of the thin musculotendinous structure of the diaphragm may cause herniation of abdominal structures into the chest, causing a life-threatening respiratory condition and potential entrapment of

abdominal organs (2). Despite advances in therapeutic techniques, the repair of hernia defects continues to be deficient and costly, resulting in chronic healthcare problems. However, hernia repair options are achieved either by surgical or conservative management (3). Generally, the DH is treated surgically (4,5). The biological or synthetic grafts are suggested in large hernial ring cases with a chronic disease (6). Polypropylene mesh, is hard and handled yet related sever complications such as adhesion, mesh extrusion, obstruction, and fistula formation with chronic pain (7,8). Therefore, some complications may lead to a recurrent hernia (9,10). To overcome these undesired weaknesses of synthetic mesh, another natural alternative biodegradable material should be tried, especially when these choices are reliably inert and resistant to microbial

colonization and chronic infection. In addition, other perfect properties of any hernial grafts should also have no initiation for adherence with internal structures, biocompatibility, no carcinogenicity, good ability to withstand pressure over a long period, suitable costs, and pain free following implantation. They should provide the cells to grow, multiply, and dissolve. However, no synthetic implants meet these demands, so the hope finding more suitable mesh materials remains (11). A high complication rate during the healing process may appear when using autogenous tissue. Therefore, different xeno and allografted tissues are indicated. The xenogenic and allogenic materials are varied in their characteristics, such as strength, cellular response, vulnerability to infection, ability to transmit diseases, and biodegradability. Besides, the mechanical properties of such grafts can be changed following *in vivo* implantation (11). The de-cellularization and crosslinking process deeply influence the long-term host response against implantation. Nearly, all kinds of biologically originated meshes elicit a mononuclear cell inflammatory infiltration with different degrees of remodeling response in the long-term (12). During the preparation process, especially the crosslinking step, the collagen of the implantation grafts is treated chemically to counterattack the degradation process by collagenases, which enhance implant strength and, at the same time, limit tissue regeneration and hence influence host responses against the implanted grafts (13). The lyophilization technique, also known as freeze-drying, is one of the most recent useful cryopreservation techniques used to preserve various biological tissues (14). Lyophilization is a procedure in which the samples water content is frozen, and then removed via sublimation and desorption. It is essential for the protection of materials (15). However, the concentration and recovery of reaction goods as lyophilization is an efficient method of drying materials without damaging them (16,17). Despite the many desired characteristics of the biological material meshes, several complications have been documented with their use, including adhesion, rejection, fistula development, stretching, function loss during early degradation, infection, and finally, secondary granulomatous inflammatory reaction (18).

Therefore, this article aims to show the effectiveness of using biodegradable xenograft materials derived from bovine skin and pericardium for diaphragmatic hernioplasty in dogs based on macroscopical and histopathological parameters.

## **Materials and methods**

### **Ethical approve**

The research was approved by the ethics of the Institutional Animal Care and Use Committee for College of Veterinary Medicine, University of Mosul.UM.VET.2022.044.

### **Animals**

Twelve (n=12), 1-3 years adult local breed dogs, from both sexes, weighing 20-25 kg. were acclimatized for seven days before the beginning of this experiment. All surgical and handling procedures were achieved in agreement with the ethical standards and animal utilization protocols. A circular diaphragmatic defect about 5 cm. diameter was created in all dogs. Then, animals were divided randomly into two equal experimental groups (n=6). In the first experiment group, a segment of the bovine lyophilized pericardium was implanted onto a diaphragmatic defect and fixed with a 2/0 polypropylene suture, using a simple interrupted suture technique. Acellular bovine skin was fixed using the same suture material and technique to close the induced diaphragmatic defect in the second group.

### **Preparation of a cellular bovine skin**

Bovine skin was taken from the local abattoir and immediately kept within ice-cold sterile phosphate-buffered saline (PBS, pH 7.4) enriched with broad-spectrum antibiotic (Amikacin-1 mg/ml), and a proteolytic inhibitor (0.02% EDTA) (Figure 1).

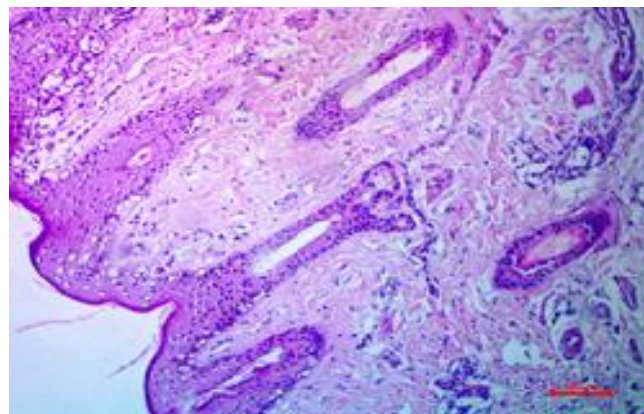


Figure 1: Microscopic picture of the normal bovine skin (H&E, 40X).

In the preparation room, the skin was shaved, and all the adherent debris and blood were washed out thoroughly using sterile PBS. De-epithelization of the skin was done using 0.25% trypsin and 2 M sodium chloride solution for 8 hours. Then, the dermis was decellularized using 2% sodium deoxycholate for 48 hours. The samples were subjected to continuous agitation in a horizontal orbital shaker at 180 rotations per minute to ensure optimal skin contact with chemicals and a better de-epithelization and decellularization process. To ensure the cellularity of the prepared skin samples, a microscopic examination following staining with Hematoxylin and Eosin (H&E) was performed (Figure 2). Then, the prepared acellular bovine skin was washed with sterile PBS solution six times (2 hours each) to remove the residual chemicals and finally stored at -20°C in

PBS solution containing 0.1% amikacin solution (19,20). Finally, the prepared cellular bovine skin was stored at 4°C in PBS containing 1% Gentamycin (21).

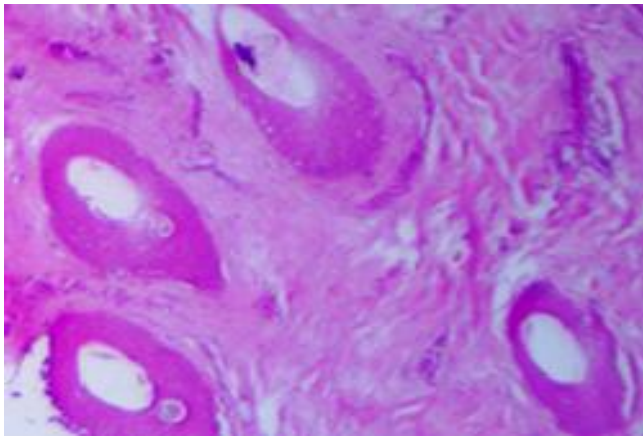


Figure 2: Microscopic picture for acellular bovine skin (H&E, 100X).

#### **Lyophilized bovine pericardium (LyoPlant)**

The lyophilized bovine pericardium was purchased from the local market. It is a biological, absorbable sheet consisting of a bilayer membrane made of pure collagen implant of lyophilized bovine pericardium manufactured by Aesculap, Inc., Center Valley, PA, B. Braun, Germany (Figure 3).



Figure 3: Photographic picture of lyophilized bovine pericardium.

#### **Anesthetic protocol**

The dogs were off feed for about twelve hours before the surgical intervention. The surgical operations were applied under general anesthesia using atropine sulfate of a dose of 0.044mg/kg as a premedication, then followed by a premixed intramuscular mixture of 10 mg/kg of Ketamine Hydrochloride and 5 mg/kg of xylazine (22). All the experimental animals were connected to the positive pressure ventilation machine to prevent lung collapse (23).

#### **Surgical procedure and creation of the diaphragmatic hernia.**

Routine surgical skin preparation on the site of the thoracic cavity was performed. The access to the diaphragmatic muscle, intercostal thoracotomy was achieved. About 10 cm skin incision in the ninth left intercostal space was made. The surgical incision was continuous through the subcutaneous tissue and thoracic muscles until it reached the pleura. Then, the pleura was opened very carefully with blunt scissors to avoid any accidental damage to the lung tissues (Figure 4) (24).

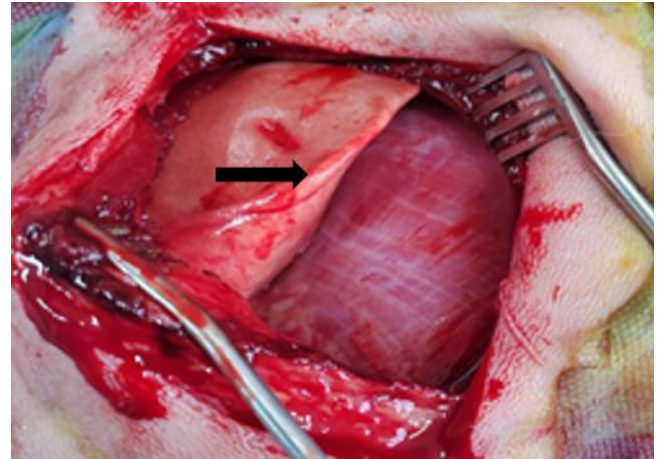


Figure 4: Photographic image for the normal approached diaphragm.

After identifying the muscular part of the diaphragm, a circular piece of about 5cm diameter of the left diaphragm muscle was demarcated and removed with a scalpel, resulting in a defect of the exact dimensions (Figure 5).



Figure 5: Photographic image of the induced diaphragmatic defect

Then, a double layer of lyophilized bovine pericardium segment was fixed and sutured into the edges of that diaphragmatic injury by 2/0 polypropylene suture using simple interrupted in the first group (Figure 6). At the same time, cellular bovine skin sheets were used for the second group (Figure 7). Finally, thoracorrhaphy was performed with simple continuous stitches made of 2/0 Vicryl.

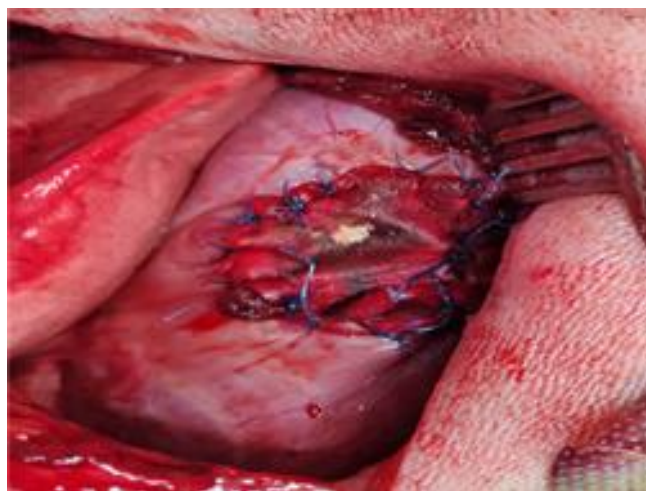


Figure 6: Photographic image for grafting the hernia with lyophilized bovine pericardium.

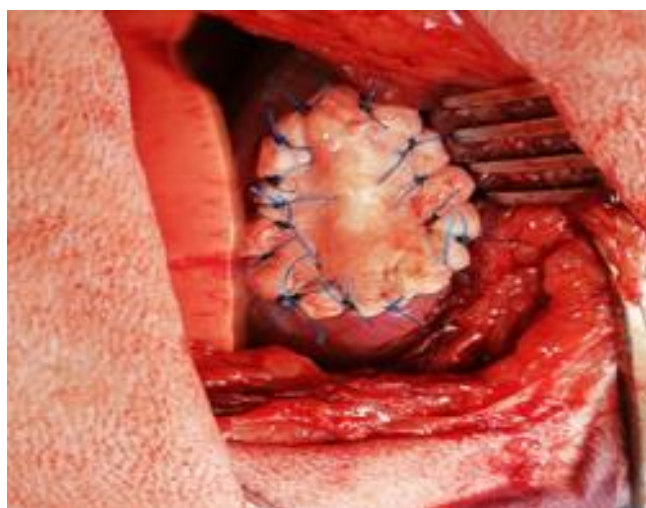


Figure 7: Photographic image for grafting the hernia with a cellular bovine skin.

#### **Postoperative care**

A daily inspection of the intercostal thoracotomy skin wounds was done for any gross signs of wound complications or dehiscence. Penicillin/streptomycin (Nederhorst den Berg, Holland) (1 ml/10 kg body weight per day for five days) and Metalgen (SPI, Saudi) (1 ml/day for five days) were administered to the dogs.

#### **Assessment of healing**

The progress of the healing process was evaluated through observation of the clinical signs post-operation during all study periods. The gross and histopathological changes were also studied on the 15<sup>th</sup> and 30<sup>th</sup> days after the operation. The experimental animals did not kill where the biopsies of the graft area were obtained for histopathological evaluation by intercostal thoracotomy procedures, and the samples were fixed in 10% formalin and stained with hematoxylin and eosin (H&E) stain.

#### **Results**

##### **Gross results**

All experimental animals survived after surgery and showed no complications post-operation. In both groups, varying degrees of adhesion were developed at the site of diaphragmatic hernial repair on the 15<sup>th</sup> day postoperatively. The degree of adhesion of the second group appeared more than that of the first group. While, on the 30<sup>th</sup> postoperative day, the adhesion was also evident at the site of diaphragmatic hernial repair, adhesion formation was much more abundant in the second group than in the first group.

##### **Histopathological findings**

In the first group (lyophilized bovine pericardium), the histopathological sections for the site of diaphragmatic hernia at the 15<sup>th</sup> day postoperative revealed the presence formation of excessive new granulation tissue, excellent profuse development of new blood vessels and mild infiltrations of mononuclear inflammatory cells (Figures 8-10). After the 30<sup>th</sup> day, the histopathological features were characterized by granulation tissue maturation with no or tiny infiltration of mononuclear inflammatory cells and reduced angiogenesis (Figures 11-13).

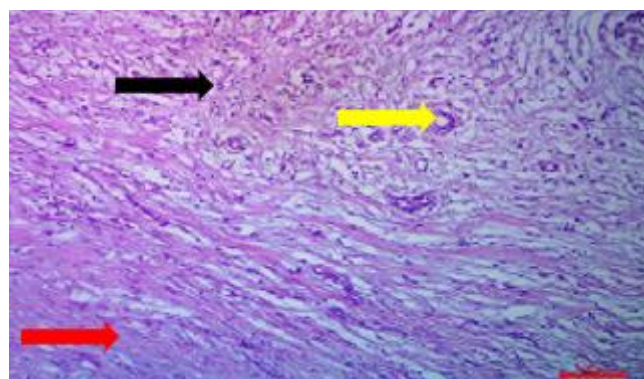


Figure 8: Micrograph at 15<sup>th</sup> day post operation in the first group showing newly granulation tissue formation (red arrow), severe angiogenesis (yellow arrow), and little infiltration of inflammatory cells (black arrow). (H&E 100X).

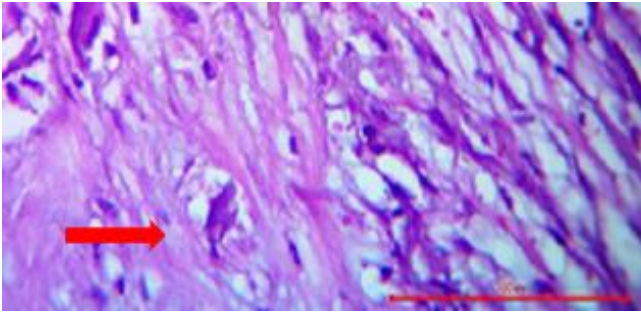


Figure 9: Micrograph at 15<sup>th</sup> day post operation in the first group showing newly granulation tissue formation (red arrow) (H&E, 400X).

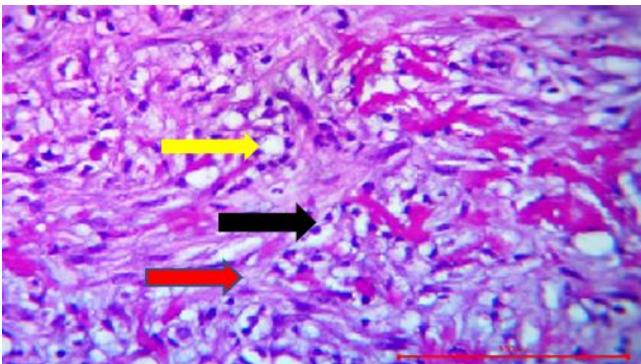


Figure 10: Micrograph at 15<sup>th</sup> day post operation in the first group showing newly granulation tissue formation (red arrow), severe angiogenesis (yellow arrow), and little infiltration of inflammatory cells (black arrow). (H&E 400X).

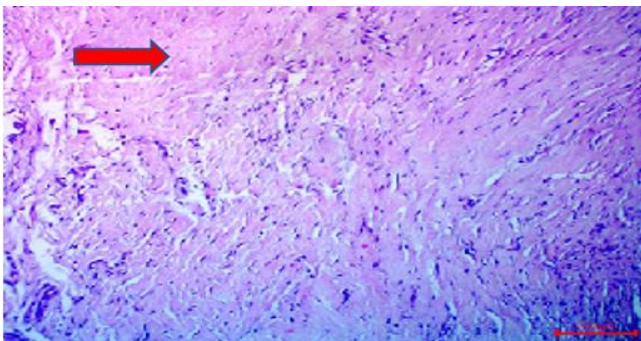


Figure 11: Micrograph at 30<sup>th</sup> day post operation in the first group showing mature granulation tissue (red arrow). (H&E 100X).

On the other hand, the histopathological features for the second group (A cellular bovine skin) after the 15<sup>th</sup> day post-implantation, showed moderate new granulation tissue formation and blood vessel development with severe infiltration of mono and multinuclear inflammatory cells (Figures 14-16). Besides, on day 30 following surgery, the

histopathological changes were characterized by less angiogenesis and the presence of inflammatory cells. The acellular part starts to rebuild its cellular components, and maturation of granulation tissue was observed (Figures 17-19).

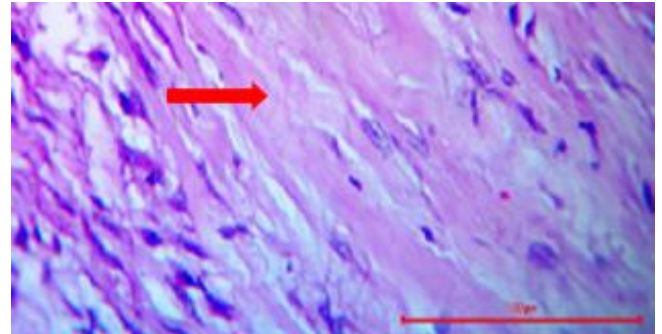


Figure 12: Micrograph at 30<sup>th</sup> day post operation in the first group showing mature granulation tissue (red arrow). (H&E 400X).

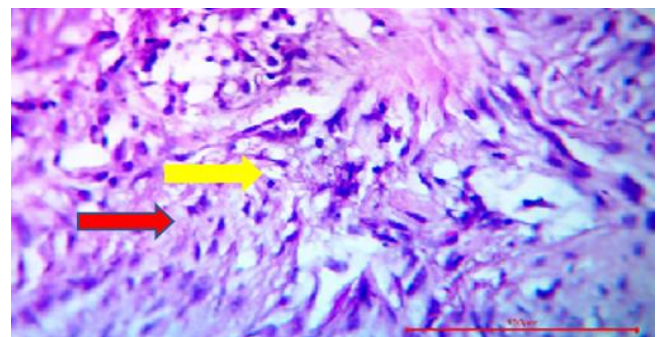


Figure 13: Micrograph at 30<sup>th</sup> day post operation in the first group showing mature granulation tissue (red arrow) and few infiltrations of inflammatory cells (yellow arrow). (H&E 400X).

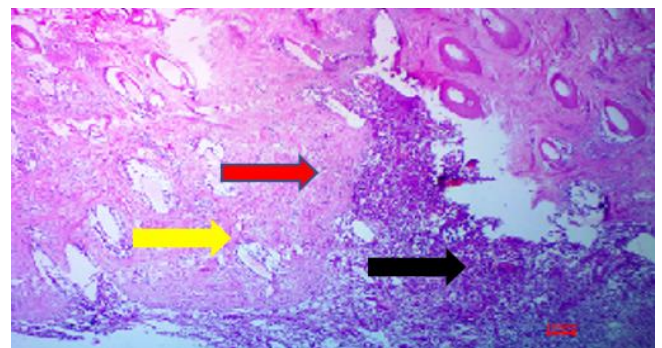


Figure 14: Micrograph at 15<sup>th</sup> day post operation in the second group showing newly granulation tissue formation (red arrow), moderate angiogenesis (yellow arrow), and more infiltration of inflammatory cells (black arrow). (H&E 40X).

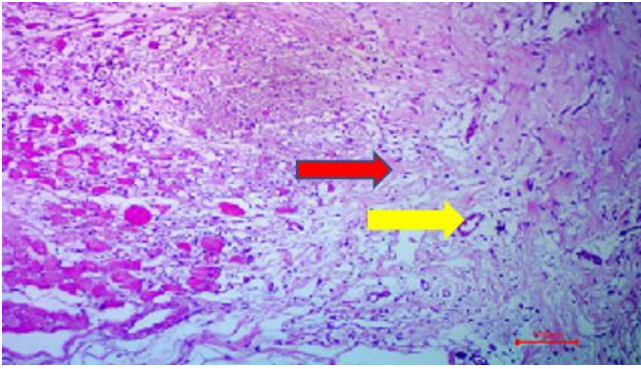


Figure 15: Micrograph at 15<sup>th</sup> day post operation in the second group showing newly granulation tissue formation (red arrow), moderate angiogenesis (yellow arrow). (H&E 100X).

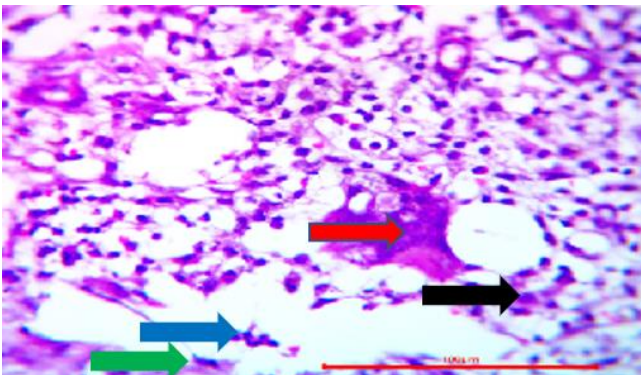


Figure 16 Micrograph at 15<sup>th</sup> day post-operation in the second group showing infiltration mono and multinucleated inflammatory cells as neutrophil (blue arrow), giant cell (red arrow),macrophage (black arrow), and lymphocyte (green arrow) (H&E, 400X).

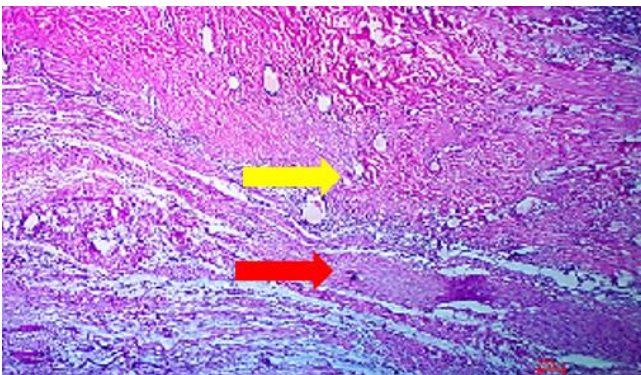


Figure 17: Micrograph at 30<sup>th</sup> day post-operation in the second group showing mature granulation tissue (red arrow) and angiogenesis (yellow arrow)(H&E 100X).

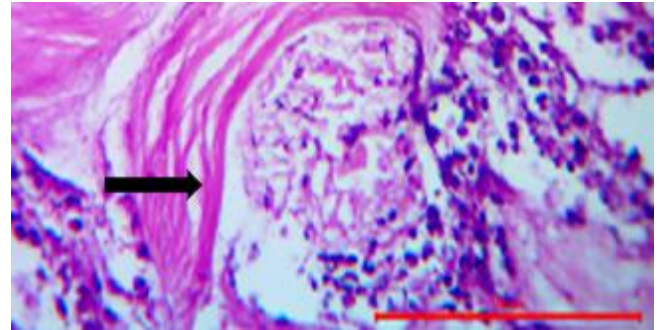


Figure 18: Micrograph at 30<sup>th</sup> day post operation in the second group showing the orientation of collagen fibers (black arrow) (H&E 400X).

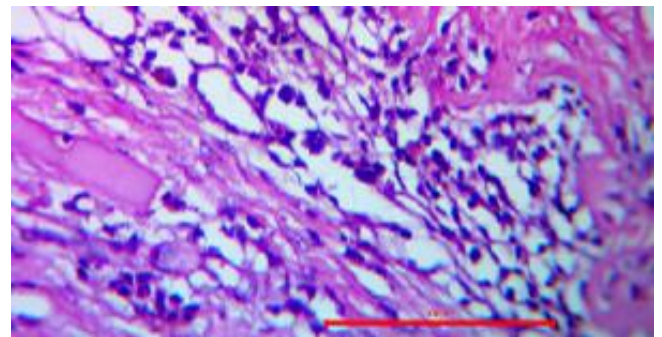


Figure 19: Micrograph at 30<sup>th</sup> day post operation in the second group showing infiltration of inflammatory cells (H&E 400X).

## Discussion

Utilizing various prostheses for hernial repair is highly recommended in cases of the presence of insufficient tissue margins for perfect closing of the defect with sutures or in chronic hernias when first reduction may cause primary postoperative dehiscence as a result of high sutures tension or intraoperative rupture (6). In this research, we used processed skin and pericardium xenografts derived from cows as a biological scaffold due to their huge bioavailability and the long history of their successful applications (25-28). The extracellular matrix (ECM) of acellular bovine skin and lyophilized bovine pericardium both supplied the host cell with the suitable basement to invade and grow as well as supported structural and functional performance that enabled the complete closure of induced hernial defects, which had been noticed since the early period following transplantation. Furthermore, these viable scaffolds have many vital proteins that preserve the health of tissue differentiation, and support the host response to tissue damage, which becomes an ideal option for replacing missing or damaged tissues (29).

In the current study, the macroscopic data following the use of lyophilized bovine pericardium and a cellular bovine

skin showed the existence of adhesion between the site of diaphragmatic hernial repair and the internal surrounding tissues of the thoracic wall following surgery. However, that adhesion was much higher with cellular bovine skin than with lyophilized bovine pericardium. This noticed adhesion may be due to foreign suture materials and initiation of inflammatory reaction following xenograft implantation (30,31). The more adhesion associated with cellular bovine skin was because it elicited a more inflammatory reaction, which was more obvious histopathologically comparable to lyophilized bovine pericardium. The less adhesion accompanied by lyophilized bovine pericardium is a good prognosis as the perfect implant should irritate less tissue reaction with good incorporation into the receipt tissue because adhesion with prosthetic subject remains an unsolved clinical problem (30). Furthermore, the adhesion associated with hernia repair continues to be one of the most common complications in about 90% of all patients undergoing hernial repair surgery (32). Other researchers even tried using some substances like Aloe vera gel to decrease the adhesion rate between hernial ring, graft, and viscera following implantation during their work for repairing induced abdominal hernia (33). Our mentioned results followed another research finding that noticed the presence of adhesion within the hernial site following its repair (30,34).

The microscopic findings following implantation of lyophilized bovine pericardium indicated the presence of slight inflammatory cell infiltration as comparable with their heavy existence associated with acellular bovine skin. The existence of inflammatory cells associated with lyophilized bovine pericardium (35), who documented the lack of inflammatory cells nor calcification associated with using of CardioCel graft, which is a material manufactured from bovine pericardium after processing through a series of tissue engineering protocol. This difference in the severity of inflammatory reaction between these two xenografts could be because the use of bovine pericardium as lyophilization decreases tissue immunogenicity and enhances its acceptance (36). Furthermore, less immunogenicity lessens cell infiltration and enhances the shelf life of the graft (37). Besides, it is highly accepted that lyophilization does not significantly influence the microstructure or the tissues expression of bioactive factors (38). Also, the less foreign body reaction associated with using lyophilized bovine pericardium in our research is in great accordance with other researchers' data who also utilized bovine pericardium to close experimentally induced abdominal defects in rats and concluded that bovine pericardium initiated a minimal foreign-body reaction and less or no macrophage response thus offering excellent mechanical support as early as two weeks after implantation (39). Other clinical studies on using a cellular bovine pericardium within contaminated tissues proved its superiority for repairing major abdominal defects because of its high tensile strength and low infection rate

(40,41). All these factors enabled the lyophilized bovine pericardium to initiate a less inflammatory reaction when compared with cellular bovine skin. On the other hand, the histopathological manifestation of a cellular bovine skin showed severe infiltration of inflammatory cells, especially mononuclear, which indicates that it elicited more immunogenicity and less acceptance. Other researchers who documented the severe cell infiltration also noticed the intense spreads of these inflammatory cells associated with cellular bovine skin (42-44). Furthermore, the infiltration of inflammatory cells with cellular bovine skin indicated the presence of foreign body reactions and chronic inflammation (45).

Another histopathological inspection of a cellular bovine skin and lyophilized bovine pericardium revealed the presence of angiogenesis, which was more developed and huge with lyophilized bovine pericardium compared to a cellular bovine skin; this revascularization developed more and more with the progress of the healing process which is considered as the key for perfect hernial repair as angiogenesis and the formation of new capillaries is the elementary phenomena within any tissue regeneration as these new formed blood vessels would transport essential nutrients and oxygen supply to the actively energetic regenerative tissue which is crucial for biologic ingrowth in hernia prosthetics as they need an adequate tremendous vascular support. The network of newly formed blood vessels seems to confirm the enhancement of the lyophilized bovine pericardium's biological features that made it resemble a regenerative scaffold (46). Our data agrees with other investigators data noticed the presence of angiogenesis following transplantation in his comparative hernioplasty in sheep using pericardium and tunica vaginalis allografts (47). Good angiogenesis following implantation helped the sound performance of lyophilized bovine pericardium comparable with a cellular bovine skin, as a slow rate of angiogenesis during the healing process is considered one of the chief drawbacks of using scaffolds.

Finally, The existence of abundant and developed granulation tissue associated with a lyophilized bovine pericardium group in comparison to a cellular bovine skin was evident post-implantation, indicating that the lyophilized pericardium incorporated into the tissue much better than cell bovine skin and indicative for the sound development of repairing process within the grafted site (18,21). Furthermore, granulation tissue was also observed with another worker (48) who noticed the presence of granulation tissue associated with irregular angiogenesis invading the implant during his study for hernial treatment.

## **Conclusions**

The macroscopic and the microscopic results of this study exhibited that repairing the diaphragmatic hernia in dogs can be done by using either lyophilized bovine

pericardium or acellular bovine skin as biological implants with superiority of the lyophilized bovine pericardium.

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### Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this article.

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## تقييم كفاءة التامور البقري المجفف بالتجميد والجلد البقري اللاخوي في علاج فتق الحجاب الحاجز في الكلاب

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### الخلاصة

تم تصميم هذه التجربة لتقييم كفاءة استخدام كلا من تامور قلب الأبقار المجفف بالتجميد وجلد الأبقار اللاخوي في إصلاح الفتق الحجابي في الكلاب. تم استخدام اثني عشر كلباً بالغاً محلياً. في جميع حيوانات التجربة تم استحداث فتحة الفتق في الحجاب الحاجز عن طريق إزالة قطعة دائرية قطرها حوالي خمس سم. ثم بعد ذلك قسمت الحيوانات إلى مجموعتين متساويتين. في المجموعة الأولى، تم إغلاق الفتق الحجابي باستخدام التامور البقري المجفف بالتجميد بينما في المجموعة الثانية تم الترقيع باستخدام جلد البقر اللاخوي. لتقييم نتائج التجربة تم الاعتماد على التغيرات العيانية والنسجية المرضية في اليومين الخامس عشر والثلاثين بعد الجراحة. أظهرت النتائج العيانية وجود التصاقات مختلفة الشدة في منطقة إصلاح الفتق حيث كانت الالتصاقات أكثر شدة مع حيوانات المجموعة الثانية مقارنة بالمجموعة الأولى. بينما أظهرت التغيرات النسيجية المرضية للمجموعة الأولى تكوين أنسجة حبيبية بكمية كبيرة وأكثر نضجاً مع القليل من ارتشاح الخلايا الالتهابية مع تكوين أوعية دموية جديدة بنسبة جيدة مقارنة بالمجموعة الثانية التي كشفت عن تكوين نسيج حبيبي متوسط وتكوين أقل للأوعية الدموية مع ارتشاح أكثر شدة للخلايا الالتهابية. الاستنتاج، يمكن استخدام كل من التامور البقري المجفف بالتجميد وجلد الأبقار اللاخوي لإصلاح فتق الحجاب الحاجز في الكلاب على الرغم من أن التامور البقري المجفف بالتجميد يعد هو الخيار الأفضل على الرغم من تكلفته العالية.