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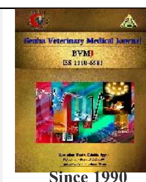
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Original Paper

Evaluation of different decellularization techniques on bovine pericardium and jugular vein for xenografts production

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ABSTRACT

This study was carried out to estimate a good decellularization protocol for pericardium and jugular vein of cattle and buffalo with preservation of the extracellular matrix. Pericardium and jugular vein were decellularized chemically using Triton X-100 (TX) plus sodium deoxycholate (SD), physically by freeze–thaw cycles +TX + SD and enzymatic protocol used TX+SD+ DNase +RNase. Untreated pericardium and jugular vein were used as control. The histological analysis was performed to evaluate the efficiency of decellularization and extracellular matrix preservation. But the immunoreaction was evaluated by subcutaneous implantation in rats. We found that no cells or cell fragments were retained, and there was no apparent tissue disruption in the decellularized tissues, except chemical group showed that the layers of pericardium were dispersed, and layers of jugular vein revealed that the elastic fibers were dispersed, degraded, and disorganized. The content of collagen and elastic fibers were affected after decellularization, in physical group showed mild detached collagen fibrils and mild fragmented elastic fibers, but enzymatic group makes sever detached collagen fibrils and sever disrupted elastic fibers. The implanted tissue showed mild-moderate inflammatory reaction and calcification in physical group, but scanty to mild inflammatory reaction and calcification in enzymatic group. These results suggested that the physical protocol showed optimal decellularization results with better extracellular matrix preservation than other methods and the enzymatic method is good in reduction the immunoreaction (mild to scanty inflammatory cells infiltration).

1. INTRODUCTION

Artificial heart valve replacement is the most common approach for treating valvular heart disease at the final stage. Every year, more than a quarter of a million of patients need heart valve replacement all over the world. Recently, the world begins to use bio-prosthetic heart valves, mechanical heart valves, and a few homogeneity biological valves (Lund and Bland, 2006).

Bovine pericardium (BP), a natural biomaterial, has several advantages when constructing a heart valve. Because of a low-cost biological scaffold, ideal anatomical structure and using as a cardiovascular implant. In addition, it preserves mechanical strength, the three-dimensional structure, and the basic ECM structures such as collagen, elastin fibers, and some glycosaminoglycan (Sacks, et al., 2006). Bovine pericardium may decellularize through different techniques as mechanical, chemical, detergent, and enzymatic methods, or a combination. Each of them has different effects upon both the resulting biologic scaffold and the associated host remodeling response and outcome. Although, the combination of the different methods effecting on the decellularized tissue by minimizing side effects on the remaining matrix constituents (Crapo et al., 2011; Keane et al., 2015) .

To get ideal tissue-engineered grafts, make decellularization to bovine jugular vein patches and their tissue stability appeared through the subcutaneous implantation into the rat (Lü et al., 2009). It is important that the decellularization technique makes removing of all cells and cell fragment in the extracellular matrix (ECM) as the cellular components which considered evidence associated with inflammation and calcification that may lead to calcified tissue deterioration and limited conduit longevity (Breymann et al., 2002; Simon et al., 2003).

Bovine pericardium is widely used for repairing congenital and acquired cardiac defects, and also to create artificial heart valves (Bielli et al., 2018). It has been used as a commercial product such as Tuto- patch® which applied in the repair of abdominal hernias, Peri-Guard® that used as a prosthesis for pericardial closure and soft tissue deficiency like abdominal defects (femoral, inguinal, scrotal, umbilical, diaphragmatic, and lumbar) along with intra-cardiac and great vessel repair (Gilbert et al., 2006; Hülsmann et al., 2012).

2. MATERIAL AND METHODS

This study mainly composed of two main parts: decellularization protocols and experimental groups. It was carried out at Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Benha University, Egypt.

2.1. Materials

2.1.1. Tissue sampling

Pericardium and Jugular vein of cattle and buffalo were obtained from a local slaughterhouse in Toukh city within 2-4 hrs. after slaughtering, then immediately rinsed with phosphate-buffered saline (PBS) to remove blood and body

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fluids, then transported from slaughterhouse to the laboratory in cold sterile phosphate-buffered saline (PBS) plus was made of phosphate-buffered saline containing 1% penicillin/ streptomycin (p/s) and 1% Gentamycin), then tissue samples were soon dissected to remove the external fat and any adherences (Gardin et al., 2015).

2.1. 2. Animals

Thirty-six healthy male Sprague-Dawley rats weighing between 280 and 351 gm were purchased from Animal Health Research Institute, Dokki, Egypt.

2.2. Methods

2.2.1. Decellularization protocols

For decellularization process, tissue samples were divided into four groups: control, chemical, physical (Li et al., 2018) and enzymatic (Lü et al., 2009 and Li et al., 2018). Pericardial samples were cut into 5 cm. square patches. Jugular vein (JV) samples were cut into 5 cm. long segments.

1- Control group: Pericardial patches and jugular vein segments of cattle and buffalo were immersed in Tris buffer (10 mM, pH 7.6) for 48 hours at 40°C.

2- Chemical group: Pericardial patches, jugular vein segments of cattle and buffalo were treated with 1% (v/v) Triton X-100 and 0.5% (w/v) SD dissolved in Tris buffer (10 mM, pH 7.6) for 48 hours 40 °C.

3- Physical group: Pericardial patches, jugular vein segments of cattle and buffalo animals were freeze–thawed (After the first thaw in Tris buffer at room temperature, samples were shock-frozen in liquid nitrogen [-196°C] for 10 min and then thawed at 37°C for 10 min.) for five cycles and rinsed with deionized water, then treated with 1% (v/v) Triton X-100 and 0.5% (w/v) SD dissolved in Tris buffer (10 mM, pH 7.6) for 48 hours 40 °C.

4-Enzymatic group: Pericardial patches, jugular vein segments of cattle and buffalo were treated with 1% (v/v) Triton X-100 and 0.5% (w/v) SD dissolved in Tris buffer (10 mM, pH 7.6) for 48 hours 40 °C. Then were treated with DNase I (30 U/ml)/RNase A (0.3 mg/ml) with 50 mmol/$MgCl_2$ for 24 hr. This step was conducted under continuous shaking condition at 37 °C.

After the first decellularization procedure, tissues samples were washed with distilled water two times at 4 °C for 12 hours. In the final stage of the decellularization procedure, it was washed in PBS for three days by changing the solution every eight hours. This step was performed to remove detergent residues.

2.2.2. Histological examination

Pericardial patches and jugular vein segments of different four groups were fixed in 4% buffered formaldehyde for 24 hours, processed into paraffin, and then sectioned at 5 µm. The tissue sections were deparaffinized and stained with Hematoxylin and eosin (H & E), Elastic von Gieson and Alizarin Red (Li et al., 2018).

2.2.3. In vivo Xeno-grafting of cattle and buffalo pericardium and jugular vein in rats by subcutaneous implantation.

Rats were maintained in standard housing conditions with food and water ad libitum, and then given at least one week for acclimatization. Those rats were used for the subcutaneous implantation of cattle and buffalo pericardium and jugular vein samples. The rats were divided into six groups: Control, Physical and Enzymatic for cattle, buffalo pericardium and jugular vein (Six rats for each group).

Rats were prepared for operative technique, anesthetized with ketamine and xylazine. The hair on the dorsal body surface was shaved, and the skin was washed with 70% alcohol and Betadine. An incision of1cm length was made in the lumbar region of each rat. Sub dermal pocket was created bluntly, and one piece of tissue sample was then inserted into each pocket smoothly against the body musculature, and the incisions were closed with sutures, Rats were allowed to recover and maintained in standard housing conditions with food and water ad libitum. At week two, tissue implants were harvested and possessed for histopathological examination (Li et al., 2018).

3. RESULTS

Histologically, the control pericardium revealed a dense tissue structure and high cellular contents within the extracellular matrix (ECM) (Fig 1.A and Fig 2.A). While the control jugular vein revealed a normal tissue structure (normal endothelial lining, intact smooth muscle layers and normal connective tissue fibers with no visible disruption of the tissue and fibril structure (Fig 1.E and Fig 2.E).

After comparing decellularized pericardium and jugular vein with control, it was found that no cells or cell fragments were retained, and there was no apparent tissue disruption in all decellularized tissue sections (Fig 1and2).But we found some exception in the decellularized tissues.

In the chemical group, the cattle pericardium showed dispersion of the outer layer (Fig 1.B), while the jugular vein showed that the elastic fibers were detached, degraded and disorganized in inner layer (Fig 1.F). The buffalo jugular vein revealed that Collagen and elastic fibers were detached in the outer and inner layers but degraded and disorganized in the inner layer (Fig 2.F).

In the physical group, the cattle pericardium showed sever wavy disruption but mild dispersion in the fibrous layer (Fig 1.C), while buffalo pericardium revealed mild wavy disruption but mild diffusion in the fibrous layer (Fig 2.C). The cattle jugular vein showed mild detached in the middle layer as well (Fig 1.G).

In the enzymatic group decellularized tissue of cattle pericardium showed mild wavy disruption (Fig 1.D) and buffalo pericardium mild wavy disruption but moderate dispersion in the fibrous layer (Fig 2.D).

Elastic von Gieson staining showed that control pericardium and jugular vein of cattle contain normal collagen fibrils and clear elastic structures (Figs 3.A and E). After comparing decellularized pericardium and jugular vein with control, it was found that the decellularized tissues, in the chemical group showed moderate detached collagen fibrils and moderate fragmented elastic structures (Figs 3. B and F), in the physical group revealed mild detached collagen fibrils and mild fragmented elastic structures (Figs 3.C and G). While in the enzymatic group, it showed sever dispersed collagen fibrils sever fragmented elastic structures (Figs 3.D and H).

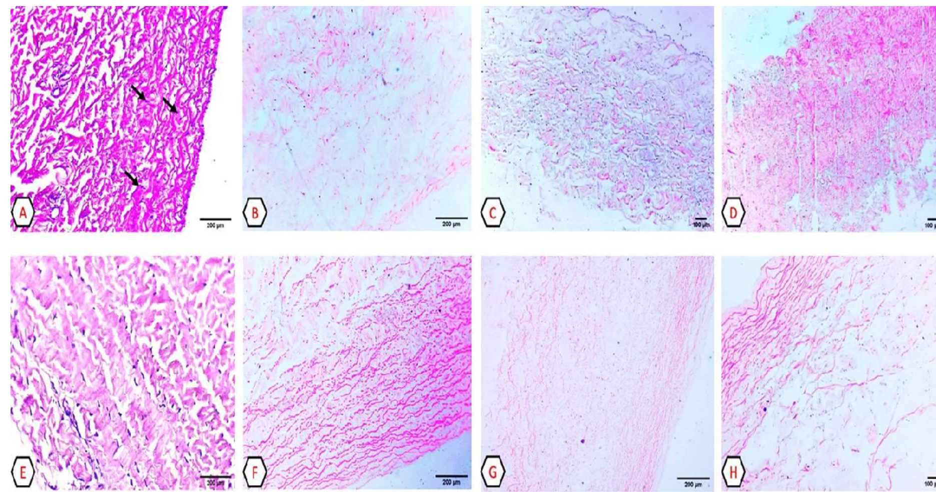

Fig. 1 Photomicrographs showed that decellularization removed the cellular components from cattle pericardium in (B,C&D) and jugular vein in (F,G,H) .Black arrows indicate cellular nuclei. A& E: normal tissue B&F: decellularized tissue by Triton X-100 (TX) + sodium deoxycholate (SD), C&G: by freeze–thaw cycles + TX + SD. D&H: by TX + SD + DNase& RNase. Hematoxylin and Eosin stain.

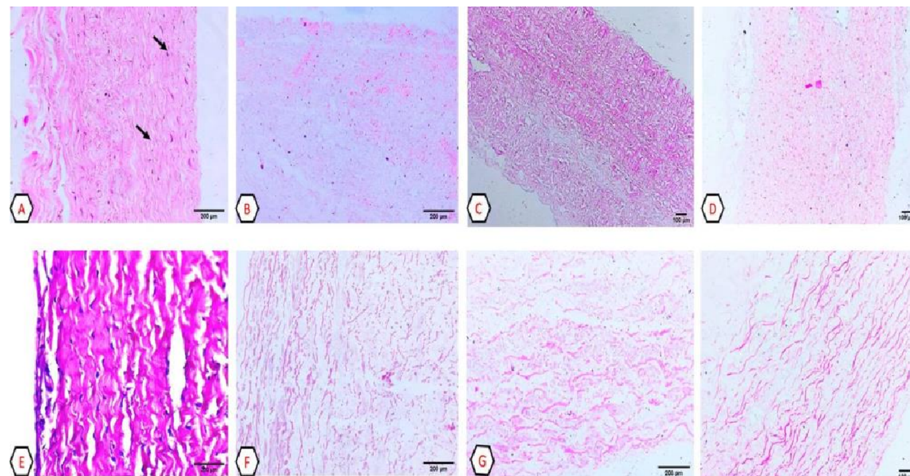

Fig. 2 Photomicrographs showed that decellularization removed the cellular components from buffalo pericardium in (B, C&D) and jugular vein in (F, G,H) .Black arrows indicate cellular nuclei. A& E: normal tissue B&F: decellularized tissue by Triton X-100 (TX) + sodium deoxycholate (SD), C&G: by freeze–thaw cycles + TX + SD. D&H: by TX + SD + DNase& RNase. Hematoxylin and Eosin stain.

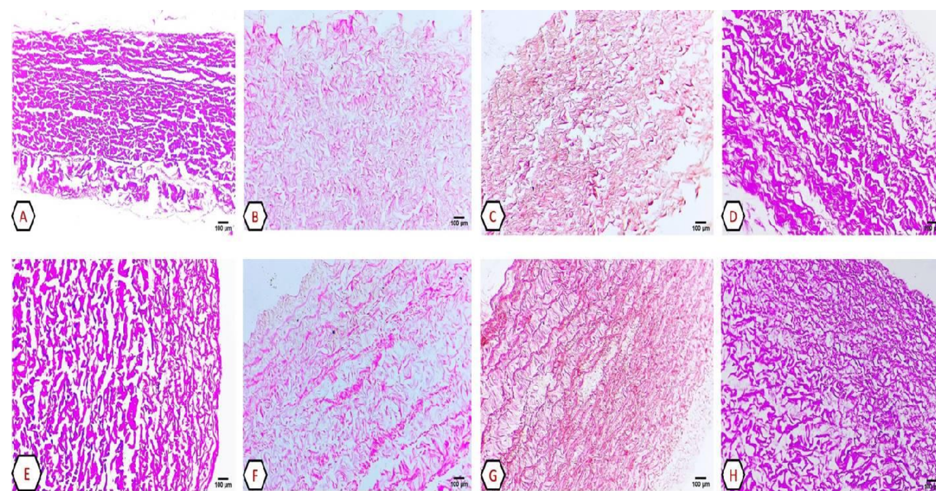

Fig. 3 Photomicrographs of cattle pericardium (A-D) &jugular vein (E-H) showing changes in elastic fibers. A& E: normal tissue B&F: decellularized tissue by Triton X-100 (TX) + sodium deoxycholate (SD), C&G: by freeze–thaw cycles + TX + SD. D&H: by TX + SD + DNase& RNase. Elastic von Gieson stain.

Histological results of implanted tissues (pericardium and jugular vein of cattle and buffalo subcutaneous implanted in rats) showed that control pericardium (without decellularization) revealed severe necrosis of the outer serosal layer associated with loss of the covering mesothelial cells, separation of the fibers and marked mononuclear cells infiltration (Fig 4. and Fig 5.A). and control Jugular vein implants showed necrotic changes, marked inflammation extended from the outer adventitial layer till the intimal layer associated with marked mononuclear inflammatory cells infiltration (Fig 4.D and Fig 5.D).

After comparing decellularized implants with control, we found that pericardial implants of physical group revealed moderate necrosis of the outer serosal layer, separation of the fibers associated with mild to moderate degree of inflammation (Fig 4.B and Fig 5.B). and the implanted jugular vein showed mild to moderate degree of inflammation in endothelial lining accompanied with loss of intimal layer, calcification and mononuclear inflammatory cells infiltration (Fig 4.E and Fig 5.E).

The pericardial implants of enzymatic group showed separation of the outer layer, focal areas of necrosis and associated with mild to scanty calcification (Fig 4.C and Fig 5.C) and the implanted jugular vein revealed mild necrosis and sloughing of the intimal layer, mild inflammatory cells infiltration with scanty calcification (Fig 4.F and Fig 5.F).

Alizarin red staining showed that implants of control group revealed sever calcification with high calcium deposition (Fig 6.A and D) then after comparing implants of decellularized group with control showed moderate deposition of calcium in the implants of physical group (Fig 6.B and E). But, in the implants of enzymatic group showed scanty to mild deposition of calcium (Fig 6.C and F).

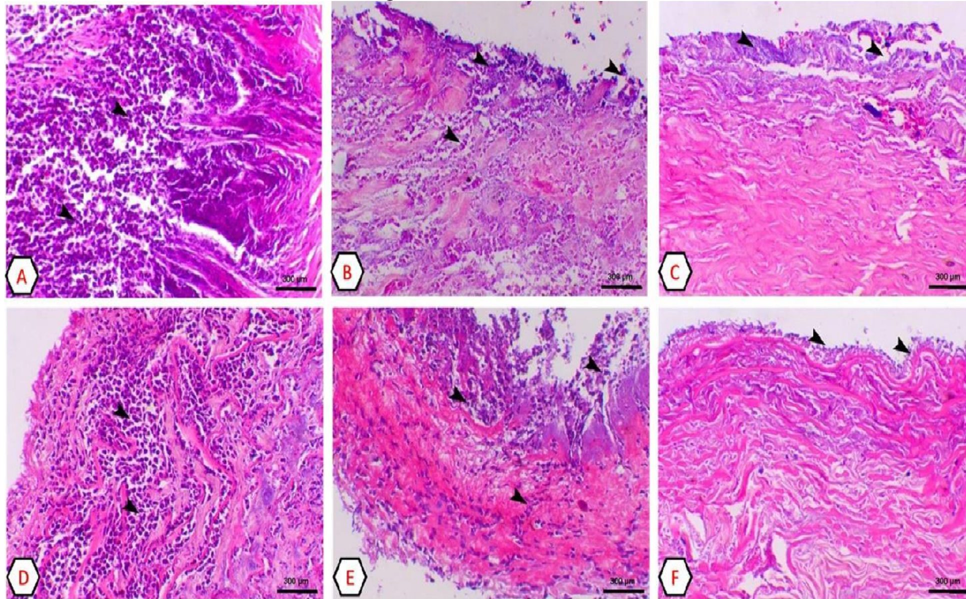

Fig. 4 Photomicrographs of cattle pericardium & jugular vein, 2 weeks later after S/C implantation showing immunological reaction on tissue implants. Arrowheads indicate mononuclear cells infiltration. A& D: normal tissue implants B&E: decellularized tissue implants by freeze–thaw cycles +Triton X-100 (TX) + sodium deoxycholate (SD), C&F: by TX + SD + DNase& RNase. Hematoxylin and Eosin stain.

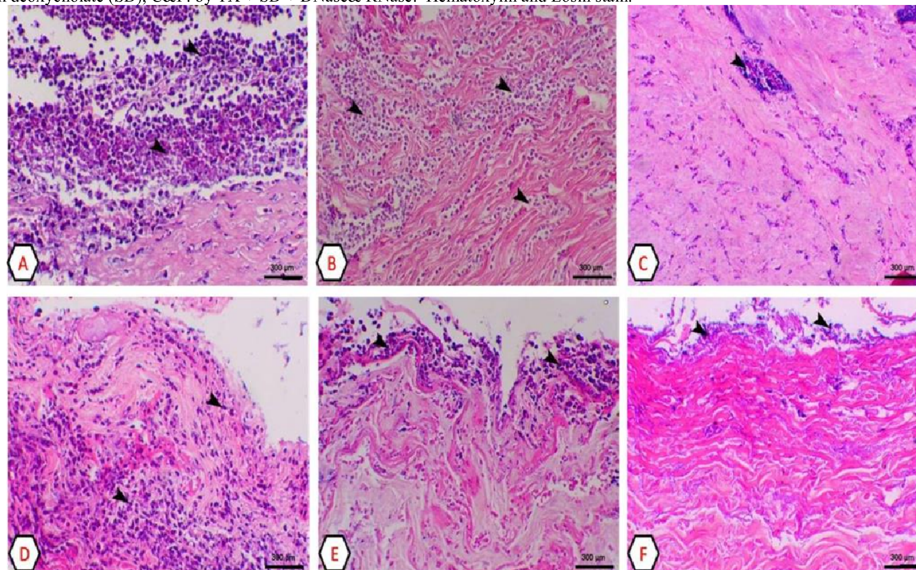

Fig. 5 Photomicrographs of buffalo pericardium & jugular vein, 2 weeks later after S/C implantation using Haematoxylin and eosin staining showing immunological reaction on tissue implants. Arrowheads indicates mononuclear cells infiltration. A& D: normal tissue implants B&E: decellularized tissue implants by freeze–thaw cycles +Triton X-100 (TX) + sodium deoxycholate (SD), C&F: by TX + SD + DNase& RNase. Hematoxylin and Eosin stain.

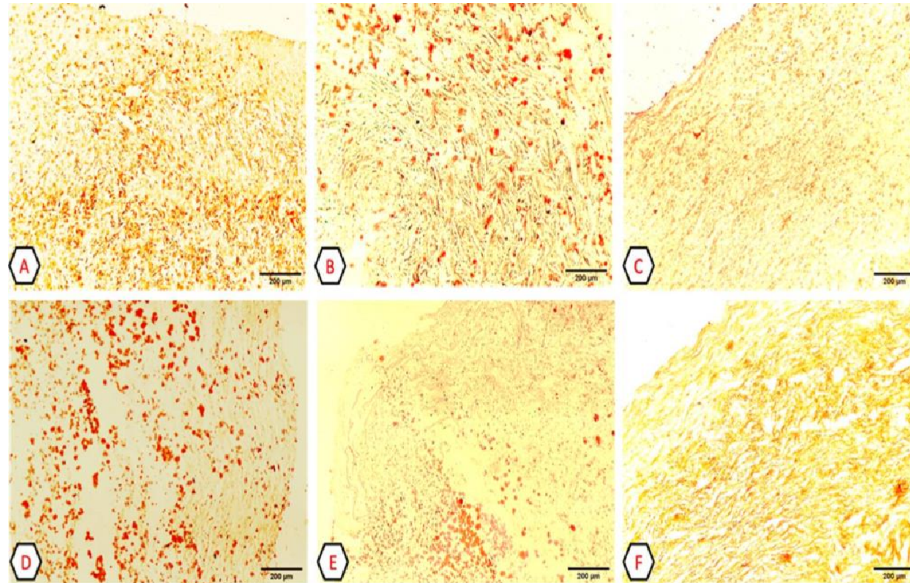

Fig. 6 Photomicrographs of cattle pericardium (A-C) &jugular vein (D-F) showed calcification. A& D: normal tissue implants B&E: decellularized tissue by freeze–thaw cycles +Triton X-100 (TX) + sodium deoxycholate (SD), C&F: by TX + SD + DNase& RNase. Alizarin red stain.

4. DISCUSSION

In this work we have compared the effects of chemical agents (TritonX-100+ Sodium deoxycholate) alone and when combined with physical (freezing -thawing) and enzymatic (DNases and RNases) agent with the control tissue sections. This agreement with Crapo et al. (2011). We found that decellularization of tissue make complete removing of cellular components. This finding agreement with Kasimir et al. (2003).

Regarding the histological examination of native bovine pericardium, our study revealed a dense tissue structure with a large number of pink collagen fibers and dark purple nuclei suggesting high cellular material within the extracellular matrix ECM. This finding in agreement with Yang et al. (2009).

The histological examination of native bovine jugular vein showed a normal tissue structure (normal intima, media and adventitia) with no visible disruption of the tissue and fibril structure. This finding in agreement with Tennant and McGeachie (1990).

In present study comparing chemical and control group of decellularized tissue sections (BP and BJV), it was found that no cells or cell fragments were retained in BP. This result in agreement with Goldstein (2005) and Bertanha et al. (2014). Moreover, we found that no apparent tissue disruption and there was dispersion of the outer layer in cattle pericardium. Also, BJV decellularized chemically revealed that no cells or cell fragments were retained, and there was no apparent tissue disruption. This result in agreement with Li et al. (2007) Moreover, we found that the elastic fibers were dispersed, degraded and disorganized in inner layer of cattle jugular vein. While the Collagen and elastic fibers were dispersed in the outer and inner layers but degraded and disorganized in the inner layer of buffalo jugular vein.

The current study made comparison between decellularized tissue sections of physical and control group we found that no cells or cell fragments were retained. This result in agreement with Li et al. (2018) Moreover, we found in the fibrous layer of cattle pericardium sever wavy disruption but mild dispersion. And in the fibrous layer of buffalo pericardium showed mild wavy disruption but mild dispersion. In addition to, we found mild dispersion in the middle layer of cattle jugular vein.

The present study compared the enzymatic group with control one of BP and BJV This finding that no cells or cell fragments were retained in BP. This result in agreement with Gardin et al. (2015) Moreover, we found mild wavy disruption but sever dispersion in the fibrous layer of cattle pericardium. And we found mild wavy disruption but moderate dispersion in the fibrous layer of buffalo pericardium.

In the current study Elastic von Gieson showed that control cattle pericardium and jugular vein revealed normal collagen fibrils and clear elastic structures. This result in agreement with Li et al. (2018) When compare chemical with control group, the decellularized tissues showed moderate dispersed collagen fibrils and moderate disrupted elastic structures. This result contradiction with Li et al. (2007) who mentioned that BJV retained collagen due to no significant differences in the contents of collagen, and elastin between the decellularized and fresh veins. The decellularized tissue of physical group compared with control one showed that mild dispersed collagen fibrils and mild disrupted elastic structures. This result partial agreement with Li et al. (2018) who mentioned that the structural preservation of collagen fibers was observed in decellularized bovine pericardium and the elastic fibers was continuous. The decellularized tissue of enzymatic group showed that sever dispersed collagen fibrils and sever dispersed elastic structures. This result contradicts with Oswal et al. (2007) who mentioned that The histoarchitecture of the collagen-elastin matrix appeared to be well preserved. But, make decellularization using sodium dodecyl sulfate with nuclease enzymes.

The present study showed the histological examination of implants was taken two weeks later after S/C implantation in rats. It was found in control group of pericardium (tissue implanted without decellularization) severe necrosis of the outer serosal layer associated with loss of the covering mesothelial cells, separation and necrosis of the fibers and marked mononuclear cells infiltration mostly lymphocytes

and macrophages. This result agreement with Li et al. (2018). Moreover, control jugular vein implants showed necrotic changes , marked inflammation in the intimal layer which associated with marked mononuclear inflammatory cells infiltration mostly lymphocytes and macrophages. We found that physically decellularized implants revealed necrosis of the outer serosal layer, separation of the fibers associated with mild to moderate degree of inflammation with mononuclear inflammatory cells infiltration mostly lymphocytes and macrophages. This finding is in partial agreement with Li et al. (2018) who stated that histological examination of FTS (freeing-thawingandTx100+SD) showed minimal evidence of inflammatory responses. Moreover, we found Mild to moderate degree of inflammation in endothelial lining accompanied with loss of intimal layer, calcification and mononuclear inflammatory cells infiltration mostly lymphocytes and macrophages.

In addition to, we found that pericardial implants of enzymatic group showed separation of the outer layer, focal areas of necrosis and scanty to mild inflammatory reaction. This result in agreement with Lü et al. (2009) Also, jugular vein implants revealed necrosis and sloughing of the intimal layer, mild inflammatory cells infiltration with scanty calcification. This may be due to nucleases enzymes (e.g. DNases and RNases)which cleave nucleic acid sequences and help in removal of nucleotides after cell lysis in tissues, Petersen et al.(2010).

The current work used Alizarin red staining for evaluating calcification in subcutaneous implants. We found sever calcification with high calcium deposition in implants of control group. This result in agreement with Li et al. (2018). When compared implants physically treated with normal one showed moderate deposition of calcium. This result contradiction with Li et al. (2018), who reported that FTS implant showed signs of calcification. Moreover, enzymatically treated implants showed scanty to mild deposition of calcium.

5. CONCLUSION

It could be concluded that the physical method is the best in preservation the integrity and durability of decellularized tissue than other methods (low dispersed collagen fibers and low disrupted elastic structures) while the enzymatic method is good in reduction the immunoreaction (mild to scanty inflammatory cells infiltration). Thus, these results suggested that the physical protocol showed optimal decellularization results with better extracellular matrix preservation. But there is no technique made complete decellularization of pericardium and jugular vein and enable to use it for xenografts production attributed to presence of inflammatory cells and calcification in all implants of decellularized tissue.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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