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THE USE OF TRITON X-100 ABSOLUTE WITH VARIATIONS OF IMMERSION TIME IN THE DECELLULARIZATION PROCESS OF INTESTINAL AND MYOCARDIAL XENOGRAFT

Fajar Shodiq Permata[1]*, Dini Agusti Paramanandi[1], Nadia Nobilla[2], Rahel Oktarina Manalu[2], and Mifta Rizqina Amalia[3]

[1]Laboratory of Anatomy, Faculty of Veterinary Medicine, Universitas Brawijaya, Malang, Indonesia
[2]Study Program of Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Brawijaya, Malang, Indonesia
[3]Master of Philosophy in Veterinary Science, School of Veterinary Science, The University of Queensland, Australia
*Corresponding author: drh.fajar@ub.ac.id

ABSTRACT

This study aims to determine the comparison of residual cells in the extracellular matrix of goat myocardium and intestinal segments after immersion in Triton X-100. A total of 20 goat myocardial segments (1x1x0.5 cm) and 20 goat intestinal segments (1x0.5x0.5cm) were divided into 4 treatment groups, namely the decellularization immersion groups for 24 hours, 48 hours, 72 hours and the control group without decellularization. In this research, the emersion technique was used by immersed the samples in 5 mL of absolute Triton X-100 solution. The samples were subjected to Hematoxylin-Eosin (HE) and Mallory staining. The HE staining showed a considerable number of nuclei remained in all of the treatment groups, in the control group, 24-hour group of treatment, 48-hour group of treatment and 72-hour group of treatment. An observation using Mallory staining showed an intact extracellular matrix in tissue samples following the immersion with absolute Triton X-100. Therefore, it can be concluded that the immersion using absolute Triton X-100 could not completely remove the extracellular matrix in the decellularization process until 72 hours of immersion.

Key words: cell nucleus, decellularization, extracellular matrix, Triton X-100, xenograft

ABSTRAK

Penelitian ini bertujuan mengetahui perbandingan sisa sel yang terlihat pada matriks ekstraseluler pasca perendaman menggunakan Triton X-100 pada otot jantung dan segmen usus kambing. Sebanyak 20 segmen jantung kambing, dimensi 1x1x0,5 cm dan 20 segmen usus kambing, dimensi 1x0,5x0,5cm dibagi menjadi empat kelompok perlakuan, yaitu kelompok deselularisasi dengan imersi selama 24, 48, 72 jam dan kelompok kontrol tanpa deselularisasi. Dalam penelitian ini digunakan teknik emersi atau perendaman sampel dalam 5 ml larutan Triton X-100 absolut. Sampel kemudian diproses pada pembuatan preparat histologi melalui pewarnaan Hematoxyline-Eosin (HE) dan Mallory. Hasil penelitian menunjukkan masih banyak ditemukan inti sel yang belum menghilang pada pewarnaan HE seluruh kelompok perlakuan perendaman Triton X-100 absolut selama 24, 48, dan 72 jam. Pengamatan histologi pewarnaan Mallory menunjukkan bahwa sampel masih memiliki matriks ekstraseluler yang utuh pasca perendaman Triton X-100 absolut. Oleh karena itu, dapat disimpulkan bahwa perendaman Triton X-100 absolut belum mampu menghilangkan sel saat deselularisasi secara menyeluruh, namun masih menjaga maktriks ekstraselular dengan baik.

Kata kunci: inti sel, deselularisasi, matriksekstraseluler, Triton X-100, xenograft

INTRODUCTION

Transplantation is one of the treatments for treating severe tissue or organ damage. Several transplant techniques have been implemented, including heart and gastrointestinal tract transplants. Every year, it is reported that >7% of the approximately 4000 heart failure patients who are on the waiting list at the United Network for Organ Sharing die due to limited availability of donor heart organs (Pierson *et al.* 2020). The limited availability of donor organs is one of the obstacles in the transplant process, while many patients need the organ immediately (Singh *et al.* 2022). Allograft is one of the ideal heart donor treatment options, however, the availability of donor organs is very limited and requires living donors with the rule that heart donors must receive a replacement heart (Lousiana 2017).

Xenograft or xenotransplantation is an alternative technique developed to overcome the problem of a shortage of donors among humans by donating animal tissue. This transplant is performed on patients with end-stage organ failure (Singh *et al.* 2022). Xenografts have been implemented with several trials with porcine-to-human xenotransplantation (Griffirth *et al.* 2022; Montgomery *et al.* 2022). An ideal biomaterial for these tissue engineering applications should be capable of cytocompatibility, maintain desired cellular function and application-specific phenotype, induce tissue growth, and provide mechanical support until absorbed and replaced by the natural extracellular matrix (Capella-Monsonís and Zeugolis 2021).

Decellularized extracellular matrix (dECM) is a biomaterial scaffold derived from animal tissue or organs, which has had its immunogenic cell components removed through a decellularization process. dECM is composed of an extracellular matrix which has a three-dimensional structure, composed of collagen, elastin, fibronectin, laminin, and matricellular proteins (Zhang *et al.* 2022). dECM is a useful biomaterial for forming complex structures of proteins, glycosaminoglycans, proteoglycans and other matrix components found in native tissue. Therefore, dECM provides a pathway for the processes of regeneration, repair and remodeling of damaged cells (Bejleri and Davis 2019). dECM formation through a decellularization process. Decellularization is a process that involves the lysis of cellular components through physical, chemical and

chemical methods and retains the original extracellular components (Capella-Monsonís and Zeugolis 2021).

Triton X-100 is a non-ionic detergent commonly used for the decellularization process. This detergent will increase the solubility of cell membranes, stretch cytoskeletal proteins from cells, and release DNA from proteins. Triton X-100 has a mechanism of action by destroying lipid-lipid and lipid-protein bonds, but does not disrupt protein interactions (Mendibil *et al.* 2020). Successful decellularization requires the removal of cell membrane epitopes, damage-associated molecular pattern molecules (DAMPs), and residual DNA from the scaffold because these components are known to induce inflammatory reactions (Balestrini *et al.* 2015). Decellularization has a number of potential benefits such as minimizing immunogenic reactions leading to graft rejection, providing optimized biomaterials without competing donor cells, and reducing the risk of disease transmission in organ transplantation (Patter 2019).

Decellularization carried out on xenograft tissue can reduce the immunogenic effect on the donor (Umashankar *et al.* 2011; Choi *et al.* 2012; Fishman *et al.* 2013; Keane *et al.* 2015; Heuschkel *et al.* 2019; Isidan *et al.* 2019; Capella-Monsonís and Zeugolis 2021), thus will provide health benefits in the future. Therefore, this research was conducted to evaluate the use of Triton X-100 in the tissue decellularization process on the structure of the extracellular matrix in the tissue engineering process.

MATERIALS AND METHODS

In this study, 20 myocardial tissue samples (1x1x0.5 cm) and 20 goat intestinal samples (1x0.5x0.5 cm) were used. The samples were divided into four groups, namely the control group (P0) (without decellularization), the immersion treatment group for 24 (P1), 48 (P2), and 72 hours (P3). This research did not involve direct treatment for animals but only collected organs samples or stored biological material from animal slaughterhouses, thus there was no risk of violating the animal ethics.

The immersion method used referred to Permata *et al*. (2012) with modifications. Control group samples were soaked in 10% formalin and treatment group samples were soaked in 5 ml of Triton X-100 (CAS No. 7646-85-7, Merck, Germany). The Triton X-100 solution was replaced every 24 hours in treatments P2 and P3. After soaking, the samples were rinsed using physiological NaCl and placed in 10 mL of 10% formalin solution.

Histology Preparations

Histological preparations for this research sample referred to Nayak (2018) using the paraffin method and routine hematoxylin staining. The samples were put into paraffin solutions I, II, III for 1 hour each. The sample was inserted into the cast for the embedding process until it hardens. The sample was embedded in paraffin to replace the position of dehydration in the tissue and as a clarifying agent through paraffin infiltration.

The sample was then cut using a 5 μm microtome and placed on a glass object fore Hematoxylin-Eosin (HE) staining process. The samples were then soaked in xylol solutions I, II, and III for 10 minutes each. After that, the sample was immersed in absolute ethanol III, II, I and graded ethanol of 95%, 90%, 80% and 70% for 5 minutes each. Next, the sample was stained using hematoxylin for 10-20 seconds. Then the sample was dipped in acidic alcohol for 4 seconds and run under running water for 20 minutes. Samples were stained using eosin for 10-20 minutes. The sample was then dipped into graded ethanol 70%, 80%, 90%, 95%, absolute I, II, and III for 4 seconds each and continued by immersion in absolute ethanol I, II, and III for 4 seconds each. 2 minutes. Then the samples were soaked in xylol solutions I, II, and III for 3 minutes each.

Mallory Staining

Mallory staining was carried out according to the instructions of Fredenburgh (2008). Myocardial tissue samples and intestinal segments that had been placed on a glass object were soaked in xylol I, II, and III solutions for 10 minutes each. After that, the samples were rehydrated by immersing them in absolute ethanol 2 times for 5 and 3 minutes each and 95% ethanol and distilled water each for 2 minutes. The samples were then stained using Mallory I dye solution (acid fuchsine) for 3 minutes. After that, the sample was rinsed using distilled water 3 times for 30 seconds each and observed under a microscope. Next, the sample was immersed in Mallory II dye solution (phospotungstic acid) for at least 5 minutes and the results were observed using a microscope. Without rinsing, the sample was immediately immersed in Malory III dye solution (aniline/blue-orange G-solution) for 2 minutes, and rinsed using distilled water 4 times, each time for 30 seconds. The staining results were observed using a microscope. After that, the samples were dehydrated using graded ethanol of 70%, 80%, and 97% for 3 minutes each and immersed in xylol I, II, and III each for 3 minutes.

Each slide preparation from each sample and each staining was examined and photographed in 5 fields of view: the upper right, upper left, middle, lower right and lower left. Microscopic images of the samples were taken at 400x magnification using an Optilab Advance camera (Indonesia) connected to an Olympus CX-21 Microscope.

Data Analysis

Cell count data and observations of residual extracellular matrix were analyzed using one-way analysis of variance (ANOVA). Mallory staining results were analyzed through qualitative and descriptive observations at 400x magnification.

RESULTS AND DISCUSSION

Histopathology and Analysis of Cell Remains

The histopathological images of cardiomyocytes in cardiac and intestinal tissue in all groups are presented in Figure 1 and Figure 2.

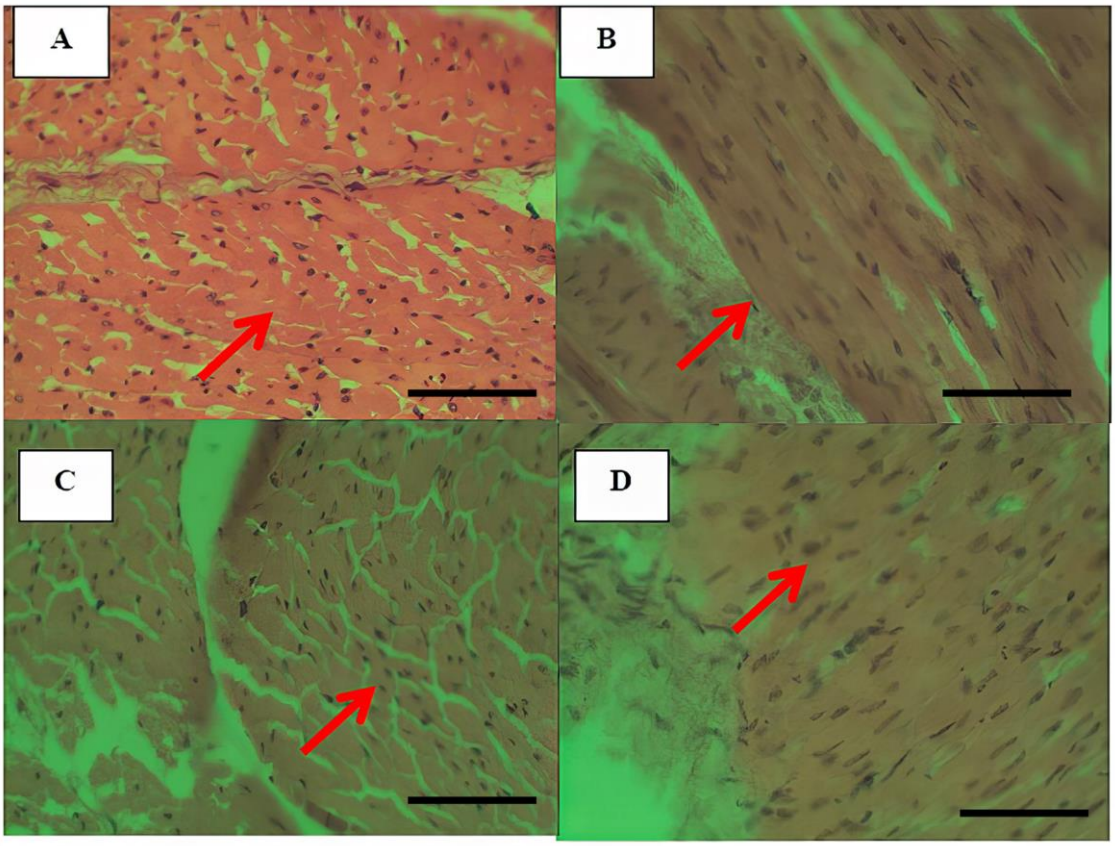

Figure 1. Histopathological image of myocardial tissue. A= negative control; B= 24-hour immersion group; C= 48-hour immersion group; D= 72-hour immersion group. All groups showed the presence of a nucleus (red arrow). HE 400x. Scale bar: 100 µm.

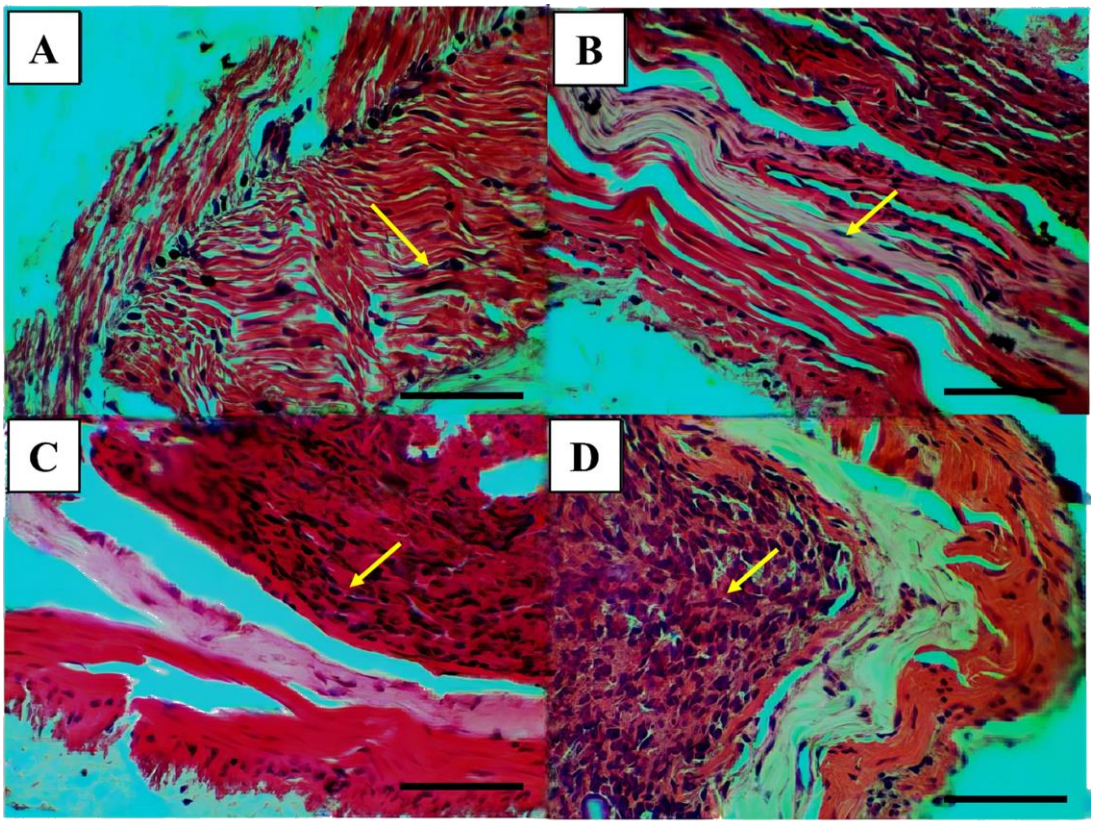

Figure 2. Histopathological images of intestinal tissue. A= negative control; B= 24-hour immersion group; C= 48-hour immersion group; D= 72-hour immersion group. Residual cells (yellow arrows) from the treatment groups and control group were similar. HE 400x. Scale bar: 100 µm

The residual myocardial cells in P0; P1, P2; and P3 were 694.1±171.60; 783.0±53.90; 760.6±106.80; and 754.0±138.40 cells, respectively. Meanwhile, the residual intestinal cells in P0; P1, P2; and P3 were 252.6±82.86; 282.6±64.20 cells; 298.0±52.24; 315.2±78.96 cells, respectively (Table 1).

An effective decellularization process depends on many factors. Based on the results obtained, the treatment group had an insignificant value with an average number of residual cells was greater than the control group. Several factors that could influence the decellularization process include tissue cellularity, tissue density, lipid content, and tissue thickness. Commonly used decellularization techniques were freezing and thawing, mechanical removal of the layer, and using the addition of lipid dissolving agents. Triton's X-100 is a commonly used non-ionic detergent for the decellularization process which act by disrupts lipid-lipid and protein-lipid bonds without affecting protein-protein bonds, thus could cause incomplete decellularization and maintain the protein structure of the cell. This was also reported by Mendibil *et al*. (2020), that Triton X-100 shows lower efficiency in the cell lysis process with insignificant cell removal.

Each organ had different structural characteristics. Myocardium was composed of myofibrils which form contractile elements in cardiomyocytes. This structure was woven, making it difficult for the decellularization solution to penetrate the myocardium completely. The myocardial mucosal tissue had a thick mass and requires a shaking or mixing technique evenly during each liquid immersion process so that it could be absorbed completely.

The extracellular matrix of intestinal tissue was also composed of various types of collagen fiber cells, especially type-I collagen and reticular fibers with glycoproteins and proteoglycans. Therefore, these tissues had different mechanical properties and will influence the choice of protocol implemented. A good decellularization process must be able to remove all cellular components from tissue without changing the histological structure and original components of the extracellular matrix. In accordance with Oliveira *et al.* (2013), who stated that apart from the protocol, preservation of the extracellular matrix structure is one of the important steps that influences the results. This is because the extracellular matrix has a main component of fibril fibers which maintain tissue integrity (especially collagen, reticular fibers and elastic fibers) and two non-fibril components (glycoproteins and proteoglycans). These non-fibril components play a role in intercellular interactions, cell adhesion, response, proliferation and cell migration, as well as maintaining tissue hydration levels. A study conducted by Alsaikh (2019), using a combination of Triton This negative result was unexpected because previous results showed effective decellularization of mouse ovaries using a mixture of both solutions in vivo. Thus, differences in organ structure and decellularization protocols must be developed systematically to optimize tissue conditions for exploration into other tissues.

Histopathology of Mallory's Staining

Observation of the structure in Mallory staining shows that the goat heart muscle tissue was not completely decellularized which could be observed by collagen fibers at the edges, myocytes and mucosal structures which were still visible (Figure 2). In addition, it could be observed that there was an extracellular matrix that did not experience decellularization (Figure 3).

The histopathological results of Mallory's staining showed that there was an intact extracellular matrix structure after immersion in Triton X-100. The extracellular matrix was an essential non-cellular component of the tissue microenvironment composed of a combination of macromolecules such as polysaccharides glycosaminoglycans (GAG) and proteins such as collagen, laminin and fibronectin (Sackett *et al.* 2018). This condition of the extracellular matrix still being intact could occur due to the working mechanism of Triton X-100. This solution works without disrupting protein-protein bonds at the cellular level. This was in accordance with Zahmati *et al*. (2017) who reported that although Triton X-100 worked to eliminate GAG from the matrix surface, decellularization of the Anterior Cruciate Ligament (ACL) showed that this solution did not have a harmful effect on GAG on the extracellular matrix surface.

The intact extracellular matrix structure could also be influenced by Triton X-100, which was a non-ionic detergent decellularization chemical agent that had a lower destructive effect compared to other groups. Non-ionic detergents could maintain the original structure of proteins well, so they were recommended for use as decellularization agents in synthetic scaffolds composed of protein components. Faulk *et al.* (2014) compared decellularization using Sodium Dodecyl Sulfate (SDS) and Triton X-100 on urinary bladder tissue. SDS was able to produce less DNA residue, but changed the structure of the collagen fibers, however Triton X-100 was well tolerated and showed a specimen architecture that was more similar to the control group.

There are several other decellularization solution options that could be used besides the non-ionic detergent group. However, some of these solutions have

Table 1. The residual cells in myocardial and intestinal tissue segment

Treatment groups	Residual myocyte cells (± SD)	Residual intestinal tissue cells (± SD)
P0 (Negative control)	694.1±171.60	252.6±82.86
P1 (24-hour immersion group)	783.0±53.90	282.6±64.20
P2 (48-hour immersion group)	760.6±106.80	298.0±52.24
P3 (72-hour immersion group)	754.0±138.40	315.2±78.96

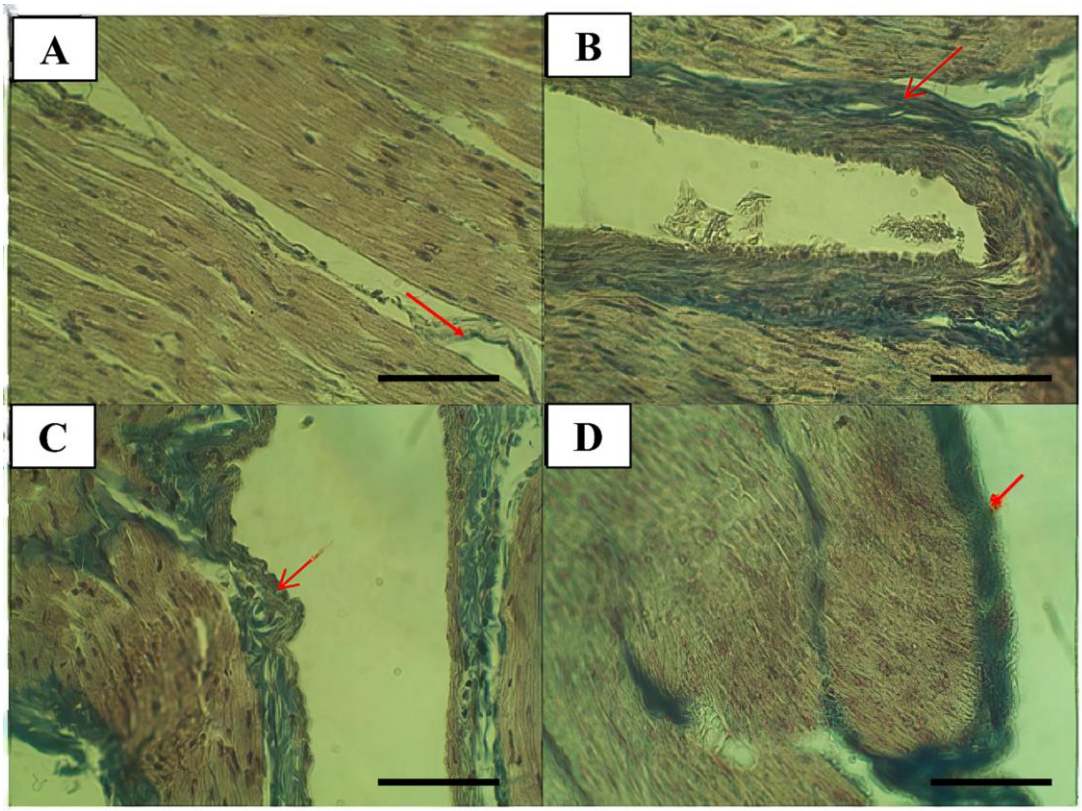

Figure 3. Histopathological image of cardiac tissue. A= negative control; B= 24-hour immersion group; C= 48-hour immersion group; D= 72-hour immersion group. Triton Mallory staining at 400x. Scale bar: 100 µm.

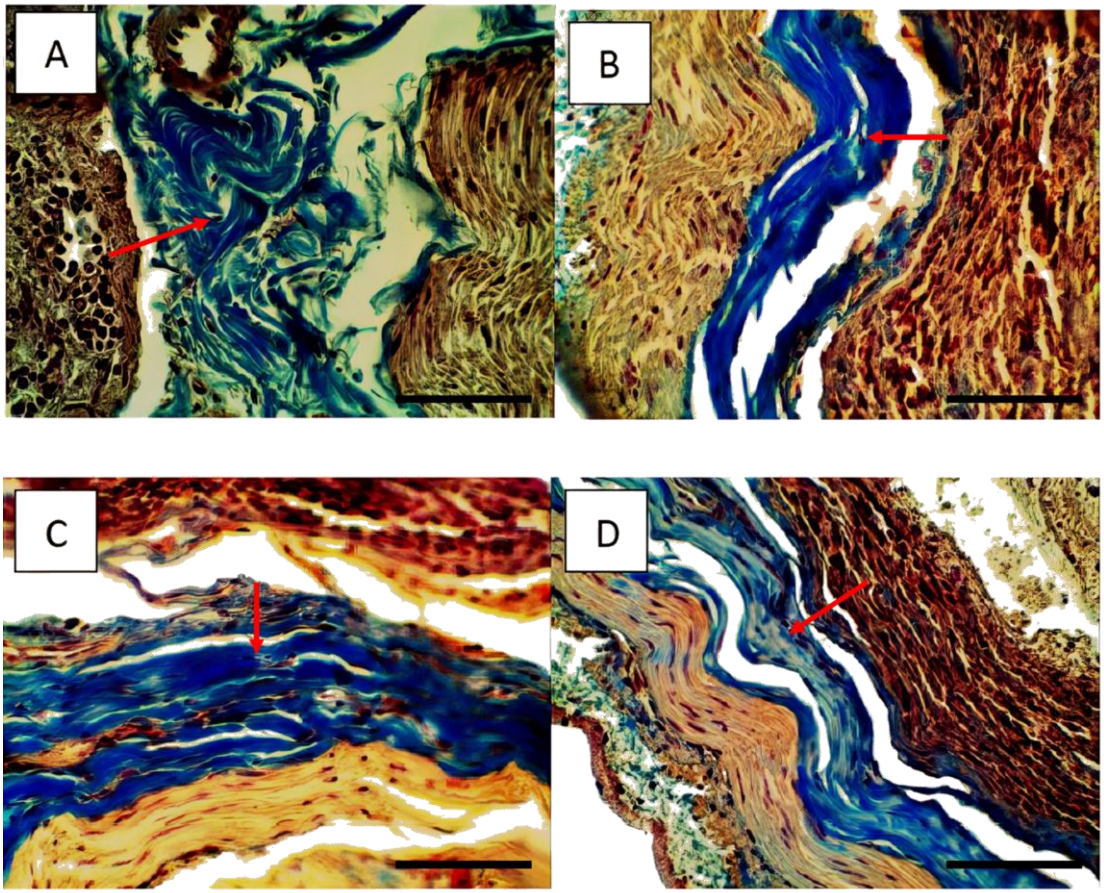

Figure 4. Histopathological image of intestinal tissue. A= negative control; B= 24-hour immersion group; C=48-hour immersion group; D=72-hour immersion group. Intestinal xenograft tissue post-immersion with Triton Mallory at 400x. Scale bar: 100 µm.

different cellular mechanisms and effects on the extracellular matrix structure. Lee *et al*. (2016) reported that there was a 15.51% decrease in mechanical strength after the decellularization process using SDS. This was indicated as a result of loss of soluble proteins or structural proteins in the extracellular matrix. The use of acid-base solutions could decompose nucleic acids and hydrolyze cytoplasmic components such as acetic acid, peracetic acid, hydrochloric acid, sulfuric acid and calcium hydroxide. However, Reing *et al.* (2010) reported that acid-base solutions could cause damage to collagen and GAG in the extracellular matrix. A report on decellularization research on myocardial fibroblasts was reported by Ye *et al.* (2016), which stated that decellularization using a solution based on SDS produces better myocardial tissue efficiency compared to Trypsin and Triton X-100 which results in complete removal of cells and better preservation of the extracellular matrix. This is different from decellularization of intestinal tissue which requires a combination of chemical and enzymatic agents (sodium deoxycholate perfusion, DNase, immersion in hypotonic solutions) which are used to remove cell components in this tissue while maintaining its structure (Maghsoudlou *et al.* 2013; Kajbafzadeh *et al.* 2018).

In research using Triton X-100 absolute it was not able to properly remove cells in myocardial and intestinal xenograft tissue but was able to reveal and maintain extracellular matrix components well. Therefore, Triton X-100 still had potential as a decellularization agent and it was recommended to verify the concentration of Triton X-100 by varying the immersion time in order to obtain the best decellularization process for myocardial and intestinal xenograft tissue.

CONCLUSION

It was concluded that the use of Triton 100 in the decellularization process of myocardial and intestinal tissue was still not able to remove the whole cell components and could maintain the matrix structure intact.

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