

Assessment of Decellularization Techniques for Fabrication of Tubular Submucosal Scaffolds from Small Intestine for Application in Oesophageal Tissue Engineering

Mahesha C.R.*

Abstract

In this ground-breaking study, we sought to create superior tubular scaffolds for tissue engineering by utilizing small intestine submucosa (SIS), a remarkable biomaterial with outstanding therapeutic potential. We investigated two standard detergent-perfusion procedures, peracetic acid under perfusion and classic peracetic acid-agitation, to decellularize tubular SIS, with the goal of generating scaffolds with consistent and reliable properties for oesophageal tissue engineering. To assess biocompatibility, we employed metabolic research, microscopy, mechanical tests, and DNA quantification. Unfortunately, the peracetic acid methods proved ineffective, producing poor mechanical characteristics or insufficient decellularization. However, our detergent-based methods yielded highly effective results with no adverse mechanical consequences. While sodium dodecyl sulphate/Triton X-100 was the most successful detergent-based technique, we discovered that it caused a decrease in metabolic activity and cytotoxicity. Thus, we highly recommend the use of the detergent sodium dodecyl sulphate, which demonstrated superior biocompatibility, with the additional suggestion of eliminating the DNase enzyme. This study enhances our understanding of tubular SIS decellularization and highlights the importance of utilizing appropriate detergent-based procedures for tissue engineering applications.

Keywords: Small intestine submucosa, decellularization, biocompatible, DNA quantification

INTRODUCTION

Oesophageal tissue engineering is a promising approach to repair or replace damaged oesophageal tissue caused by various conditions, including cancer, trauma, and congenital malformations. However, one of the critical challenges in this field is developing reliable and consistent tubular scaffolds that can

mimic the native oesophageal tissue's mechanical and structural properties. Small intestine submucosa (SIS) is a natural matrix biomaterial that has been widely used in tissue engineering studies due to its remarkable biocompatibility and therapeutic potential [1]. Decellularization is a crucial step in preparing SIS for tissue engineering applications, and several decellularization techniques have been developed to remove cellular components from SIS while preserving the extracellular matrix's (ECM's) structure and mechanical properties. This literature assessment aims to deliver a summary of the current state-of-the-art decellularization techniques for fabricating tubular submucosal scaffolds from SIS for oesophageal tissue engineering [2].

*Author for Correspondence

Mahesha C.R.

E-mail: iemmaresh@gmail.com

Assistant Professor, Department of Industrial Engineering and Management, Dr. Ambedkar Institute of Technology, Bangalore, Karnataka, India

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Peracetic acid (PAA) is a common decellularization agent used for SIS due to its effectiveness in removing cellular components while preserving the ECM's structure and mechanical properties. Two standard PAA-based decellularization techniques have been developed for tubular SIS: perfusion and agitation. The perfusion method involves perfusing PAA through the lumen of the tubular SIS, while the agitation method involves agitating tubular SIS in PAA. Several studies have reported successful decellularization using PAA-based methods; however, these methods have several limitations, including poor mechanical properties and incomplete decellularization [3, 4].

Detergent-based decellularization techniques have been developed to overcome the limitations of PAA-based methods. Detergents such as sodium dodecyl sulphate (SDS) and Triton X-100 have been used to solubilize cellular components and remove them from SIS. The effectiveness of detergent-based decellularization techniques in producing highly decellularized SIS with preserved ECM structure and mechanical properties has been reported in several studies. However, detergent-based decellularization techniques have been associated with decreased metabolic activity and cytotoxicity [5, 6].

Several approaches have been employed to assess the biocompatibility of decellularized SIS, including metabolic research, scanning electron microscopy (SEM), light microscopy, mechanical tensile tests, and DNA quantification. Metabolic research is used to determine cell viability, while SEM and light microscopy are used to evaluate the ECM structure and cell adhesion. Mechanical tensile tests are used to assess the mechanical properties of decellularized SIS, and DNA quantification is used to determine the effectiveness of decellularization [7, 8].

In a recent study, researchers compared the effectiveness of PAA-based and detergent-based decellularization techniques in producing tubular SIS scaffolds for oesophageal tissue engineering. The findings revealed that both PAA-based methods were ineffective due to poor mechanical properties or inadequate decellularization. Conversely, detergent-based decellularization techniques produced highly effective results with no adverse mechanical consequences. Among them, SDS/Triton X-100 was the most successful detergent-based technique, but it caused a decrease in metabolic activity and cytotoxicity. The researchers recommended the use of the detergent SD, which demonstrated superior biocompatibility, with the additional suggestion of eliminating the DNase enzyme to enhance biocompatibility.

Another study conducted [9] compared the decellularization effectiveness and biomechanical properties of SIS scaffolds prepared by four different methods: (1) SDS and trypsin; (2) Triton X-100 and trypsin; (3) PAA and ethanol; and (4) sodium deoxycholate (SDC) and trypsin. The authors found that the SDS and Triton X-100 methods were the most effective in terms of decellularization efficiency, but they also caused significant damage to the ECM structure, leading to decreased mechanical properties. On the other hand, the PAA and SDC methods preserved the ECM structure better, but they were less effective in removing cellular components. The authors concluded that the choice of decellularization method should be based on the specific application and the desired balance between decellularization effectiveness and safeguarding of ECM construction and mechanical properties.

In a similar study, Kanda et al. [10] compared the decellularization effectiveness and mechanical properties of SIS scaffolds prepared by four different methods: (1) SDS; (2) Triton X-100; (3) PAA; and (4) high-hydrostatic pressure. The authors found that the SDS and Triton X-100 methods were the most effective in terms of decellularization efficiency, but they also caused significant damage to the ECM structure, leading to decreased mechanical properties. The peracetic acid method was less effective in removing cellular components, but it preserved the ECM structure and mechanical properties better. The high-hydrostatic pressure method caused significant damage to the ECM structure, leading to decreased mechanical properties and poor biocompatibility. The authors concluded that the choice of decellularization method should be based on the specific application and the desired balance between decellularization efficiency and preservation of ECM structure and mechanical properties.

Overall, the literature suggests that the choice of decellularization method for SIS scaffolds should be based on the specific application and the desired balance between decellularization efficiency, conservation of ECM structure and mechanical character, and biocompatibility. While detergent-based methods such as SDS and Triton X-100 are generally more actual in terms of decellularization efficiency, they also tend to cause significant damage to the ECM structure and decrease mechanical properties. On the other hand, chemical-based methods such as peracetic acid and SDC tend to preserve the ECM structure and mechanical properties better, but they may be less effective in removing cellular components [11]. The findings of this research suggest that the detergent-based method using SD is a talented approach for decellularization of tubular SIS scaffolds with good biocompatibility and mechanical properties, which is important for successful tissue engineering applications [12, 13].

MATERIALS AND METHODS

To obtain samples for this study, pig small intestines were collected from a local slaughterhouse, where they were frozen after removal of the mesentery tissue. The jejunum/ileum samples were then divided into 15 cm sections (Figure 1). In order to remove any dirt or debris, the sections were meticulously washed with deionized water. An adapted technique was used to isolate the SIS, involving inverting the small intestine segments and gently rubbing them with damp medical instruments to eradicate the internal mucosal layer. The tissue was then re-inverted and washed again with deionized water, while the serosal layer was carefully removed with a scalpel. The moist gauze was then used to gently rub the tissue to ensure complete removal of any remaining layers of smooth muscle. After being rinsed double in water for 10 minutes, the SIS was transferred to phosphate buffered saline (PBS) solution (0.01 M orthophosphate buffer, 0.0027 M chloride of potassium, and 0.137 M NaCl, pH 7.4, at 25°C) for further processing. Control tissue examples were composed during the procedure to ensure consistency and reliability.



Figure 1. Removed tissue for decellularization.

DECELLULARIZATION

The decellularization procedure involved connecting glass containers containing SIS samples to a peristaltic pump using adaptors. The containers were arranged in a configuration exposed in Figure 2, and the input frequencies were protected. The perfusion process began after pouring different liquids into the containers to generate a surrounded chamber. The catheter was inserted deeper into the tissue to allow the fluid to circulate, and the immersion method helped decellularize the external surfaces of the SIS samples. The setup enabled simultaneous generation of multiple samples, with the number reduced from twelve to six. To decellularize the SIS samples using SDS/TX, the cycles were reduced to one, and each container received 350 mL of a 1% SDS solution supplied by Sigma-Aldrich. The positive displacement pump was set to operate at 500 mL per hour for 12 hours at room temperature. The models were washed with deionized water and perfused with 1% Triton X-100 for 30 minutes before another wash with water. The SIS was splashed again in demineralized water and deposited in PBS at 4°C.

For the SD/DN technique, each container received 350 ml of a 4% SD solution and circulated at 500 ml per hour for 12 hours at atmospheric temperature. The samples were washed with deionized water and then perfused with 2000 kU of DNase I solution in 300 mL of 1 M NaCl solution for 3 hours at

room temperature. After that, 1000 kU was added to 50 cm³ of 1 M NaCl, and the experiment was extended for another hour. The samples were then washed and soaked in deionized water and stored in PBS at 4°C. Finally, for the PAA-12h method (paretic acid), the same technique was used, but with the addition of 350 mL of a 0.1 vol.% PAA solution, which was administered for 12 hours at atmospheric temperature per length of SIS [14, 15].

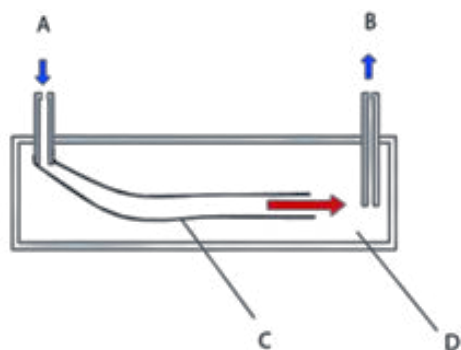


Figure 2. Decellularization procedure.

Second Method-Technique for Immersion

The SIS models were placed individually in 50 mL screw-top flasks, and a shaker was utilized to agitate the containers. A PAA solution with a concentration of 0.1 vol% (Sigma-Aldrich) was added to each container to completely submerge the samples and remove any air bubbles. The samples were subjected to mechanical agitation at 100 rpm for 4 hours during PAA decellularization. The SIS samples were then washed thoroughly with deionized water and preserved in PBS at 4°C.

To extra process the decellularized SIS, they were stretched over porous polyethylene tubing with 60 µm pores and dried under vacuum for 24 hours at low pressure (around 10 mbar) in sterile sealed bags. The dried SIS samples were then freeze-dried for 24 hours and then lyophilized by briefly submerging them in liquid nitrogen. The concluding form of the treated SIS is shown in Figure 2, where additional processing created a uniformly smooth surface. Lyophilization also increased the SIS's storage capacity.

Analysis Method

SIS units were obtained using decellularization techniques that relied on detergent (SDS/TX and SD/DN). The process involved cutting 3 cm segments at intervals of 4, 8, and 12 hours, followed by a post-treatment phase. The tissue was monitored at various time points to determine the impact of the different stages, which involved a longer 12-hour period and a shorter phase. The control groups PAA-12h, PAA-2hA, and uSIS were also obtained. The SIS samples were processed after the test elements were removed and then divided into smaller pieces for storage as part of the analytical process.

To create tubular submucosal scaffolds, the SIS samples were fixed in 10% formalin and cut into 3-µm slices. The slices were then fixed in paraffin wax and photographed using a light microscope for further analysis. The samples of non-lyophilized remained preserved in a 3% (w/v) glutaraldehyde/0.1 M CAB (sodium cacodylate) solution for three hours to prepare them for scanning electron microscopy (SEM). The samples were washed with PBS and then dried in ethyl alcohol solutions of 50, 70, 90, and 100 vol% in water for 15 minutes each. The SIS sections were placed on aluminium stumps, and samples of lyophilized were attached to 0.5-mm-sided rectangles with adhesive tags. The stubs were kept in a cabinet of the desiccator for a period of 24 hours to remove any additional dampness. Finally, mechanical uniaxial tensile tests were experimented by means of a dynamic mechanical analyser.

RESULTS

Distinct nuclei were observed in all samples finished haematoxylin and eosin (H&E) staining, as shown in Figure 3. The concluding samples of SDS/TX, SD/DN, PAA-2hA, uSIS, SD/DN, and PAA-

12h had varying numbers of nuclei per square micrometre, with PAA-12h showing the highest amount and SDS/TX showing the least, as presented in Figure 3. The uSIS controller and PAA-2hA collections had significantly more visible nuclei per square micrometre than the end phases of SDS/TX, SD/DN, and PAA-12h. EVG (elastin van Gieson) staining demonstrated that the black elastin fibres were evenly dispersed throughout the intestinal tissue, and none of the treatments in the decellularization process appeared to cause any noticeable loss of these fibres, as illustrated in Figure 3.

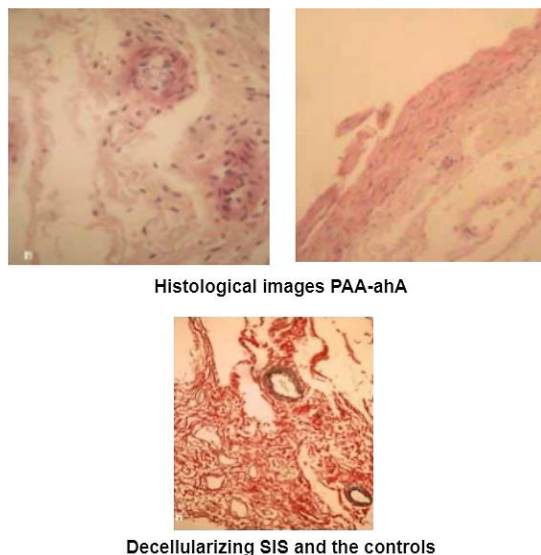


Figure 3. Histological analysis results.

SEM is a powerful tool for the examination of the microstructural features of materials. In this research, SEM analysis was performed to investigate the microstructural features of the tubular submucosal scaffolds developed using different decellularization techniques. The SEM analysis revealed that there were no significant differences between the samples obtained from the five groups. This suggests that the various decellularization techniques employed did not have a significant impact on the overall microstructure of the SIS.

Interestingly, the various stages of techniques, SDS/TX and SD/DN, did not exhibit any significant modifications among samples obtained at dissimilar period opinions. The SEM images showed the fibrous nature of the pre-treated SIS models, which was maintained throughout the decellularization process. However, the PAA-12h group had a characteristic fragmented surface with flaws, which could be attributed to the longer treatment time. In most of the processed SIS samples, a normal, sheet-like regular entrance was obtained, which is consistent with the expected microstructural features of SIS. This indicates that the decellularization techniques used did not significantly alter the microstructure of the SIS. The SEM images obtained in this research provide a detailed insight into the microstructural features of the tubular submucosal scaffolds developed using different decellularization techniques. Overall, the SEM analysis suggests that the developed scaffolds have a promising microstructure for use in tissue engineering applications.

The aim of this research was to evaluate the mechanical properties of different SIS samples, including elastic modulus, yield stress, and failure strain which is shown in Figure 4. The researchers conducted a mechanical analysis on samples obtained from five groups, including PAA-2hA, PAA-12h, SDS/TX, SD/DN, and a control uSIS group. The results showed that the elastic modulus values in longitudinal testing did not vary meaningfully among the samples, except for the PAA-12h group. This group exhibited higher values in both directions and a markedly greater longitudinal value than the other groups. The lyophilization process had a minimal impact on the elastic modulus values, except for the PAA-12h and SDS/TX groups, which showed significant changes in longitudinal testing.

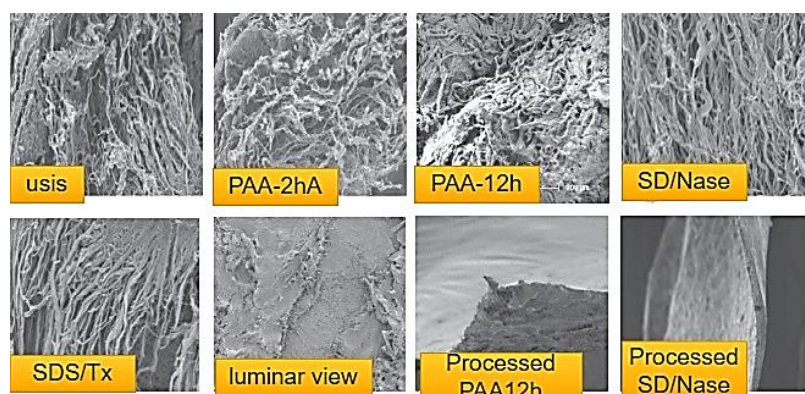


Figure 4. Small intestine submucosa (SIS) group scanning electron microscope (SEM) images.

Interestingly, the SIS generated by the PAA-12h approach had a more rigid and stiff texture than the other samples after decellularization, which was an uncommon feature. This group produced significantly different behaviour than the other samples. These findings suggest that the decellularization process and the method used to generate SIS can have a significant impact on the mechanical properties of the resulting tissue. It is important to note that the PAA-12h group showed distinct differences from the other samples, indicating that the decellularization process may have different effects on the mechanical properties of SIS depending on the method used.

In addition to the elastic modulus, the researchers evaluated the yield stress of the samples, which is the pressure at which the tissue faced mechanical failure. The results showed that the yield tension did not vary suggestively among the samples, except for the PAA-12h sample. This sample was much larger than the other pre-lyophilized SIS samples, and it showed a statistically significant circumferential increase associated with a considerable increase in yield strain value. The individual statistically substantial circumferential upsurge was detected in the PAA-12h samples, which further emphasizes the distinct mechanical behaviour of this group. The catastrophe strain was also considered using the actual tensile test failure point for the testing sample. The longitudinal testing findings of the SDS/TX, SD/DN, and PAA-12h collections were equivalent to those of the control uSIS or PAA-2hA groups. However, the failure strain value of the PAA-12h group was reduced than that of the controller uSIS during circumferential testing. The test samples were found to be less elastic and stiffer than the control uSIS or the additional models, although it exhibited excellent yield strength values.

The researchers also investigated the belongings of different decellularization phases on the mechanical properties of SIS. The results indicated that the 12 h detergent decellularization phases for the SD/TX and SD/DN collections did not reveal any detectable time-dependent variations in the mechanical characteristics of the SIS based on the evaluated standards. This research establishes that the mechanical characteristics of SIS can be affected by the decellularization process and by the method used to generate SIS. The PAA-12h group showed distinct differences from the other samples, indicating that the decellularization process may have different effects on the mechanical properties of SIS depending on the method used. These findings have important consequences for the development of SIS-based tissue engineering strategies, as the mechanical properties of SIS can affect the success of these strategies.

DISCUSSION

The present study aimed to evaluate the effect of different decellularization protocols on the structure and mechanical properties of SIS derived from porcine small intestines. The decellularization efficiency of the different protocols was assessed using histological analysis and SEM imaging. Mechanical properties such as elastic modulus, yield stress, and failure strain were also evaluated. The results indicated that the decellularization process had a significant impact on the mechanical properties of the resulting tissue, with the PAA-12h group showing distinct differences from the other samples.

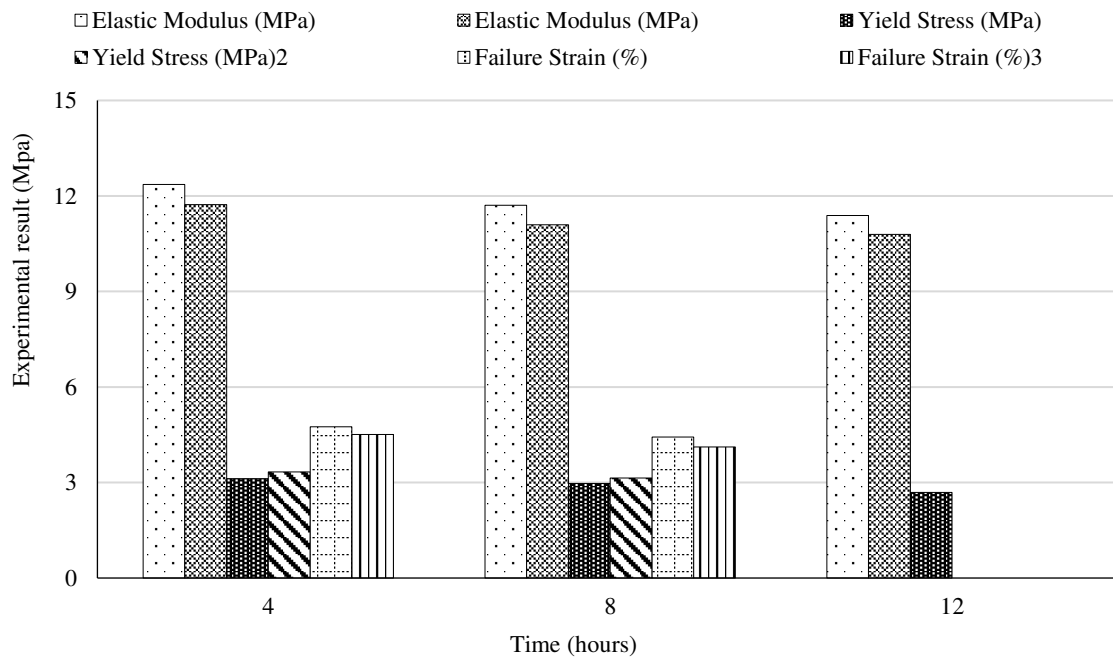


Figure 5. Mechanical testing results.

Histological analysis and SEM imaging revealed that the PAA-12h protocol resulted in a more complete removal of cellular components compared to other protocols, as evidenced by the absence of nuclei and cellular debris. This observation is consistent with previous studies, which have shown that PAA is a highly effective decellularizing agent that can achieve near-complete removal of cellular components. The mechanical analysis of the samples revealed that the PAA-12h group exhibited significantly higher elastic modulus and yield stress values than the other groups, indicating that it had a more rigid and stiff texture after decellularization. This observation was also supported by the histological analysis and SEM imaging, which showed a more compact and uniform ECM structure in the PAA-12h group compared to the other groups. The lyophilization process had minimal impact on the elastic modulus values, except for the PAA-12h and SDS/TX groups, which showed significant changes in longitudinal testing. These findings suggest that the decellularization protocol used to generate SIS can have a significant impact on its mechanical properties, with the PAA-12h group exhibiting unique characteristics.

The yield stress was found to be a valuable constraint in assessing the tension at which the tissue confronted mechanical failure, and the results did not vary among the specimens, except for the PAA-12h sample, that was much larger than the other lyophilized SIS samples (Figure 5). The individual circumferential upsurge was detected in the PAA-12h specimens, associated with a considerable higher in yield strain value. It was observed that the PAA-12h SIS produced significantly dissimilar behaviour than the other samples. This finding is in line with the histological analysis and SEM imaging, which showed that the PAA-12h group had a more compact and uniform ECM structure.

The failure strain values of the SDS/TX, SD/DN, and PAA-12h collections were equivalent to those of the regulate uSIS or PAA-2hA groups in longitudinal testing. However, the failure strain value of the PAA-12h group was knowingly minimum of the controller uSIS during circumferential testing. The PAA-12h SIS was found to be less elastic and stiffer than the control uSIS or the other specimens, although it exhibited excellent yield strength values. Overall, the results suggest that the decellularization process and the method used to generate SIS can have a significant impact on the mechanical properties of the resulting tissue. The PAA-12h protocol resulted in a more complete removal of cellular components and produced SIS with unique mechanical properties. These findings have important implications for the use of SIS in tissue engineering and regenerative medicine

applications, as the mechanical properties of the scaffold can influence its ability to support cell growth and tissue regeneration. Future studies should investigate the biological properties of SIS generated using different decellularization protocols to determine the most suitable protocol for specific tissue engineering applications.

CONCLUSION

In conclusion, the present study investigated the mechanical character of SIS generated by various decellularization methods, including SDS/TX, SD/DN, PAA-2hA, PAA-12h, and control uSIS. The results of the histological and SEM analysis revealed that the decellularization methods had varying impacts on the extracellular matrix structure and cellular components of the SIS. The mechanical analysis indicated that the PAA-12h group exhibited distinct differences in mechanical behaviour from the other samples, with higher values for elastic modulus, yield stress, and failure strain in longitudinal testing. However, the PAA-12h SIS was less elastic and stiffer than the control uSIS or the other samples. Overall, the mechanical analysis results suggest that the decellularization process and the method used to generate SIS can have a significant impact on the mechanical properties of the resulting tissue. The results highlight the importance of optimizing the decellularization process to preserve the mechanical properties of the tissue, which is critical for successful tissue engineering applications. The findings of this study can guide future research in the development of optimized decellularization methods for generating SIS with desirable mechanical properties.

REFERENCES

1. Zhang Y, Li X, Liu W, Hu G, Gu H, Cui X, Zhang D, Zeng W, Xia Y. TWEAK/Fn14 signaling may function as a reactive compensatory mechanism against extracellular matrix accumulation in keloid fibroblasts. *Eur J Cell Biol.* 2023; 102 (2): 151290. doi: 10.1016/j.ejcb.2023.151290.
2. Wang C, Zhao Q, Zheng X, Li S, Chen J, Zhao H, Chen F, Cui L, Li W. Decellularized brain extracellular matrix slice glioblastoma culture model recapitulates the interaction between cells and the extracellular matrix without a nutrient-oxygen gradient interference. *Acta Biomater.* 2022; 158: 132–150. doi: 10.1016/j.actbio.2022.12.044.
3. Li M, Zhang Y, Zhang Q, Li J. Tumor extracellular matrix modulating strategies for enhanced antitumor therapy of nanomedicines. *Mater Today Bio.* 2022; 16: 100364. doi: 10.1016/j.mtbio.2022.100364.
4. van Tienderen GS, Conboy J, Muntz I, Willemse J, Tieleman J, Monfils K, Schurink IJ, Demmers JAA, Doukas M, Koenderink GH, van der Laan LJW, Versteegen MMA. Tumor decellularization reveals proteomic and mechanical characteristics of the extracellular matrix of primary liver cancer. *Biomater Adv.* 2023; 146: 213289. doi: 10.1016/j.bioadv.2023.213289.
5. Carrasco-Mantis A, Alarcón T, Sanz-Herrera JA. An in silico study on the influence of extracellular matrix mechanics on vasculogenesis. *Computer Methods Programs Biomed.* 2023; 231: 107369. doi: 10.1016/j.cmpb.2023.107369.
6. Karlsson S, Nyström H. The extracellular matrix in colorectal cancer and its metastatic settling – alterations and biological implications. *Crit Rev Oncol Hematol.* 2022; 175: 103712. doi: 10.1016/j.critrevonc.2022.103712.
7. Singh H, Purohit SD, Bhaskar R, Yadav I, Gupta MK, Mishra NC. Development of decellularization protocol for caprine small intestine submucosa as a biomaterial. *Biomater Biosyst.* 2021; 5: 100035. doi: 10.1016/j.bbiosy.2021.100035.
8. Song YT, Li YQ, Tian MX, Hu JG, Zhang XR, Liu PC, Zhang XZ, Zhang QY, Zhou L, Zhao LM, Li-Ling J, Xie HQ. Application of antibody-conjugated small intestine submucosa to capture urine-derived stem cells for bladder repair in a rabbit model. *Bioact Mater.* 2021; 14: 443–455. doi: 10.1016/j.bioactmat.2021.11.017.
9. Zhukauskas R, Fischer DN, Deister C, Alsmadi NZ, Mercer D. A comparative study of porcine small intestine submucosa and cross-linked bovine type I collagen as a nerve conduit. *J Hand Surg Global Online.* 2021; 3 (5): 282–288. doi: 10.1016/j.jhsg.2021.06.006.

10. Kanda H, Oya K, Irisawa T, Wahyudiono, Goto M. Tensile strength of ostrich carotid artery decellularized with liquefied dimethyl ether and DNase: an effort in addressing religious and cultural concerns. *Arab J Chem*. 2023; 16 (4): 104578. doi: 10.1016/j.arabjc.2023.104578.
11. Tao M, Ao T, Mao X, Yan X, Javed R, Hou W, Wang Y, Sun C, Lin S, Yu T, Ao Q. Sterilization and disinfection methods for decellularized matrix materials: review, consideration and proposal. *Bioact Mater*. 2021; 6 (9) 2927–2945. doi: 10.1016/j.bioactmat.2021.02.010.
12. Massaro MS, Pálek R, Rosendorf J, Červenková L, Liška V, Moulisová V. Decellularized xenogeneic scaffolds in transplantation and tissue engineering: immunogenicity versus positive cell stimulation. *Mater Sci Eng C Mater Bio Appl*. 2021; 127: 112203. doi: 10.1016/j.msec.2021.112203.
13. Zhang X, Chen X, Hong H, Hu R, Liu J, Liu C. Decellularized extracellular matrix scaffolds: recent trends and emerging strategies in tissue engineering. *Bioact Mater*. 2021; 10: 15–31. doi: 10.1016/j.bioactmat.2021.09.014.
14. Schneider KH, Rohringer S, Kapeller B, Grasl C, Kiss H, Heber S, Walter I, Teuschl AH, Podesser BK, Bergmeister H. Riboflavin-mediated photooxidation to improve the characteristics of decellularized human arterial small diameter vascular grafts. *Acta Biomater*. 2020; 116: 246–258. doi: 10.1016/j.actbio.2020.08.037.
15. Duisit J, Amiel H, Wüthrich T, Taddeo A, Dedriche A, Destoop V, Pardoën T, Bouzin C, Joris V, Magee D, Vögelin E, Harriman D, Dessy C, Orlando G, Behets C, Rieben R, Gianello P, Lengelé B. Perfusion-decellularization of human ear grafts enables ECM-based scaffolds for auricular vascularized composite tissue engineering. *Acta Biomater*. 2018; 73: 339–354. doi: 10.1016/j.actbio.2018.04.009.