Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62935R2
Full Title:	Decellularization for the Preparation of Highly Preserved Human Acellular Skin Matrix for Regenerative Medicine
Corresponding Author:	Clotilde Castaldo Universityof Naples Federico II Naples, ITALY
Corresponding Author's Institution:	Universityof Naples Federico II
Corresponding Author E-Mail:	clotilde.castaldo@unina.it
Order of Authors:	Veronica Romano
	Immacolata Belviso
	Domenico Cozzolino
	Anna Maria Sacco
	Fabrizio Schonauer
	Daria Nurzynska
	Franca Di Meglio
	Clotilde Castaldo
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TITLE:

Decellularization for the Preparation of Highly Preserved Human Acellular Skin Matrix for Regenerative Medicine

AUTHORS AND AFFILIATIONS:

Veronica Romano[1,*], Immacolata Belviso[1,*], Domenico Cozzolino[1], Anna Maria Sacco[1], Fabrizio Schonauer[1], Daria Nurzynska[2], Franca Di Meglio[1], Clotilde Castaldo[1]

[1]Department of Public Health, University of Naples Federico II, Naples, Italy
[2]Department of Medicine, Surgery and Dentistry, Scuola Medica Salernitana, Baronissi, Italy

*These authors contributed equally.

Email addresses of co-authors:

Veronica Romano (veronica.romano@unina.it)
Immacolata Belviso (immacolata.belviso@unina.it)
Domenico Cozzolino (cozzolino.domen1994@libero.it)
Anna Maria Sacco (annamaria.sacco@unina.it)
Fabrizio Schonauer (fabrizio.schonauer@unina.it)
Daria Nurzynska (dnurzynska@unisa.it)

Corresponding authors:

Franca Di Meglio (franca.dimeglio@unina.it)
Clotilde Castaldo (clotilde.castaldo@unina.it)

SUMMARY:

Decellularized human skin is suitable for tissue regeneration. A major issue of decellularization is the preservation of the native architecture, along with the appropriate content of structural proteins, glycosaminoglycans (GAGs), and growth factors. The method proposed allows fast and effective decellularization, producing decellularized skin with well-preserved native features.

ABSTRACT:

Extracellular matrix (ECM) provides biophysical and biochemical stimuli to support self-renewal, proliferation, survival, and differentiation of surrounding cells due to its content of diverse bioactive molecules. Due to these characteristics, the ECM has been recently considered a promising candidate for the creation of biological scaffolds to boost tissue regeneration. Emerging studies have demonstrated that decellularized human tissues could resemble the native ECM in their structural and biochemical profiles, preserving the three-dimensional (3D) architecture and the content of fundamental biological molecules. Hence, decellularized ECM can be employed to promote tissue remodeling, repair, and functional reconstruction of many organs. Selecting the appropriate decellularization procedure is crucial to obtain acellular tissues that retain the characteristics of the ideal microenvironment for cells.

The protocol described here provides a detailed step-by-step description of the decellularization

method to obtain a reproducible and effective cell-free biological ECM. Skin fragments from patients undergoing plastic surgery were scaled down and decellularized using a combination of sodium dodecylsulfate (SDS), Triton X-100, and antibiotics. To promote the regular and homogeneous transport of the solution through the samples, they were enclosed in embedding cassettes to ensure protection from mechanical insults. After the decellularization procedure, the snow-white color of skin fragments indicated complete and successful decellularization. Additionally, decellularized samples showed an intact and well-preserved architecture. The results suggest that the proposed decellularization method was effective, fast, and reproducible and protected samples from architectural damages.

INTRODUCTION:

The ECM serves as a scaffold for cells, supporting them through an intricate architecture maintained by different components, and it is one of the major factors responsible for the mechanical properties of the heart and cardiac tissue function[1,2]. Increasing evidence suggests that ECM plays an active role in tissue remodeling, making the conventional assumption that the ECM is a passive component obsolete[3,4,5,6]. The role of the ECM is to provide biophysical and biochemical cues to resident cells. It is well-established that these signals can influence many fundamental cell behaviors, impacting their contractile function, proliferation, migration, and differentiation potential[7,8,9]. Thus, ECM is increasingly being employed in tissue engineering and regenerative medicine as a therapeutic support tool[9,10,11,12,13].

The ECM consists of several proteins such as collagen, elastin, fibronectin, proteoglycan, and laminin, along with ECM-bound growth factors, all involved in regeneration mechanisms, such as cell recruitment, migration, and differentiation, as well as cell alignment and proliferation[14]. The mechanical properties of the tissues also have great relevance in the physiopathology of the organs. Indeed, changes in mechanical properties are often associated with the onset and the evolution of several diseases. The reason resides in the fact that when the ECM is modified, signals coming from the environment induce changes in gene and protein expression, leading to functional impairment[15,16].

Regenerative therapies for organ repair are currently focused on replicating the sophisticated microenvironment of the native tissue to heal the organ where the body fails. Despite the rapid pace of many tissue engineering approaches, the tissues still cannot be reproduced accurately in their entirety and complexity by artificial procedures. Synthetic materials have been largely employed so far, as they can be appropriately tuned to simulate the mechanical and biochemical properties of the cellular microenvironment. Nevertheless, they have limits, such as the inability to mimic the numerous interactions within the native tissue, the cost of technologies to produce them, and the fact that they are less natural and biocompatible than native tissue[17,18,19]. Additionally, their composition, primarily in terms of proteins and soluble factors, greatly differs from the natural one, which is extremely difficult to replicate[20].

The cutting-edge approach in regenerative medicine to reduce the gap between the patients in need and organ transplants is to produce scaffolds made of decellularized extracellular matrix (d-ECM) and repopulate them with the appropriate cell types to regenerate the damaged organs.

Decellularization is the process in which the ECM is isolated from its native cells and genetic material to produce a natural and biomimetic scaffold, able to avoid the immune response and rejection once implanted in the patients[21,22,23]. The ECM thus obtained can then be repopulated to produce functional tissue. The major issue when developing a d-ECM is the method. For any decellularization technique, the primary goal remains the preservation of the native ECM composition, stiffness, and 3D structure, and all strategies have both benefits and drawbacks. Because the elimination of cellular content and DNA from the tissue requires the use of chemical or physical agents, or the combination of both, each decellularization procedure causes, to different degrees, the disruption of the ECM. Hence, it is crucial to minimize the damage to the ECM[24,25,26].

Native ECM utilization as a platform for the reconstitution of native ECM *in vitro* is highly desirable. For this purpose, several decellularization protocols have been applied to a wide range of tissues[27,28,29,30]. In fact, since the early stages of decellularization research and ECM development, several tissues, such as arteries, aortic valves, and peripheral nerves from both animals and humans, have been decellularized, and some d-ECMs are still commercially available and used for tissue replacement or wound healing[31,32,33]. Recently, human skin has also emerged as a suitable candidate to produce decellularized scaffolds for cardiac repair owing to its composition and mechanical properties—able to boost the regenerative potential of cardiac progenitor cells (CPCs) and adapt to cardiac contractility[34]. This paper describes a simple and fast protocol to produce decellularized scaffolds from adult human skin, allowing the development of a d-ECM with well-preserved architecture.

PROTOCOL:

The specimens from human tissue were collected according to the principles of the Declaration of Helsinki and observing University Hospital Federico II guidelines. All patients involved in this study provided written consent forms.

1. Preparation of solutions

1.1. Preparation of 1200 mL of 1% decellularizing solution

1.1.1. Prepare 600 mL of 2% Triton X-100 solution by measuring 588 mL of double-distilled water in a graduated cylinder and transferring it to a 1 L beaker. Using a serological pipette, add 12 mL of Triton X-100 and a stir bar to the beaker. Place the beaker on a magnetic stirrer and mix the solution until the Triton X-100 is completely dissolved.

1.1.2. Stop the agitation. Remove the stir bar using a stir bar retriever. Store at room temperature.

1.1.3. Prepare 600 mL of 2% SDS solution by measuring 550 mL of double-distilled water in a graduated cylinder and transferring it to a 1 L beaker.

1.1.4. Weigh 12 g of SDS powder in a plastic weighing boat and transfer it to the beaker containing the double-distilled water in step 1.1.3.

NOTE: Step 1.1.4 must be performed under a chemical hood, and the user should wear personal protective equipment.

1.1.5. Add a stir bar, place the beaker on a magnetic stirrer, and mix the solution until the SDS is completely dissolved.

1.1.6. Stop the agitation process. Remove the stir bar using a stir bar retriever.

1.1.7. Transfer the solution to a graduated cylinder and adjust the volume to 600 mL by adding double-distilled water.

1.1.8. Pour 2% Triton X-100 solution prepared in step 1.1.1 into a 2 L cylinder.

1.1.9. Pour 2% SDS solution prepared in steps 1.1.3-1.1.7 into the same 2 L cylinder to obtain a total volume of 1200 mL.

1.1.10. Cover with parafilm and mix gently by inversion to obtain a homogeneous solution.

1.1.11. Transfer the mixed solution to a 2 L bottle using a funnel to avoid the formation of foam. Store the solution at +4 °C.

1.2. Preparation of 1x phosphate-buffered-saline (PBS)

1.2.1. Prepare 500 mL of 1x phosphate-buffered-saline (PBS) by dissolving 0.1 g of potassium phosphate monobasic, 0.1 g of potassium chloride, 4.0 g of sodium chloride, and 0.575 g of sodium phosphate dibasic in sterile double-distilled water. Check the pH value (7.4).

1.2.2. Store at +4 °C until use.

1.3. Preparation of antibiotic solution

1.3.1. Weigh 625 µg of amphotericin B in a plastic weighing boat.

1.3.2. Pipette 8 mL of a penicillin and streptomycin (pen/strep) mixture into a 15 mL tube.

1.3.3. Add the weighed amphotericin B to the pen/strep mixture, and adjust the volume to 10 mL by adding penicillin and streptomycin (pen/strep) mixture. Cap the tube and dissolve the amphotericin B by shaking vigorously.

2. Day 1—start the decellularization procedure.

2.1. Pour 400 mL of the decellularizing solution into a 500 mL beaker.

2.2. Add 2.0 mL of the antibiotic solution.

3. Preparation of skin samples

3.1. Wash the human skin sample from the abdomen of patients undergoing abdominoplasty in a plastic tray by shacking and turning it upside down in a 0.9% NaCl isotonic physiological solution to remove excess blood and other biological fluids.

3.2. Remove hair using fine forceps and fat using large surgical scissors.

3.3. Place the skin sample on the dissecting board and dissect it using a scalpel, with the graduations on the dissecting board as a reference to obtain 3 cm x 2.5 cm fragments (length by width), avoiding scars and dirty or burned areas of the tissue.

3.4. Place each fragment in an embedding cassette, close it, and make sure all the cassettes are appropriately locked to avoid leakage of the sample during the decellularization procedure.

3.5. Place a maximum of four embedding cassettes with samples in a beaker with the decellularizing solution. Add a stir bar. Cover with aluminum foil, writing on the top all the information to identify the sample and the starting time of the procedure.

3.6. Place the beaker on the magnetic stirrer and start the agitation at a rotational velocity of 150 rpm. Stop the agitation. Remove the aluminum foil covering the beaker and take the embedding cassettes out with long forceps.

NOTE: After stopping the agitation, check the clarity of the solution. Modify the duration of the agitation (less or more than 8 hours) based on the degree of the clouding due to the release of cellular debris.

3.7. Place each cassette in a 100 mm dish. Replace the decellularizing solution by repeating steps 3.5–3.6 overnight.

4. Day 2—check the state of the skin samples.

4.1. Stop the agitation process. Remove the aluminum foil covering the beaker and take the embedding cassettes out with long forceps.

4.2. Place each cassette in a 100 mm dish and open it. Check the color of the sample.

NOTE: The samples might show a color change from the native beige to snow-white.

4.3. Remove residual hair and detach the epidermis from the dermis. Replace the

decellularizing solution by repeating steps 3.5–3.6.

4.4. Stop the agitation after 8 h. Repeat steps 4.1–4.2 until the sample appears snow-white. Stop the decellularizing procedure.

NOTE: The time of exposure to the action of detergents can be extended if needed.

4.5. Measure 400 mL of 1x PBS in a 500 mL cylinder, pour it into a 500 mL beaker, and add 2 mL of the Pen Strep/Amphotericin B mixture. Add a stir bar.

4.6. Place a maximum of four cassettes containing the samples in each beaker with the 1x PBS/antibiotics solution. Cover with aluminum foil and write all the information on the top as in step 3.5.

4.7. Place the beaker on a magnetic stirrer and start the agitation at a rotational velocity of 150 rpm overnight, at room temperature.

5. Day 3—a final wash of the samples

5.1. Stop the agitation. Replace the 1x PBS solution with 400 mL of double-distilled water. Add a stir bar. Cover with aluminum foil and write all the information on top as in step 3.5.

5.2. Place the beaker on a magnetic stirrer and start the agitation at a rotational velocity of 150 rpm for 30 min.

5.3. Stop the agitation process. Remove the aluminum foil covering the beaker and take the embedding cassettes out with long forceps.

5.4. Place each embedding cassette containing the decellularized skin sample in a 100 mm dish. Open the cassettes and gently dry the skin samples by dabbing them on laboratory wipes.

5.5. Place each decellularized skin sample in a weighing boat previously tagged with the information about the sample. Cover the boat with aluminum foil and write all the information on the top to identify the sample. Store at – 80 °C.

REPRESENTATIVE RESULTS:

The aim of the protocol was to obtain a skin d-ECM sample from biological tissue, maintaining a well-organized 3D structure and well-preserved content of biological molecules (**Figure 1**). This method is primarily based on the constant stirring of the samples in a solution containing the combination of two detergents, Triton X-100 and SDS, thus preserving the biological and structural features typical of the native tissue and reducing the time of exposure during the decellularization process. Upon receipt, the samples were washed and prepared for the procedure, obtaining 3 cm x 2.5 cm clean skin fragments, free from hair or fat tissue (**Figure 2** and **Figure 3**). The use of embedding cassettes allowed the minimization of tissue disruption

(**Figure 3**), resulting in samples with a more intact architecture. The decellularized skin did not show signs of significant mechanical or chemical damage after macroscopic observation. The epidermis was detached during the decellularization procedure (**Figure 4A,B**), and the samples changed in color from beige, typical of the whole native tissue, to snow-white, indicating complete and successful decellularization (**Figure 4C**).

FIGURE AND TABLE LEGENDS:

Figure 1: **Protocol workflow**.

Figure 2: Representative images of the skin sample preparation upon receipt. (**A**) Incoming sample. (**B, C**) Macroscopic evaluation of the sample placed on dissecting board. (**D–F**) Removal of the fat tissue using large surgical scissors.

Figure 3: **Skin sample dissection and placement in embedding cassettes**. (**A**) Hair removed by fine forceps. (**B–E**) Skin samples dissected using a scalpel to obtain 3 cm x 2.5 cm fragments to fit into the embedding cassettes. (**F, G**) Skin fragment placed in embedding cassette.

Figure 4: **Checking the state of the sample during decellularization**. (**A, B**) Detachment of the epidermis from the dermis. (**C**) Sample color change indicating the occurrence of decellularization. Scale bars = 3 cm.

DISCUSSION:

Although the protocol described above has been optimized and improved compared to previously published protocols, it presents a few critical steps that need attention and precision. The formation of foam must be avoided during the preparation of the decellularizing solution to prevent incorrect dilution of the detergents. This could be addressed by gently pouring the solutions and making them flow along the inner side of the cylinder. Furthermore, care must be taken when manually removing fat tissue from the samples, as decellularization using detergents, such as Triton X-100 and SDS, does not eliminate fat, and lipidic remnants could negatively affect the effectiveness of the method. Another critical step is the removal of the epidermis from the samples. During the decellularization, the epidermis gradually detaches from the skin surface. In this phase, it is crucial to peel it away with forceps only when it is completely swept off, thus avoiding tearing or shredding it and causing the entrapment of debris within the samples.

This protocol focuses on reducing the impact of decellularization on the samples by two major strategies: the shortening of exposure time to the procedure by using two detergents in combination[28,35,36] and the protection of the samples from mechanical insults by enclosing them in embedding cassettes[37]. Further, the procedure has yielded successful outcomes when applied to samples other than skin, such as human and porcine myocardium and blood vessels. The evaluation of the effectiveness of the protocol described here is primarily done by a macroscopic observation of the samples, which would show a remarkable change in color, from beige to snow-white. Certainly, some other aspects must be assessed to assess the quality of d-ECM, such as the removal of cellular debris and residual genetic material, the preservation of structural

proteins and biomolecule content, and the retention of mechanical properties, as described elsewhere[34,37,38]. The removal of cellular debris and genetic material is crucial to reduce the immunogenicity of the construct. Hence, some criteria for assessing the efficacy of removing these components are well-established in the literature: the d-ECM must have not more than 50 ng of double-stranded DNA (dsDNA)[39] per mg of ECM dry weight, and no nuclear material must be microscopically visible.

To meet these criteria, residual DNA content evaluation and staining with hematoxylin and eosin must be performed, as described by Di Meglio et al.[38] to support the macroscopic data. Moreover, the evaluation of ECM preservation by detecting structural proteins, such as collagen, fibronectin, and laminin, together with glycosaminoglycans and growth factors, is also appropriate[34]. Finally, the mechanical properties of the d-ECM should match those of the native tissue[40]. Although the efficacy and feasibility of this procedure have been demonstrated to obtain desirable decellularized tissue, the method has some limitations. For example, it allows the decellularization of only a few samples at a time (4 for each beaker), leading to considerable wastage of solutions. On the one hand, the use of embedding cassettes helps protect and preserve the samples; on the other hand, it forces the operators to decellularize only samples of small sizes that fit into the cassettes. Another limitation is the possibility of microbial contamination of the d-ECM obtained. Although the procedure involves the addition of antibiotics, it is highly recommended to sterilize the d-ECM by UV prior to use. Finally, the incubations required throughout the entire procedure must be accurately calculated and organized to optimally manage the time.

This protocol was developed to address the drawbacks observed while testing other protocols in the laboratory. Existing protocols allow a varying degree of decellularization without guaranteeing adequate integrity of the tissue, thus involving a considerable loss of essential biological molecules and mechanical properties[41]. The preservation of the ECM structure and composition after the decellularization are fundamental aspects, as d-ECM may act as the scaffold, that once repopulated, will support cellular regenerative mechanisms within the organ. By optimizing the decellularization process, biological scaffolds derived from human skin may be utilized in regenerative medicine, helping to minimize the gap between donors and patients in need of organ transplants. In addition, advancements in recellularization methods and applications of human induced pluripotent stem cell-derived cells will improve the distribution of the proper cell types[42,43] to enable the regeneration process[44].

ACKNOWLEDGMENTS:
None

DISCLOSURES:
The authors have no conflicts of interest to disclose.

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Figure 1

Figure 2

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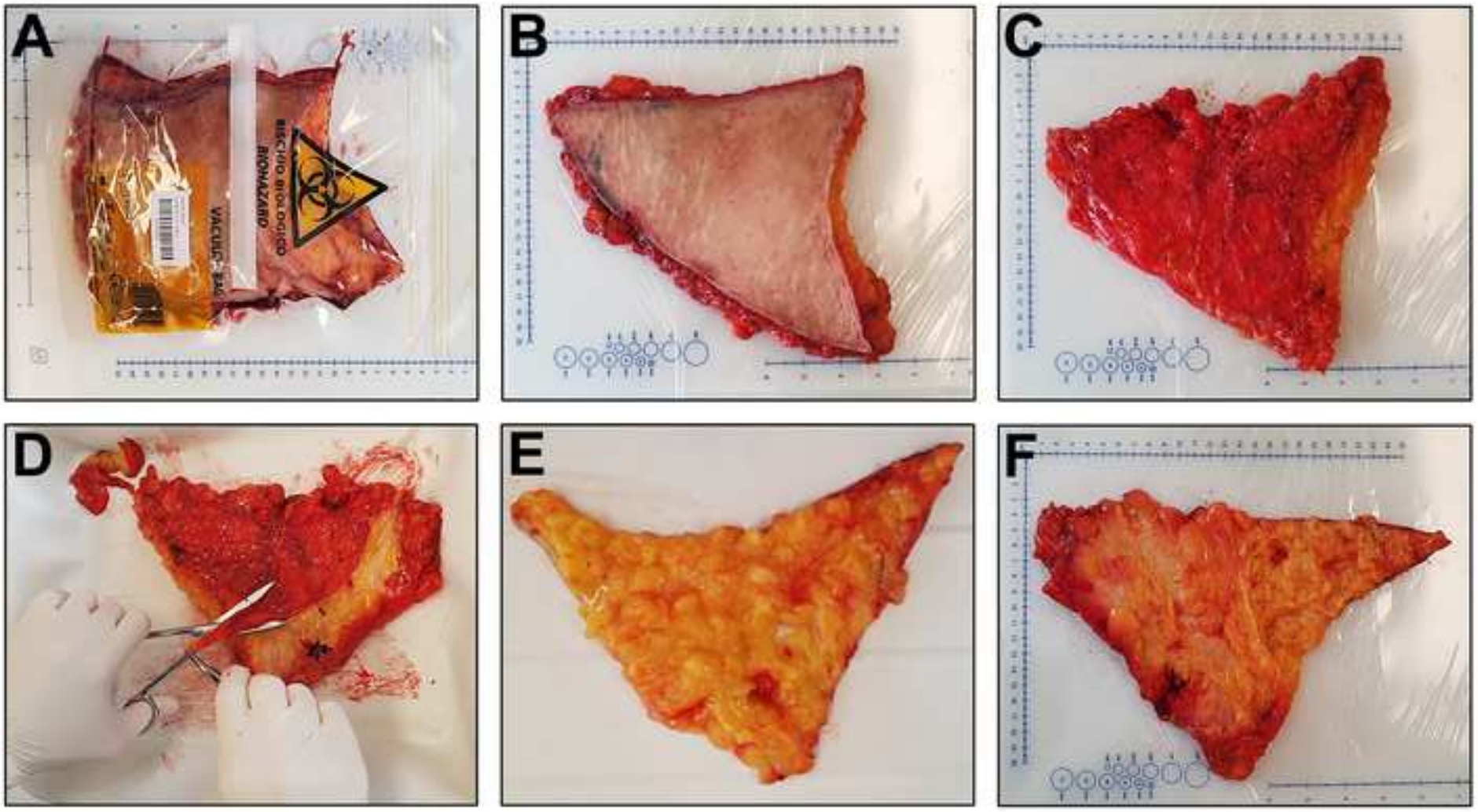

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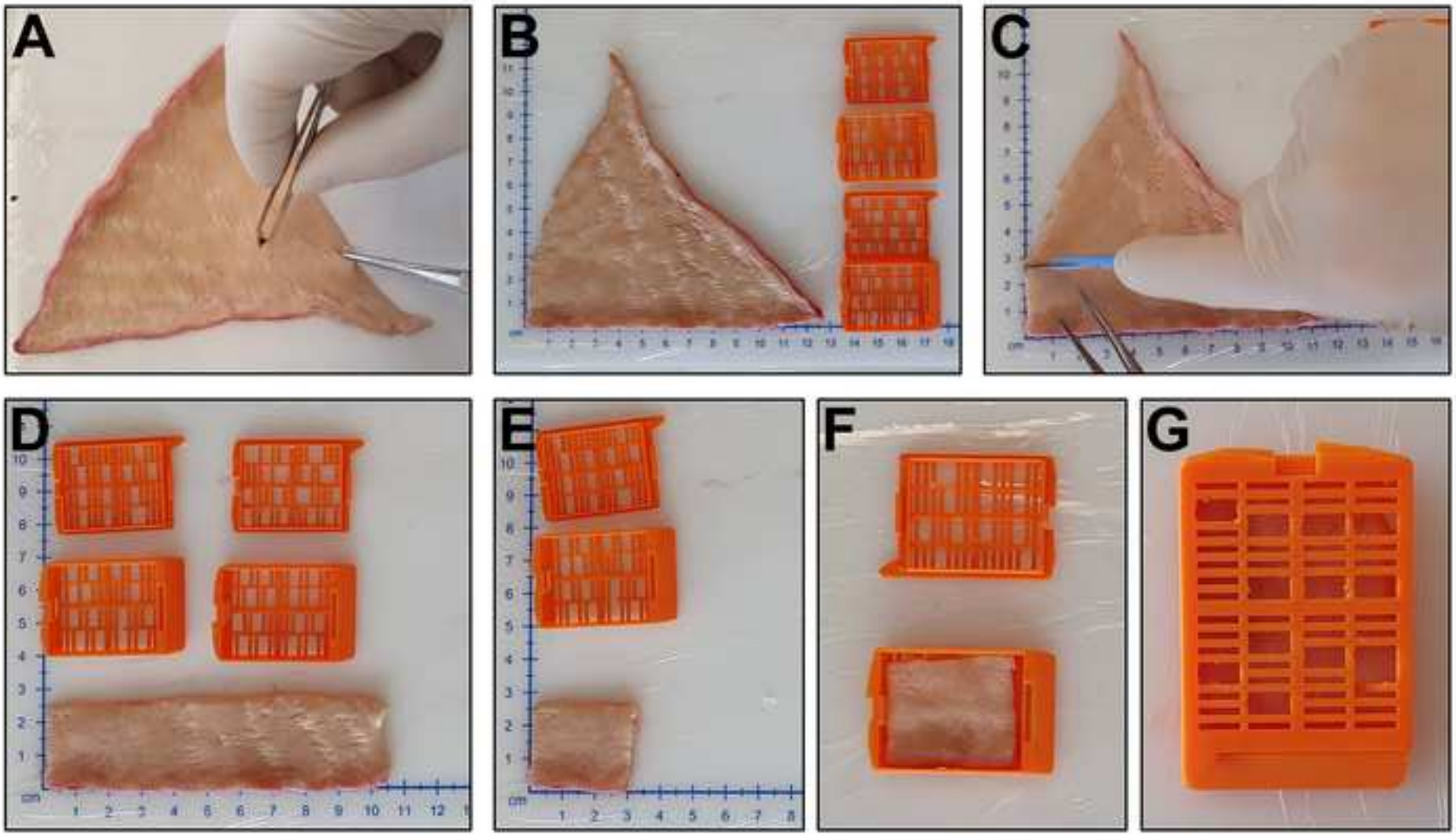
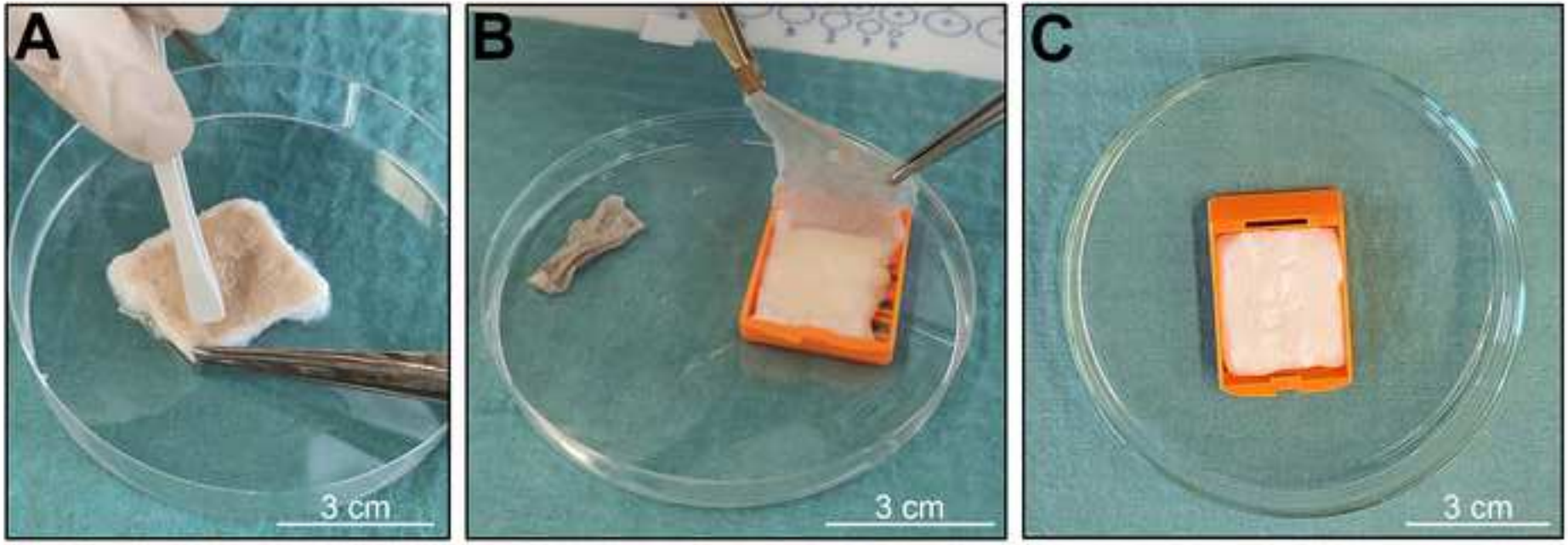


Figure 4



Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

As pointed in the Editor's comment, we proofread the manuscript and revised spelling and grammar issues.

2. Please revise the following lines to avoid previously published work: 324-326

We thank the Editor for the suggestion. We rephrased the sentence as follows "Moreover, the evaluation of ECM preservation by detecting structural proteins like collagen, fibronectin and laminin, together with glycosaminoglycans and growth factors, is also appropriate".

3. Line 119-128/132-147: Please refrain from using bullets. Please move the list of surgical and consumables to the Table of Materials.

As suggested by the Editor, we moved all tools and consumables required for the protocol to the Table of Materials.

4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We reviewed the protocol to ensure that all details were given.

5. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

One-line space was inserted between steps and steps needed for the video were highlighted.

6. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We appreciate the kind suggestion of the Editor, and we implemented the discussion section adding content to cover all the indicated paragraphs.

7. Figure 1: Please label the figure to make it more informative.

We thank the Editor for the suggestion. We labeled the figure 1 in order to increase its readability.

_____

Reviewers' comments:

Reviewer #1:

I read the manuscript and I found it very interesting, moreover, very ussefull and well explicated. I think it´s really a good paper to publish

We thank the Reviewer for his/her positive feedback on our manuscript

Reviewer #2:

Accept.

We thank the Reviewer for appreciating our manuscript.

Reviewer #3:

Manuscript Summary:

The manuscript of Romano et al. describes a decellularization method of human skin. The final product a decellularized extracellular matrix (ECM) can be stored frozen and used on demand as a biological scaffold for regenerative medicine applications, such as cardiac repair.

Overall the manuscript is clear and the step by step protocol contain enough details.

Minor Concerns:

Overall, the experiments are performed under a culture hood?

We do apologize to the Reviewer for the missing information. It is not mandatory to perform the proposed protocol under a culture hood, but it is necessary the use of antibiotics (penicillin, streptomycin, amphotericin B) during the entire procedure to reduce the risk of microbial contaminations and to sterilize the d-ECM with UV light prior to use, as well.

1/line 119: Addition of References/Providers of tools used will help readers.

As suggested by the Editor, all tools and consumables were moved to the Table of Materials and suppliers for each one of them were provided.

2/ line 147: PBS is prepared later in the protocol. You should detail here the powder to be used to prepare PBS.

Since all tools and materials have been moved to the Table of Materials, chemicals used for PBS have been listed in the same table and the step for the preparation of PBS mentioned by the Reviewer has been deleted.

3/ line 161: The author may specify how to remove the stir bar. If a specific tool is used, it should be indicated earlier in the list of materials.

The stir bar is removed using a stir bar retriever. As suggested by the Reviewer, this tool has been added to the Table of Materials.

4/ line 174: Why the authors indicate the necessity to use 1L cylinder when the final volume is 1.2 L? The use of a bigger one would be a problem?

We thank the Reviewer for highlighting the incorrect information. The final volume of the solution obtained mixing Triton X-100 and SDS is equal to 1.2 L, therefore the cylinder required should be a 2L cylinder. We replaced the 1L cylinder with a 2L cylinder.

5/ line191: concentration of Pen/strep and AmphoB in stock solution?

The volume of the antibiotic mixture required at each decellularizing solution replacement and for the overnight wash in PBS is equal to 2 mL. For the whole procedure, including three replacements steps at 2.2, 3.12, 4.6 and a single wash in PBS at step 4.11, the total volume is equal to 8 mL, thus we usually prepare an exceeding volume of antibiotic mixture (10 mL), by adding powdered Amphotericin B to Pen/Strep solution to obtain a final working solution concentrated at 62.5 ug/mL. We do not use a stock solution, as we prepare the antibiotic mixture required extemporaneously.

6/ line 199: agitation until use or for a specific duration?

During the step 2 the antibiotic solution prepared in step 1.3 is added to the decellularizing solution and agitation is not required, therefore we eliminated the sentence “Add a stir bar”.

7/ line 203: origin of human skin: what type of surgery, from cadavers,…?

We do apologize for the missing information. The step 3.1 has been implemented adding details on the origin of human skin and the statement was rephrased as “Human skin was obtained from abdomen of patients undergoing abdominoplasty”.

8/ line 207: it may be useful to specify to dissect the skin using the graduations on the dissecting board. It was not clear to me without seeing the corresponding figure.

We thank the Reviewer for the suggestion. We rephrased the sentence as follows: “Place the skin sample on the dissecting board and dissect it by the scalpel, using the graduations on the dissecting board as a reference to obtain 3cm x 2.5cm fragments (length by width) avoiding scars, dirty or burned areas of the tissue”.

9/ line 214: stir bar already added in line 199?

We thank the Reviewer for her/his remark: we eliminated the sentence “Add a stir bar” in the step 2, as during the step 2 the agitation is not required, and a stir bar will be added during the following step 3.6.

10/ line 218: 8h: is it a precise duration or you can add a time interval?

The time of agitation process depends on the clarity of the decellularizing solution. It can vary from less than 8 hours to more, based on the degree of the clouding due to the release of cellular debris into the solution. To clarify this step, we added a NOTE.

11/ line 222: 8h or overnight?

We thank the Reviewer for highlighting the missing information. The step 3.12 was implemented adding the incubation time “overnight”.

12/ line 232: a precision here would be useful: what type of color is expected or not?

Recognizing the lack of clarity, we introduced the following NOTE for the step 4.4 “at step 4.4 the samples might show a color change from the native beige to snow white”.

13/ line 236: if not translucent, a new incubation can be added?

The time of exposure to the action of the decellularizing solution can be extended until the color of the samples results snow white, this information has been reported in the NOTE of the step 4.

14/ line 263: will it remain sterile in such container at -80°C?

Although the procedure involves the addition of antibiotics in the decellularizing solution, it is highly recommended to sterilize by UV exposure the d-ECM stored at -80°, prior to its use.

15/ line 307: shortening of exposure time to detergents: specify the duration of other protocols compared to yours.

This protocol has been developed based on the comparison to previously published decellularizing procedures, and improving the drawbacks noticed testing them in our laboratory. Among protocols tested are:

- Ott H C et al. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. Nat Med, 14, 213, 2008. doi: 10.1038/nm1684. (duration of decellularizing procedure: 136.75 hours).

- Greco K V et al.: Characterisation of porcine dermis scaffolds decellularised using a novel non-enzymatic method for biomedical applications. J Biomater Appl, 30(2):239-253, 2015. doi:10.1177/0885328215578638. In this study authors tested four different protocols of decellularization: protocol 1 (duration of 101 hours), protocol 2 (duration of 205 hours), protocol 3 (duration of 98 hours) and protocol 4 (duration of 77,5 hours).

- Brouki M P et al.: Decellularization and preservation of human skin: A platform for tissue engineering and reconstructive surgery. Methods, 171:62-67, 2020. doi: 10.1016/j.ymeth.2019.07.005. (duration of decellularizing procedure: 104 hours).

16/ line 334: you mean hiPSC derived cells not hipsc.

As suggested by the Reviewer, we modified the sentence as follows “human induced pluripotent stem cells derived cells” instead of “human induced pluripotent stem cells”.

17/ figure 4 : scale bars missing.

As suggested by the Reviewer, we added scale bars to figure 4.