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COMPARISON OF THREE DIFFERENT METHODS FOR PROCESSING ACELLULAR DERMAL MATRIX

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Abstract. Tissue engineering is an inter-related and a multi-disciplinary field that blend of biology and engineering efforts that attempt to address clinical problem of skin damage by many reason including burn, trauma etc. Decellularization of allogeneic tissues witn subsequent combened of the decellularized tissue with autologous cells and/or the processed of hybrid structures plays a pivotal role in current tissue engineering approaches for temporary or constant closure of skin defects. The purpose of this study was to compare methods for processing a decellularized dermal matrix and to determine the optimal protocol for obtaining a decellularized dermal matrix scaffold. As a result of a comparative analysis, it was determined that all three skin decellularization protocols make it possible to obtain a product with desired properties by the absence of cellular and nuclear material. However, the use of various detergents for decellularization leads to some structural features of the resulting material. The significance of differences in the structural characteristics of the obtained matrices should be evaluated in future studies in terms of stimulation of cell migration and proliferation.

Key words: acellular dermal matrix; decellularization; burn injury; collagen; regenerative medicine.

СРАВНЕНИЕ МЕТОДИК ПОЛУЧЕНИЯ АЦЕЛЛЮЛЯРНОГО ДЕРМАЛЬНОГО МАТРИКСА

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Резюме. Развивающиеся технологии тканевой инженерии, несомненно, представляют интерес для разработки материала для закрытия дефектов кожных покровов. Одним из перспективных направлений является децеллюляризация аллогенных тканей для временного закрытия дефектов кожных покровов, насыщения децеллюляризированной ткани аутологичными клетками и/или создания гибридных конструкций. Цель данной работы состояла в сравнении методик получения децеллюляризированного дермального матрикса и определении оптимального протокола для последующей разработки тканеинженерной кожи. Для достижения цели была проведена оценка

трех различных методов получения ацеллюлярных дермальных матриксов путем децеллюляризации кожи. В результате сравнительного анализа было определено, что все три протокола децеллюляризации кожи позволяют получить продукт с заданными свойствами: отсутствие клеточного и ядерного материала. Однако использование для децеллюляризации различных детергентов приводит к некоторым структурным особенностям полученного материала. Значимость различий в структурных характеристиках полученных матриксов должна быть оценена в дальнейших работах с точки зрения стимуляции миграции и пролиферации клеток.

Ключевые слова: ацеллюлярный дермальный матрикс; децеллюляризация; ожоговая травма; коллаген; регенеративная медицина.

INTRODUCTION

Currently, the search for new optimal means of surgical treatment of skin lesions of various genesis is an extremely urgent task [5, 7]. It is necessary to achieve the maximum possible combination of therapeutic properties that improve the patient's condition and increase the quality of subsequent life, ease of use in routine clinical practice, facilitating the widespread implementation of the method. It is also important to create the possibility of relatively simple production of a large volume of medical devices, which will ensure the economic feasibility of their use in comparison with other possible options [2]. One of the areas of action to complete this task may be the development of medical products using biomedical technologies. A variant of such a multifunctional product may be a tissue processing product that preserves a number of properties necessary in the clinic: acellular dermal matrix (ADM) obtained by detergent-based decellularization. The application of this object seems to be promising in the treatment of deep skin lesions, including burns. The main advantages of this biological material are the composition and structural organization of the resulting product, corresponding to the patient's own dermis. This allows it to be used as a temporary biological coating that promotes the migration of the patient's cells and the regeneration of functionally competent skin, while effectively performing the functions of a wound dressing. After a full processing cycle, the ADM obtained from donor skin does not contain immunogenic factors that are caused by cellular structures and can lead to rejection of the donor material. At the same time, the native structure and composition of the extracellular matrix of the dermis are preserved [3]. This product can be considered both as an independent medical device and as a stage for further development of a tissue-engineered skin substitute. At present, a is a lot of practical experience has been accumulated in skin treatment for the purpose of decellularization. The wide range of reagent base for these tasks, as well as methods for assessing the obtained result, has been determined [4]. In addition, there is no perfect method approved in clinical recommendations and widely used. At the moment, the expansion of the range of methods and their technological development are especially relevant. This is due to the fact that in modern conditions logistical problems with the availability of various reagents are possible.

AIM

The aim of the study is to determine the optimal method of skin decellularization based on a comparative analysis of acellular dermal matrices obtained by different techniques.

MATERIALS AND METHODS

Skin area from the abdominal region was obtained during cosmetic surgery (tummy tuck or abdominoplasty) in a 37-year-old female patient and delivered to the Research Department of Medical and Biological Research of the Research Center of the Military Medical Academy named after S.M. Kirov of the Ministry of Defense of the Russian Federation for experimental work. Without breaking sterility, a fatty layer of subcutaneous tissue was mechanically removed using a surgical instrument. The resulting skin layer was divided into 9 equal parts, which were washed abundantly with sterile distilled water. 3 groups of samples were formed (3 in each group). The area of each sample was ≈7.78 cm^2. Then the skin layers were placed in a low temperature refrigerator for storage at a temperature of –80 °C for a period of one day (Angelantoni Life Science Platinum 340 V, Italy). In all cases, samples were thawed at room temperature for 4–6 hours.

Decellularization was then performed using three different protocols shown in Table 1.

Each sample was placed in a glass container with 200 ml of the corresponding detergent solution and subjected to shaking on an orbital shaker at 200 rpm at 37 °C for decellularization. The solutions were changed according to the protocols (Table 1). After each stage of detergent use, samples were thoroughly washed with sterile distilled water under the same conditions.

In accordance with the protocol No. 1, the samples were first placed in the Versen solution (Biolot, Russian Federation) after thawing, where they were kept for 16 hours. The next stage was to treat the samples with a 0.5% sodium dodecyl sulfate solution (SDS, LenReaktiv, Russian Federation) for 48 hours. The solution was changed to a fresh one every 12 hours.

Table 1

Stages of technologies for processing decellularized dermal matrix using three different methods

Processing stage number	Protocol No. 1	Protocol No. 2	Protocol No.3
1	Freezing–thawing	Freezing–thawing	Freezing–thawing
2	Versen solution (16 hours)	3% Tween 20 (16 hours)	1,8 M NaCl (3 hours)
3	Washing with sterile distilled water (15 minutes)	Washing with sterile distilled water (15 minutes)	Washing with sterile distilled water (15 minutes)
4	0.5% SDS (24 hours, 2 solution changes). ***Histological examination*** 0.5% SDS (24 hours, 2 solution changes)	0.5% SDS (48 hours, 4 solution changes)	3% Tween 20 (16 hours)
5	Washing solution ***Histological examination***	Washing solution ***Histological examination***	Washing with sterile distilled water (15 minutes)
6	–	–	0.5% SDS (48 hours, 4 solution changes)
7	–	–	Washing solution ***Histological examination***

Protocol No. 2 repeated method No. 1, except that the 3% Tween 20 solution (PanReac, Spain) was used instead of the Versen solution.

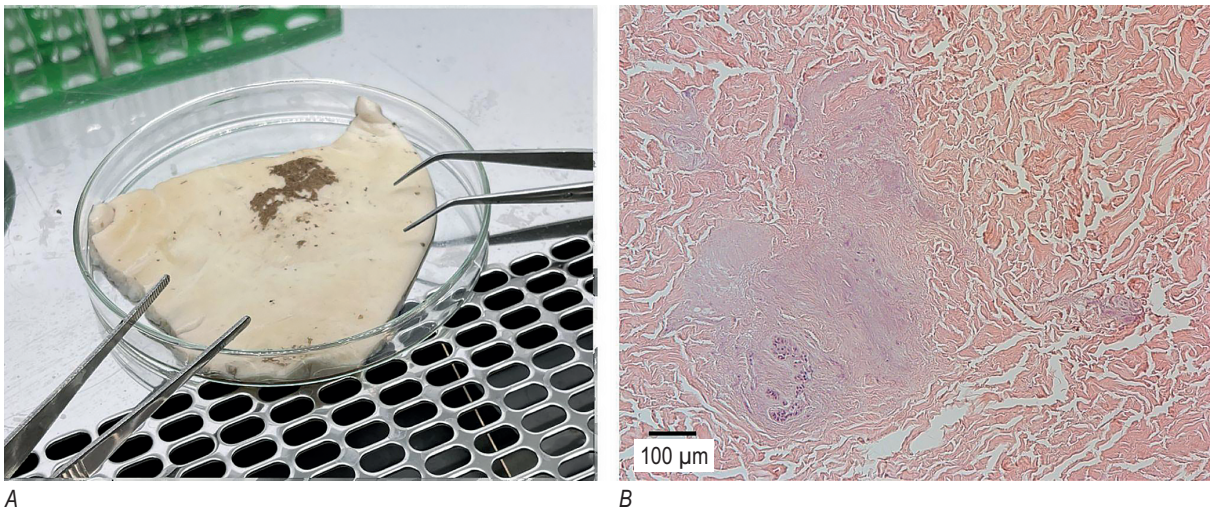
Protocol No. 3 differed from the previous one only by adding an additional stage after thawing the skin areas — a 3-hour treatment with 1.8 M NaCl (LenReaktiv, Russian Federation).

After all stages of detergent treatment, the samples were thoroughly washed with phosphate-buffered saline (Biolot, Russian Federation). The appearance of the samples was noted throughout the treatment. Then, upon completion of decellularization, a histological examination of the central and marginal areas of each sample carried out. When performing protocol No. 1, the histological examination was carried out twice (Table 1). The material was fixed in 10% formalin, dehydrated and embedded in paraffin blocks. 2–3 μm thick sections were stained with hematoxylin and eosin, according to Van Gieson (Biovitrum, Russian Federation), as well as with the nuclear-specific fluorescent dye DAPI (4',6-diamidino-2-phenylindole).

The study did not contradict the provisions of the Ethical principles for medical research involving human subjects of the World Medical Association of Helsinki. The postoperative material (skin flap) was transferred with the patient's approval after signing a written voluntary informed consent.

RESULTS

During protocol No. 1, the separation of the epidermal layer was detected only at the SDS processing stage and



A *B*

Fig. 1. Morphological study of acellular dermal matrix in incomplete decellularization of the skin: macroscopic (*A*) and microscopic (magnification 100×, hematoxylin and eosin staining) (*B*) images

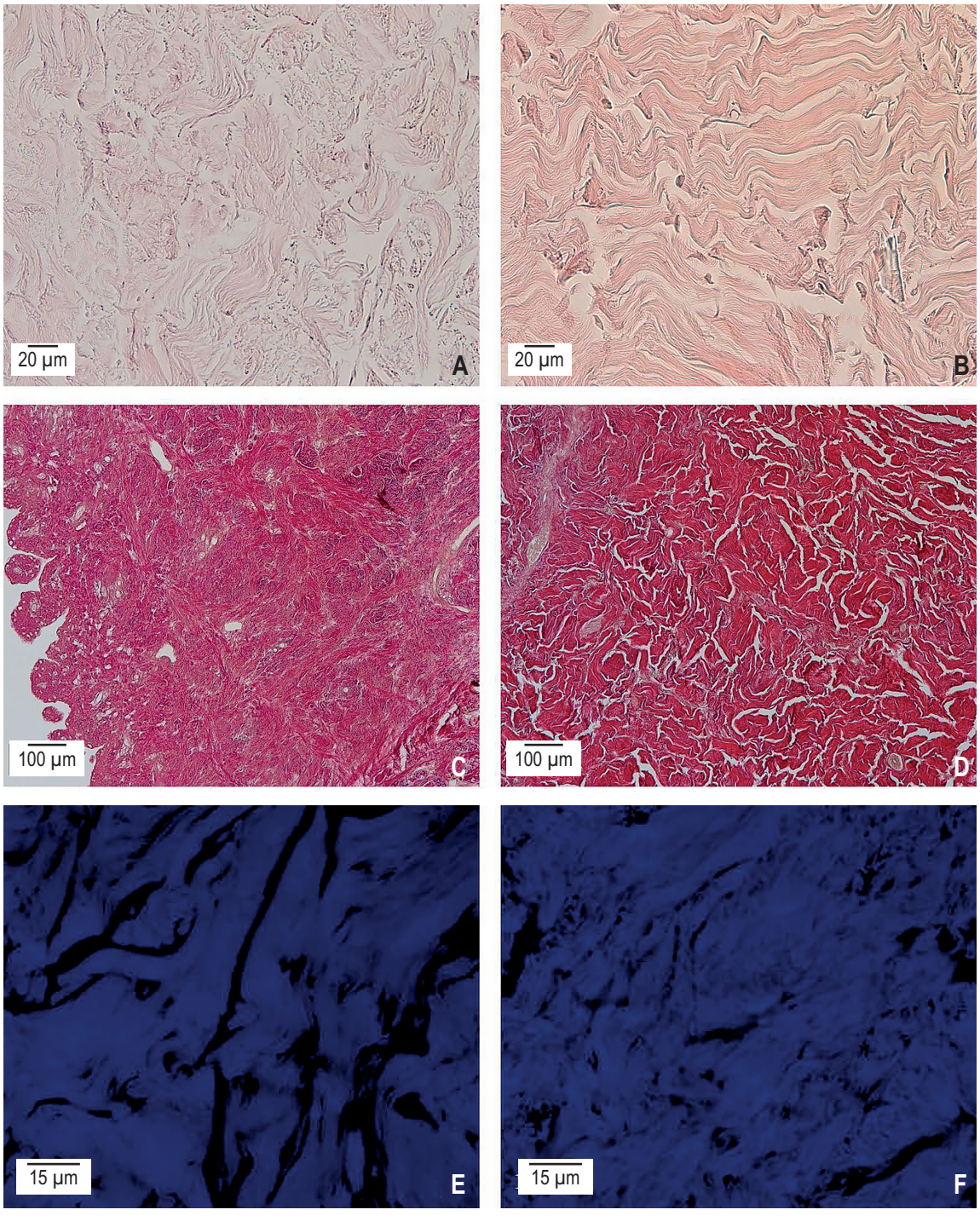


Fig. 2. **Acellular dermal matrix obtained according to protocol No. 1. A, C, F — marginal area; B, D, F — central area. Hematoxylin and eosin staining (*A*, *B*), van Gieson's staining (*C*, *D*), DAPI staining (*E*, *F*)**

was completed at the end of the protocol. The decellularized tissue was quite flexible and pliable, smooth on the epidermal side, but noticeably fibrous on the reverse side.

In the middle of the protocol (stage 4, Table 1), remnants of the epidermis were observed, mosaically located over the entire surface of the material. Histological examination also showed cellular structures in the central region of the samples (Fig. 1). No nuclei or other cellular elements were detected at the end of the protocol (Fig. 2).

Protocol No. 2 differed from Protocol No. 1 in that a softer detergent, Tween 20, was used for the initial treatment. In this case, the removal of epidermis from the dermis was slower, but the result (in terms of removing cellular elements and the epidermal layer) at the end of the protocol was similar.

However, there was a significant external difference in the resulting sample — there is no fiber unweaving on its reverse side, mechanically it was somewhat denser and more elastic.

The use of a hypertonic NaCl solution in Protocol No. 3 allowed for faster initiation of the process of separating the epidermal layer from the dermis. This process began almost immediately, but was completed closer to the end of the protocol. Thus, the additional stage did not provide any advantages, and at the end of the protocol, the result was similar to that obtained using Protocol No. 2, including in terms of appearance.

The study of ADM micropreparations obtained by all the methods researched showed complete removal of cellular elements. It was confirmed by staining with hematoxylin and eosin (Fig. 2, *A*, *B*) and the nuclear-specific dye DAPI — only autofluorescence of extracellular matrix proteins was visualized during microscopy (Fig. 2, *E*, *F*). Van Gieson's staining of sections made it possible to verify the integrity of collagen fibers (Fig. 2, *C*, *D*).

Morphological differences between the samples obtained by three different methods during microscopy of histological preparations were not detected. However, the use of Versen solution led to some fiber unweaving of the collagen matrix on one side, which may also have a positive effect on the migration of recipient cells.

DISCUSSION

Based on the obtained data on the quality assessment of the obtained cell-free material, it can be stated that all three protocols presented by us are quite effective in obtaining the final product with the required biological characteristics. The obtained ADM retains the structural organization necessary for the implementation of the therapeutic effect in clinical use. At the same time, there is no cellular component that creates an immunogenic potential that can cause rejection of the sample when placed on the wound surface. The physicochemical properties of the product ensure ease of handling and direct use in the treatment of skin lesions of various etiologies. At the same time, the production process is well-established, does not require complex technological solutions and expensive reagents, does not impose too high requirements on the qualifications of personnel. This makes it relatively easy to implement at the scientific and production base of the Military Medical Academy named after S.M. Kirov of the Ministry of Defense of the Russian Federation. The combination of these facts opens up great prospects for extensive production and clinical use of ADM in the treatment of skin defects of various geneses. Further definition of patient categories and the state of their wounds for the use of ADM seems quite logical.

Differences in the skin decellularization protocols had no significant effect on the final result. Protocol No. 3 took 3.5 hours longer than protocols No. 1 and 2, but no significant increase in the effect was found. Thus, there was acceleration of epidermal layer separation, which did not affect further processing. It can be assumed that to increase the efficiency of skin treatment with a hypertonic NaCl solution, a longer exposure with periodic change of the latter is necessary.

The same amount of reagents, time and labor is needed to execute protocols 1 No. 1 and 2. However, in the first case, the material was softer, with some fiber unweaving on one side. It is probably a consequence of the effect on the collagen matrix of ethylenediaminetetraacetic acid (EDTA), which is the basis of the Versen solution. A similar effect has been shown in the decellularization of corneal lenticular tissue with trypsin-EDTA solution [1]. In the case of skin, this effect may be useful, because there is no significant destruction of the extracellular matrix. But the increase in porosity and, accordingly, a decrease in the density of the material will facilitate the migration of the patient cells into the material [6].

CONCLUSION

1. All three protocols studied allow obtaining an acellular dermal matrix with the specified properties of absence of cellular and nuclear material.
2. Protocol No. 1 leads to fiber unweaving of the matrix, which may be a positive factor for the migration of recipient cells.
3. Acellular matrix obtained as a result of protocols No. 2 and 3, on the contrary, retained its density, while remaining elastic.
4. The features of the structural characteristics of the obtained matrices should be assessed in further studies in terms of their impact on the success of clinical application.

ADDITIONAL INFORMATION

Author contribution. Thereby, all authors made a substantial contribution to the conception of the study, acquisition, analysis, interpretation of data for the work, drafting and revising the article, final approval of the version to be published and agree to be accountable for all aspects of the study.

Competing interests. The authors declare that they have no competing interests.

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Consent for publication. Written consent was obtained from the patient for publication of relevant medical information within the manuscript.

ДОПОЛНИТЕЛЬНАЯ ИНФОРМАЦИЯ

Вклад авторов. Все авторы внесли существенный вклад в разработку концепции, проведение исследования и подготовку статьи, прочли и одобрили финальную версию перед публикацией.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии внешнего финансирования при проведении исследования.

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