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TECHNIQUES FOR OBTAINING DERMAL EXTRACELLULAR MATRIX SCAFFOLD

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Despite advancements in modern surgery in the treatment of cutaneous injuries, the search for new methods of ensuring faster and more effective wound healing appears especially urgent today. Tissue engineering is undoubtedly of interest when it comes to developing such technologies. **Objective:** to determine the optimal protocol for obtaining a decellularized dermal matrix scaffold for subsequent development of tissue-engineered skin. **Materials and methods.** One Landrace piglet was used as the experimental animal. After preliminary skin treatment with dermatome, 0.3 cm thick samples were taken. Two decellularization protocols were considered: protocol No. 1 was based on the use of Triton X-100 and deoxycholate, protocol No. 2 was only based on deoxycholate. There were 5 processing cycles in total for the 2 protocols. After treatment, acellular matrix scaffolds were examined through histological analysis and quantitative determination of DNA concentration. Next, static recellarization of the matrix scaffolds was carried out with porcine dermal fibroblasts. After that, the matrix scaffolds were tested for cytotoxicity using XTT test and differential staining test to differentiate between live and dead cells. **Results.** Comparative analysis of the two protocols for porcine dermis decellularization showed that both protocols effectively remove cells and nuclear material, while maintaining the architectonics of the intercellular substance intact, since fibrous structures are not destroyed. But when assessing the biocompatibility of matrix scaffolds based on analysis of cell viability according to data obtained from XTT test and cell–matrix adhesion, the matrix scaffold processed under protocol No. 1, shows advantages. **Conclusion.** In this study, a decellularization protocol based on Triton X-100 and deoxycholate was noted. The results obtained mark the first stage towards developing a tissue-engineered skin.

Keywords: regenerative medicine, skin, decellularization, tissue-engineered graft.

INTRODUCTION

The skin is the largest organ in the human body and performs such several vital functions as barrier, immune, and sensory. It has the ability to self-regulation and possess a number of other features [1]. Loss of skin integrity due to injuries or diseases can lead to acute physiological imbalance and ultimately to significant disability or even death [2].

Skin damages are diverse and can be caused by burns, injuries or be caused by trophic disorders due to venous hypertension, arterial insufficiency, diabetes mellitus and other reasons that lead to ulceration of the skin. A number of hereditary diseases associated with a violation of the structural proteins of the epidermis or dermis can also cause the development of extensive skin wounds and chronic erosion [3].

As a rule, skin damages are associated with a number of complex biochemical processes aimed at wound healing. In superficial wounds, where defects are limited by the epidermis or upper dermal layer, regeneration of the epidermis only is needed, which leads to quick healing and minimal risk of scar formation. Nevertheless, in wounds that penetrate deeper through the dermal layer, with peeling the skin or subcutaneous fat, such complications as infection develop more often, and scars usually remain even after the wound has completely healed. Actually, the duration of the wound healing tends to vary in different individuals and depends on the varying severity of damage [4].

Therapeutic interventions to restore skin surface and functions are an important long-term direction of both traditional and translational medicine where several key achievements and clinical advantages have been noted in recent years [3]. Choosing the right wound healing strategy is critical to their successful closure. This choice determines the rate of healing of the wound surface, the likelihood of complications and scar formation. Currently, various methods are used to close wound defects. Cadaveric allografts, xenografts, synthetic materials are used as wound surface coatings. Autodermoplasty re-

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mains the gold standard in the treatment of most skin wounds.

Regenerative medicine is currently actively developing due to the integration of engineering disciplines with biological sciences [5]. The prospects of using cellular technologies, additional biological factors that are aimed at stimulating tissue regeneration are under consideration. Experimental work is underway to create a tissue-engineering full-fledged skin [6]. Such developments are quite exciting, and undoubtedly can be useful, especially in cases where there is a significant deficit of the skin and autodermoplasty is impossible.

The tissue-engineering approach to the creation of artificial organs involves the use of extracellular matrices, stem cells and biologically active substances. One of the methods for producing matrices is the decellularization of the native tissue or organ. The choice of the optimal method to produce an extracellular matrix is one of the main challenges for tissue engineering. In the present study, the comparative analysis of two protocols for the preparation of the acellular dermal matrix for the subsequent creation of tissue-engineering skin was performed.

MATERIALS AND METHODS

All the experiments were carried out in accordance with the Rules for the use of experimental animals (order of the USSR Ministry of Health No. 755 of 12.08.1972) and the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (Strasbourg, 1986), after the approval of the research protocols by local ethics committee. The experiments were performed in the Central Research Laboratory of fundamental studies in the field of regenerative medicine of the Kuban State Medical University. The native dermis sampling took place under sterile operating conditions in the vivarium. The experimental animal was one Landrace breed porcine (age 12 weeks, weight 22 kg). After pretreatment of the skin with a dermatome, 0.3 cm thick dermis samples were taken.

Decellularization of the dermal matrix with subsequent assessment of the skin matrix quality

Two different processing protocols were proposed for the dermis decellularization. At the first stage, 2×1×0.3 cm samples were frozen at –80 °C, followed by 18 h treatment with Trypsin–Versene solution (1:1, Biolot, Russia) in a thermo-shaker-incubator at +37 °C. The next step was the processing of the matrix with detergent solutions on a rotating platform at 170 rpm. For protocol No. 1, an alternation of 1% solution of X100 triton (Sigma-Aldrich, USA) and 4% sodium deoxycholate solution (Sigma-Aldrich, USA) in combination with 0.002 M Na2-EDTA, 2 h each was used. There were five treatment cycles. The detergent treatment in Protocol No. 2 included only 4% sodium deoxycholate solution in combination with 0.002 M Na2-EDTA for 4 h, the number of cycles was also five. After each treatment cycle, the matrices were washed in deionized water for 30 minutes. The final processing stage in both protocols consisted of exposure to porcine pancreatic DNase I (Sigma-Aldrich, USA, 2000 IU was dissolved in 200 ml of phosphate-saline buffer Ca^{2+}/Mg^{2+}) for 4 h in a thermal shaker-incubator at +37 °C.

The obtained samples of native and decellularized dermis were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin by the standard technique. 4 μm thick sections were obtained with a microtome. For a general histological evaluation of the preparations, the sections were hematoxylin and eosin stained (Sigma-Aldrich, USA). Cell nuclei were visualized using fluorophore (4',6-diamidino-2-phenylindole) DAPI (Sigma-Aldrich, USA). Micropreparations were studied with Olympus CX 41 microscope (Japan).

DNA content quantification was determined with NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., USA) with (Dneasy Blood and Tissue Kit, Qiagen, Sweden) reagent kit according to the manufacturer's protocol.

Recellularization of the dermal matrix followed by assessment of biocompatibility with cells *in vitro*

To assess the biocompatibility and the ability of the cells to adhere to the dermal matrix, the static recellularization of dermal fibroblasts isolated and cultured from porcine dermis was used. A standard enzymatic protocol based on 0.1% collagenase solution was used to isolate cells from the dermis. Cells were cultured using complete culture medium consisting of DMEM solution (Gibco), 10% fetal bovine serum (Gibco) and 1% solution of antibiotic-antimycotic (Gibco) in a CO_2 incubator. With the confluence achieved, up to 80% of the cells were subcultured with Trypsin solution (Biolot, Russia) and continued to cultivate until the 2nd passage. Then, the obtained number of cells was used for the XTT test and the test for differential staining of living and dead cells. An XTT reagent working solution was prepared in accordance with the manufacturer recommendations (Cell proliferation assay XTT, AphliChem GmbH, Germany). Initially, 0.3×0.3 cm samples of decellularized dermal matrices were placed in a 96-well plate, then the samples were seeded with fibroblasts suspension at the rate of 25,000 cells per scaffold with a pipettor. After 72 h of cultivation, 200 μl of XTT working solution was added to each well, with the incubation time of 4 h. The cells that were freely cultured in the plate were taken for a positive control. The results were evaluated with FilterMax F5

multifunctional reader (Molecular Devices, USA) under standard conditions at 450 nm wavelengths according to the preset protocols of the device manufacturer.

To visually assess the ability of the matrices to support cell growth and their cytotoxicity, the living and dead cells were stained differentially using fibroblasts for the decellularized scaffolds of the dermis with LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes, USA) with AM calcein and ethidium homodimer according to the manufacturer instructions. The micropreparations were studies with Olympus IX 51 fluorescence microscope (Japan).

Statistical analysis

The obtained material was statistically processed with MS Excel v6.0, GraphPadPrism version 6.04 (www.graphpad.com). The results were assessed by Student's t-test. The confidence interval was calculated according to the Student's distribution table. Significant differences correspond to $p < 0.05$.

RESULTS AND DISCUSSION

The detergent-enzymatic method was chosen for the decellularization of the dermis, which allows the most complete removal of cells from tissues, while time sparing the extracellular matrix fibers with respect to proteins. The difference in the composition of the protocols was dictated by the desire to reduce the time of exposure of tissues to sodium deoxycholate and to evaluate the difference in the effect of exposure in comparison with the protocol that uses the milder Triton X100 detergent on the extracellular matrix. At the start of finalizing the protocols, one of the reagents of choice at the preparatory stage was sodium dodecyl sulfate solution. However, the high concentration of the substance necessary for the destruction of the cells of the papillary dermis had a significant damaging effect on the extracellular matrix fibers. The inability to completely eliminate the active solution from the matrix led to significantly worse cell growth and to their death. The obtained data forced us to abandon the further use of sodium dodecyl sulfate solution, which corresponded to the literature data [7].

Also, at the preparatory stage, we encountered the dermis taken simultaneously with the epidermis was less susceptible to decellularization. Perhaps, this is due to the complexity of detergent penetration through the thick epithelial layer and, undoubtedly, the dense basement membrane [8]. To solve the arisen complexity of skin decellularization was to use Trypsin–Versene solution, which, according to the literature, effectively acts on squamous cells, removing them, while loosening the dermis itself and making it more susceptible to detergents.

Hematoxylin and eosin staining after each cycle of detergent treatment showed that the preserved cells and cell nuclei in the matrix were absent already after the 2nd treatment cycle in protocol No. 1 and after the 1st treatment cycle in protocol No. 2. However, a large number basophilically stained matrix filaments present in the sections and a high quantitative DNA content in tissues dictated the need to increase the number of treatment cycles to five in both cases (Fig. 1).

The trend was confirmed by the DAPI fluorophore staining of the nuclear material of the preparations. In the native dermis, cell nuclei actively fluoresced and were detected in large numbers. In decellularized samples, only a slight autofluorescence of extracellular matrix fibers was found after the fifth treatment cycle (the data not shown).

Determination of residual DNA content is an important stage in the evaluation of the resulting matrix after the performed decellularization. The development of adverse reactions on the part of the recipient's organism depends on the DNA presence, since many decellularized tissues are obtained from xeno- and allogeneic sources and there are reasons to concern that this DNA may be included in the recipient's cells. Quantitative

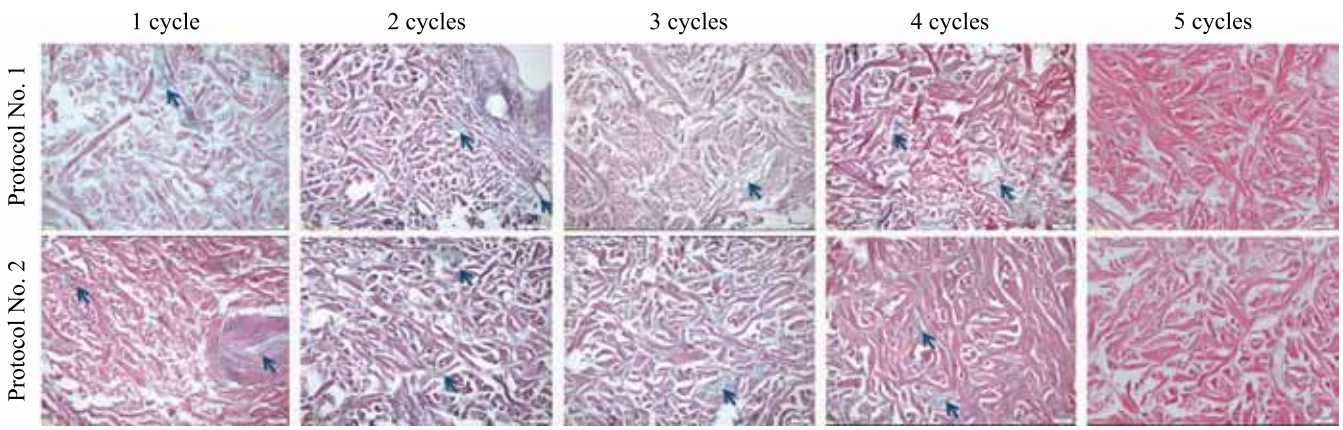

Fig. 1. Histological analysis. Dynamics of successive changes in the structure of the extracellular matrix during decellularization. Pointers indicate basophilically colored bands containing residual nuclear material. ×200

analysis showed that the DNA content in the decellularized dermis after the 5th treatment cycle decreased to 19.8% (65.4 ng/mg of the tissue) and 12.1% (40.1 ng/mg of the tissue) according to protocols Nos. 1 and 2, respectively, in comparison with the DNA content in the native dermis (330.4 ng/mg of the tissue). The obtained results testified to the effectiveness of decellularization, after which the matrix was largely ($p = 0.0011$) purified of nuclear material (Fig. 2).

After the main criteria for evaluating the effectiveness of decellularization proposed in the literature [9] were considered, a necessary condition for choosing the optimal protocol was to assess the effect of the resulting matrix on the cells after recellularization. The significance of this stage is that it allows to select the matrix that will best promote the adhesion and proliferation of cells while being the least toxic to tissues due to the possible preservation of residual detergents.

The XTT test after static matrix recellularization showed living, metabolically active cells in both test samples. However, cell viability was higher in the matrix decellularized according to the protocol No. 1 in contrast to the protocol No. 2, where viability was statistically lower ($p < 0.005$).

When comparing the samples from the experimental and control groups, it was found that the results of the XTT test in wells seeded with cells only differ from the experimental ones and significantly exceed them ($p < 0.001$), which is explained by the experimental conditions and the proven cells ability to easily attach to plastic. In general, when comparing all groups according to the XTT test results, it was found that the matrices

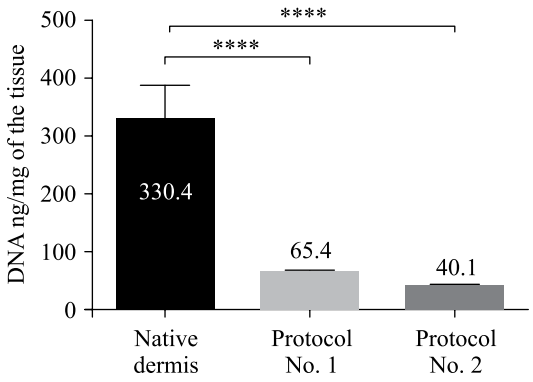

Fig. 2. Quantitative analysis of the DNA content in the native and decellularized porcine dermis

obtained according to protocols Nos. 1 and 2, seeded with cells, are biocompatible and not cytotoxic (Fig. 3).

Differential detection of living (calcein fluorescence) and dead (ethidium homodimer fluorescence) cells based on active calcein transport into living cells and passive ethidium homodimer transport into dead cells, allowed additional visualization of living cells at the static scaffold recellularization (Fig. 4). Cultured cells were found to retain their viability on both matrices obtained for 72 h. It was shown that 80 ± 10% of the cells remained viable on matrix No. 1 and 55 ± 10% on matrix No. 2. The formation of a monolayer of living cells during cultivation on the matrix obtained according to the protocol No. 1 was also noteworthy. These results showed not only the ability of cells to adhere, but also low cytotoxicity of the resulting scaffolds. The difference in results can be associated with a twofold difference in the time of treatment with sodium deoxycholate solution and, as

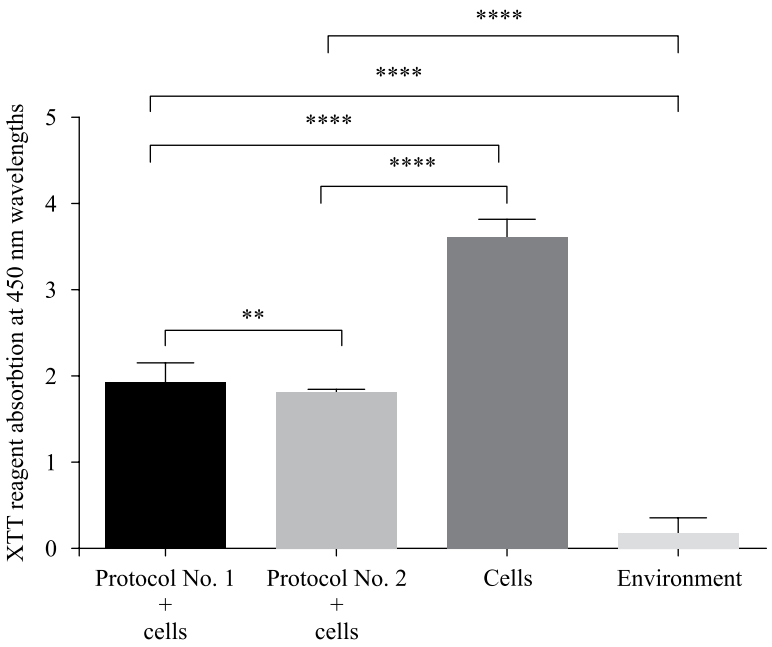

Fig. 3. Results of cytotoxic tests of biological matrix of porcine dermis. Optical density during the XTT test

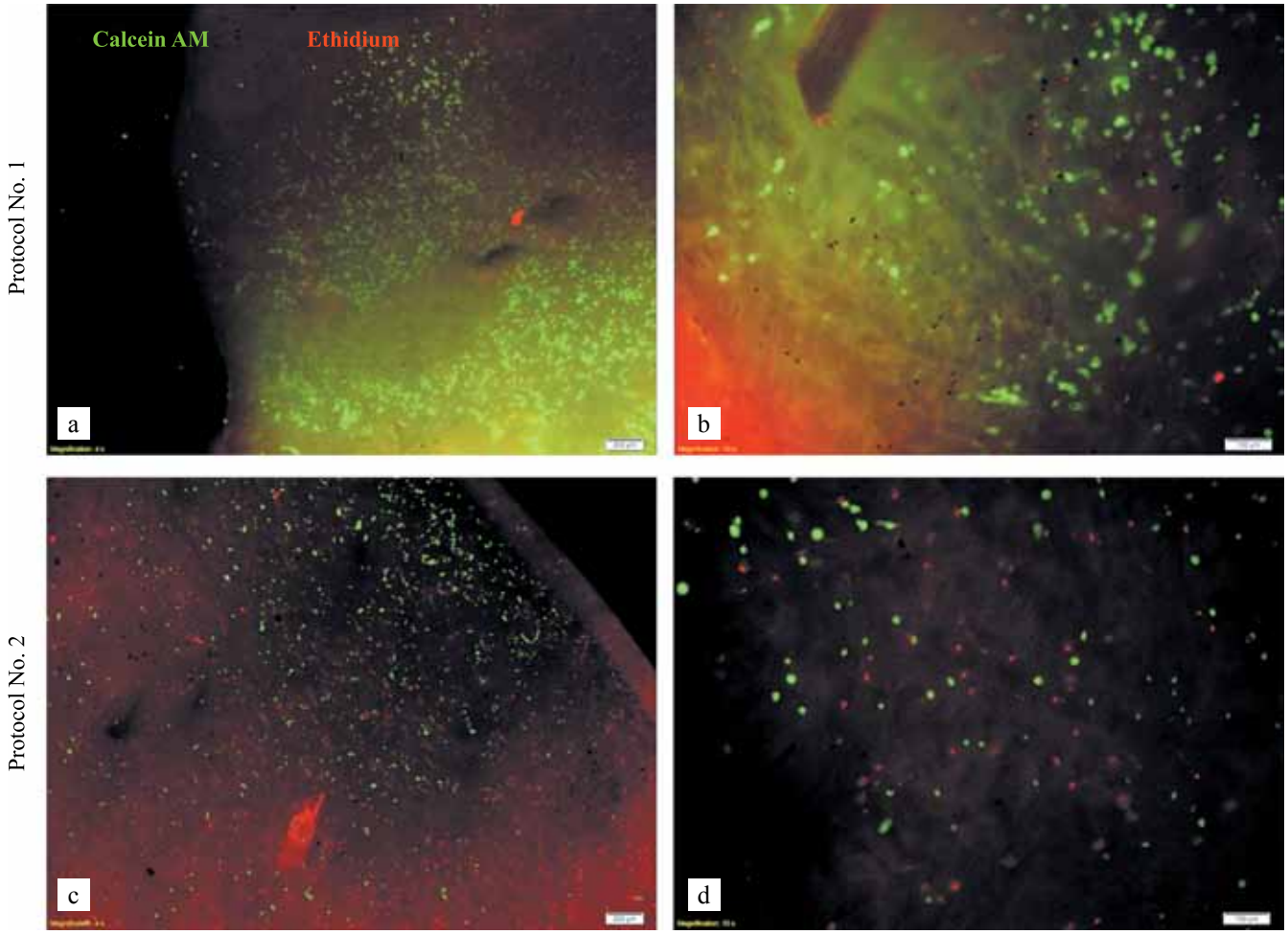

Fig. 4. Evaluation of the viability of fibroblasts settled on decellularized matrix of porcine dermis. Living cells – positive calcein AM (green) staining, dead ones – positive ethidium homodimer (red) staining. a, c – ×40; b, d – ×100

a result, its lower residual content in the matrix when the protocol No. 1 was implemented.

Thus, a comparative analysis of two protocols for porcine dermis decellularization showed that both protocols effectively remove cells and nuclear material, while the architectonics of the intercellular substance remains intact since fibrous structures are not destroyed. However, when assessing the biocompatibility of matrices based on the analysis of cell viability and their adhesion to the matrix, the matrix processed according to the protocol No. 1 had the advantages which made us note that the protocol based on the use of Triton X100 and deoxycholate is more promising for further research, considering Triton X100 is a softer detergent for decellularization.

CONCLUSION

The search for the optimal tissue decellularization protocol is fundamental in tissue engineering, which is primarily conditioned by the development of a matrix that would maximally correspond to the native extracellular matrix, at all levels biocompatible and actively functional. In the present study, the determination of the decellularization method and the initial characteristics of the resulting matrix is the first step for the further development of tissue-engineering skin.

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The authors declare no conflict of interest.

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