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COMPARATIVE ANALYSIS OF PROTOCOLS FOR DECELLULARIZATION OF CORNEAL LENTICULAR TISSUE

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Shortage of donor corneas is a burning issue in ophthalmology. That is why there is a search for new alternative ways for treating corneal diseases. Decellularization technologies make it possible to create corneal tissue-engineered constructs that can adrress the issue of donor corneal shortage. **Objective:** to conduct a comparative analysis of effective methods for treating the corneal lenticula and to create an optimized and standardized decellularization protocol. **Materials and methods.** Corneal stromal lenticules obtained after ReLEx SMILE surgery were chosen for the study. Lenticule parameters: thickness 77–120 microns, diameter 6.5 mm. We used 3 protocols for the treatment of lenticules: 1) treatment with 1.5 M sodium chloride with nucleases (NaCl); 2) 0.1% SDS (SDS); 3) treatment with Trypsin-EDTA solution, followed by double washing in a hypotonic Tris buffer solution with nucleases (Trypsin-EDTA). Optical properties of lenticles were determined spectrophotometrically, where the samples before decellularization served as a control. Fluorescence imaging of nuclear material in the original cryosections was performed using Hoechst dye. The state of collagen fiber ultrastructure was assessed by scanning electron microscopy. The quantitative DNA content in fresh lenticules and in lenticules after treatment was analyzed. **Results.** All three decellularization protocols effectively removed nuclear and cellular material; the residual DNA content was <50 ng/mg. However, the Trypsin-EDTA protocol led to significant damage to the extracellular matrix structure, which negatively affected the transparency of corneal tissue-engineered constructs. Transparency of samples for the NaCl protocol was close to native lenticules. Conclusion. To create a corneal tissue-engineered construct, NaCl decellularization protocols appear to be optimized and can be used to treat various corneal diseases.

Keywords: cornea, tissue engineering, decellularization, lenticula.

INTRODUCTION

Technical advances in refractive surgery have led to the development of a femtosecond laser vision correction method using ReLEx SMILE technology (Refractive Lenticule Extraction & Small Incision Lenticule Extraction). During operation, the surgeon first uses a laser to form a disc-shaped portion of the corneal stroma (the lenticule), which is then extracted through a micro incision. Such lenticule parameters as thickness and diameter depend on the initial corrected myopia or myopic astigmatism [1].

Lenticule reimplantation has been shown to restore stromal volume and refractive anomalies after surgery (refractive lenticule extraction), as shown in monkey and rabbit models [2, 3]. Correction of farsightedness and keratoconus with intrastromal lens implantation has also been reported [4–6].

It is worth noting that the current severe shortage of donor material underlies the search for new directions in the treatment of corneal disorders. It is generally recognized that tissue engineering is an alternative to allotransplantation, therefore, the use of lenticules seems to be a promising and effective technique. However, residual cellular components of the lenticules place this graft in the category of true grafts, which, as is known, can lead to rejection reaction.

The modern view of allogeneic transplantation implies the preparation of suitable acellular, non-immunogenic tissues for subsequent transplantation to the recipient. Decellularization is a promising direction in tissue engineering, allowing for maximum removal of cells and genetic material, thereby reducing the risk of graft immunity reaction. However, it should be noted that damage to the extracellular matrix (ECM) should be minimized, since preservation of the framework and structural and functional properties of ECM is fundamental to effective use of decellularized organs and tissues [7, 8]. Various decellularization protocols described in the literature employ physical, enzymatic, and chemical methods to release ECM from cells [9].

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In the studies by Gary Hin-Fai Yam et al., the authors used donor corneas as a source of 70 μm thick stromal lenticules created by femtosecond laser [10]. The authors compared decellularization protocols, both with isolated application of 0.1% sodium dodecylsulfate (SDS), 0.1% Triton X-100, 1.5 M NaCl, and in combination with nucleases of various concentrations. By spectrophotometry, a transmittance similar to the control was obtained in the 1.5 M NaCl group followed by the 0.1% SDS group. When evaluating immunohistochemistry data, it was found that after treatment with 0.1% SDS, there was no luminescence of the nuclei, while in the groups using 0.1% Triton X-100, 1.5 M NaCl and 1.5 M NaCl with nucleases 2 U/ml, luminescence was preserved. DNA content in the 0.1% SDS group was 20.71 ± 4.3 ng DNA per 1 mg dry weight of the sample; the length of extracted DNA fragments was less than 200 base pairs, which corresponded to the indicative criteria for the effectiveness of organ and tissue decellularization suggested by Crapo et al. [11].

In an experiment on rabbits, Chinese researchers created a construct of decellularized lenticules glued together with fibrin glue for carrying out anterior lamellar keratoplasty [12]. These stromal lenticules were extracted from the human cornea by ReLEx SMILE surgery (with ≥100 μm thickness and 6.6 mm diameter). The treatment protocol used was 1.5 M NaCl with 5 U/ml DNase and 5 U/ml RNase solution. This protocol was borrowed from the work of Shafiq et al, where the authors reported its successful application on whole donor human corneas for cell removal [13].

In a recent study, Huh M.I. et al. used donor corneas from which stromal lenticules were extracted using ReLEx SMILE (100 μm thickness, 8 mm diameter). Decellularization protocols used included washing in Triton X-100, SDS or trypsin-EDTA solution under various concentrations: 0.1%, 0.25%, 0.5%. Then washing was done in hypotonic, isotonic, and hypertonic tris-buffer solutions followed by washing in nuclease solution. Finally, the wash cycle was repeated in Tris-buffer solutions [14].

Of the protocols, decellularization with hypotonic 0.25 and 0.5% trypsin-EDTA followed by washing in hypotonic tris-buffer with nucleases showed the lowest DNA contents. Spectrophotometry revealed that the group using 0.5% trypsin-EDTA and hypotonic Tris-buffer had the best transparency. Scanning electron microscopy data in this group revealed that the ECM after treatment was undisturbed.

Thus, we can conclude that there are many corneal lenticular decellularization protocols today. However, despite all the diversity, there is still no clear understanding of the preferred protocol.

PURPOSE OF THE STUDY

In our study, we compared effective lenticular treatment methods in order to optimize and standardize the decellularization protocol.

MATERIALS AND METHODS

Before the Smile operation, voluntary informed consent for further use of donor lenticular tissue was obtained from all patients. The mean age of patients was 27.3 ± 5.4 years. All patients underwent SMILE surgery for myopia and complex myopic astigmatism. The spherical equivalent before the SMILE surgery was –4.72 ± 0.86 diopters. For decellularization, lenticules with 77–120 μm thickness and 6.5 mm diameter were used.

The material was taken in the operating room at the head office of Fyodorov Eye Microsurgery Federal State Institution in Moscow. The ReLex SMILE eye surgery was carried out under local drip anesthesia using a VisuMax femtosecond laser (500 kHz pulse frequency, 160 nJ pulse energy). First, the base of the lenticule was formed, and then its "lid". The lenticule diameter was 6.5 mm, while the lid diameters were 7.5 mm and 7.6 mm. The resulting lenticule was transferred into a pre-prepared vial containing dispersed viscoelastic (DV) containing 3.0% sodium hyaluronate and 4.0% chondroitin sulfate weighing 600,000 Daltons. Next, the vial with samples was transferred into a container and transported to the laboratory at the Center for Fundamental and Applied Biomedical Problems, Fyodorov Eye Microsurgery Federal State Institution. In the laboratory, studies were carried out under sterile conditions in vitro (n = 145). Table 1 shows the distribution of lenticules in the experiment.

Table 1

Distribution of lenticules by type of work

Lenticule assessment methods	Number of lenticules
Spectrophotometry	45
Histology	20
Immunohistochemistry	20
Scanning electronic	20
Microscopy	40
DNA analysis	145

Decellularization of the lenticule

Before decellularization, the lenticules were washed of DV in PBS solution (three times) for 5 minutes each. Three solution variants were used for decellularization. They differed in components, exposure time, and concentration. As a result, four groups were formed, where

three groups were experimental and one was the control (Table 2).

Transparency of native and decellularized lenticules

Spectral transmission of lenticule from 380 to 780 nm wavelength was determined using a Multiskan GO spectrophotometer (Thermo Scientific, USA); data were collected in 10 nm increments. Spectral transmission for the same samples was performed in two stages. At the first stage, native lenticules were measured – the control group. At the second stage, the transparency of the three experimental groups after treatment was investigated.

According to literature, glycerol (glycerin) is used to eliminate nonspecific lenticular edema after decellularization. In this study, DV approved for clinical use in ophthalmology was used for this purpose. After dehydration in DV for 1 hour, the samples were transferred to a 96-well plate starting from the second well, with the first well containing no lenticule. A volume of 250 μl of DV was added to each well and evenly distributed along its bottom. Then the 96-well plate was inserted into a spectrophotometer chamber to measure the transmittance K_p (%). As a calculated value for statistical analysis of the data, we used the average for the entire group of 41 transparency points of the obtained spectrum. For this, K_o was first calculated as follows:

$$K_o = \frac{K_1}{K_2} \times 100\ \%,$$

where K_o – transmittance for each of 41 spectral points within one sample in the group; K_1 – transmittance in the well containing the sample; K_2 – transmittance in the well not containing the sample.

K_c was then calculated as follows:

$$K_c = \frac{\Sigma K_o}{N},$$

where K_c – average transmittance for each of 41 spectral points of all samples in the group; ΣK_o – aggregate value of measurements of each sample in the group; N – total number of samples in the group.

K_p was then calculated as follows:

$$K_п = \frac{\Sigma K_c}{41},$$

where K_p – average transmittance for all 41 spectral points of the group as a whole; ΣK_c – aggregate value of the average transmittance; 41 – number of spectral points, which corresponds to a 380 to 780 nm wavelength, in 10 nm increments.

Histological evaluation of native and decellularized lenticules

The lenticules were fixed in a 10% solution of neutral formalin, then washed with running water, dehydrated in alcohols of ascending concentration, and embedded in paraffin. Next, a series of 2–3 μm thick histological sections were performed using hematoxylin and eosin stain, Van Gieson's stain, and alcian blue stain to assess the content of glycosaminoglycans in the tissues. The preparations were studied on an ix81 inverted microscope (Olympus, Japan) at 40× magnification, followed by photographing.

Using immunohistochemistry (IHC), we studied the expression of collagen types I, III, V and VI, characteristic of the corneal stroma and being the main ECM component. For this purpose, native and treated samples were first placed in Shandon Cryomatrix medium (Ther-

Table 2

Description of decellularization protocols

№	Active components	Description of method
1	Native lenticules	
2	0.1 % SDS (SDS)	Incubation in 0.1% sodium dodecyl sulfate (SDS) solution (Sigma-Aldrich) for 24 hours at room temperature. Samples were then washed in phosphate-buffered saline (PBS) with continuous shaking in a shaker and at 4 °C temperature, with PBS replacement every 24 hours
3	1.5 M NaCl + DNAase 5 U/ml and RNase 5 U/ml (NaCl)	Incubation in 1.5 M sodium chloride solution for 48 hours, with replacement of NaCl solution every 24 hours. Samples were then incubated in a DNase 5 U/mL (Sigma-Aldrich) and RNase 5 U/mL (Sigma-Aldrich) solution for 48 hours. Samples were then washed in PBS solution for 72 hours, with replacement every 24 hours. The treatment procedure was carried out at room temperature and with continuous shaking in a shaker
4	0.25% Trypsin-EDTA (Thermo fisher) + hypotonic Tris buffer solution (pH 7.2) + DNAase 50 U/mL and RNase 1 U/mL + hypotonic Tris buffer solution (Trypsin-EDTA)	Incubation in 0.25% Trypsin-EDTA solution for 48 hours, then 1 hour in a hypotonic Tris buffer solution (pH 7.2), then in a DNase 50 U/mL and RNase 1 U/mL solution for 24 hours. The samples were then washed in hypotonic Tris buffer solution (pH 7.2) for 1 hour. The decellularization procedure was performed at 37 °C temperature and with continuous shaking in a shaker

mo scientific, uk) and frozen at –30 °C temperature in an HM525 NX cryostat (Thermo scientific, UK). Then 10 μm-thick cryostat sections were made and transferred to Polysine slides (Thermo Scientific, UK), at the rate of four sections per slide. The following primary antibodies were used: type I collagen (Rabbit 1:200, ab34710, Abcam), type III collagen (Mouse 1:100, ab6310, Abcam); collagen type V (Rabbit 1:100, ab114072, Abcam); collagen type VI (Rabbit 1:200, ab6588, Abcam). Secondary antibodies Alexa Fluor 488 (1:250, ab150077, Goat Anti-Rabbit IgG, Abcam) and Alexa Fluor 594 (1:250, ab150116, Goat Anti-Mouse IgG, Abcam) were used to identify the above markers. After removal of secondary antibodies, the Hoechst dye (O150, PanEko) was used to assess nuclear staining. The results were evaluated using a laser scanning confocal microscope "Fluo View FV10i" (Olympus, Japan) 100×.

Evaluation of the ultrastructure of collagen fibers by scanning electron microscopy

Samples were dehydrated in acetone solution at an ascending concentration of 10%; 30%; 50%; 70%; 90%; 100% (three times) for 10 minutes each. The samples were then subjected to critical drying using a desiccant dryer (Critical Point Dryer Qurum k850, Quorum Technologies, UK). Then the samples were sputtered with gold (5 nm layer thickness, assay 999) using a sputtering device (Smart Coater SPI, SPI Supplies, USA) and analyzed using a scanning electron microscope – 6000plus (Jeol, Japan). The samples were analyzed at ten randomly selected points in a high vacuum mode 1000× (10 kV power).

Measurement of DNA content

DNA was extracted from samples using the DNeasy Blood & Tissue Kit (QIAGEN, Germany) according to the manufacturer's recommendations. The DNA content was counted using a Qubit 2.0 fluorometer (Invitrogen, USA) and a Qubit dsDNA HS (High-Sensitivity) Assay Kit (Invitrogen, USA). Measurement was carried out according to the manufacturer's instructions.

Statistical analysis

The mean with standard deviation (M ± SD) and median with interquartile range (Me (1–3 quartiles)) were used as descriptive statistics for variables. Normality of distribution of variables was assessed using the Shapiro–Wilk test. Homogeneity of variances was assessed using the Bartlett's test.

Independent samples were compared using the Kruskal–Wallis test followed by Dunn's post-hoc test or Welch's t-test for non-normally and normally distributed samples (with heterogeneous variances), respectively. In all cases, the Holm's correction for multiple comparisons was used. Results were considered statistically significant at $p < 0.05$.

Data was statistically processed using statistical computing environment R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). The data was visualized using GraphPad Prism 8.4.3 (GraphPad Software, Inc., USA).

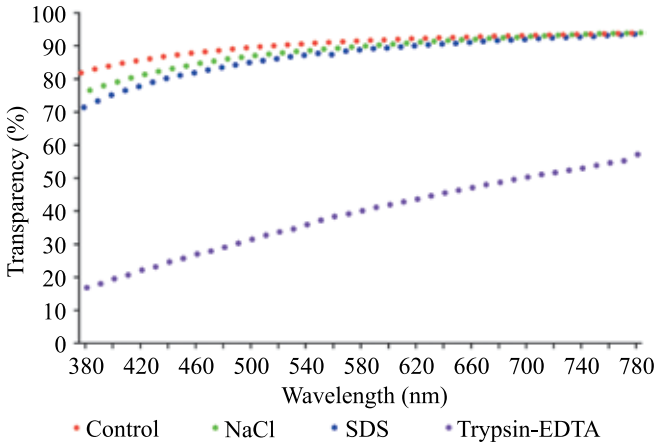

Fig. 1. Average spectral transmittance (%) at 380 to 780 nm wavelength

RESULTS

Transparent properties of the lenticule

The study showed that transparency was significantly reduced in the Trypsin-EDTA group compared to the control group, and the results in the NaCl group were the closest to the control (Fig. 1). There were statistically significant differences in groups SDS/Control (86.85 ± 3.34 vs. 90.39 ± 5.11; (87.46 (84.33–89.63)) vs. (91.49 (86.64–93.80)); $p < 0.03$); Trypsin-EDTA/Control (38.70 ± 8.78 vs. 90.39 ± 5.11; (43.74 (33.56–44.86)) vs. (91,49 (86,64–93,80)); $p < 0.0001$); also when comparing NaCl/Trypsin-EDTA (88.63 ± 2.56 vs. 38.70 ± 8.78; (89.12 (88.59–90.06)) vs. (43,74 (33,56–44,86)); $p < 0.0001$); SDS/Trypsin-EDTA (86.85 ± 3.34 vs. 38.70 ± 8.78; (87.46 (84.33–89.63)) vs. (43.74 (33.56–44.86)); $p < 0.003$).

Histological staining of lenticules

Histological staining with H&E, Van Gieson and alcian blue revealed complete removal of cells, cell nuclei and the absence of significant structural damage in the resulting acellular matrix in the SDS and NaCl groups. In the Trypsin-EDTA group, there was noticeable significant damage to the matrix structure against the background of complete absence of cells and cell nuclei (Fig. 2).

Immunohistochemical staining of lenticules

Fluorochromation of nuclei with Hoechst dye showed that the lenticules that were treated stained negatively. Meanwhile, a characteristic luminescence of the nuclei was determined in the control group. Also, according to IHC data, there was positive expression of collagen types I, III, V and VI in the control and experimental groups (Fig. 3).

Results of scanning electron microscopy

When analyzing the thickness of collagen fibers of the samples, differences were found both between the experimental groups and the control, as well as when comparing the experimental protocols in pairs with each other. In the NaCl/Control pair comparison, the thickness was (1.96 ± 0.46 vs. 2.30 ± 0.40; (1.9 (1.69–2.21)) vs. (2.26 (2.03–2.63)); p = 0.00013). Groups SDS/Control (1.36 ± 0.25 vs. 2.30 ± 0.40; (1.36 (1.21–1.47)) vs. (2.26 (2.03–2.63)); p < 0.0001). For Trypsin-EDTA/Control protocols (4.19 ± 0.56 versus 2.30 ± 0.40; (4.13 (3.85–4.54)) versus (2.26 (2.03–2.63)); p < 0.0001). Pairwise comparison of the protocols for NaCl, SDS, Trypsin-EDTA between each other revealed statistically significant differences (p < 0.0001). In Fig. 4, it can be seen that the SDS group is mainly represented by frayed fibrils, due to lack of sufficient volume of collagen fibers. In the Trypsin-EDTA group, there is a noticeable thickening of collagen fibers, which is caused by gross disturbance of their ultrastructure. Slight changes in the state of collagenous fibers were detected in the NaCl group.

DNA analysis

Pairwise comparison of groups by Welch's t-test (with Holm's correction) revealed a statistically significant difference, both between the experimental groups and the control, and between the experimental groups: NaCl/Control (39.34 ± 8.65 vs. 132.18 ± 44.17; (41.99 (38.57–44.67)) vs. (128.5 (102.02–154.48)); p < 0.0002); SDS/Control (37.07 ± 6.19 vs. 132.18 ± 44.17; (39.74 (36.72–42.75)) versus (128.5 (102.02–154.48)); p <

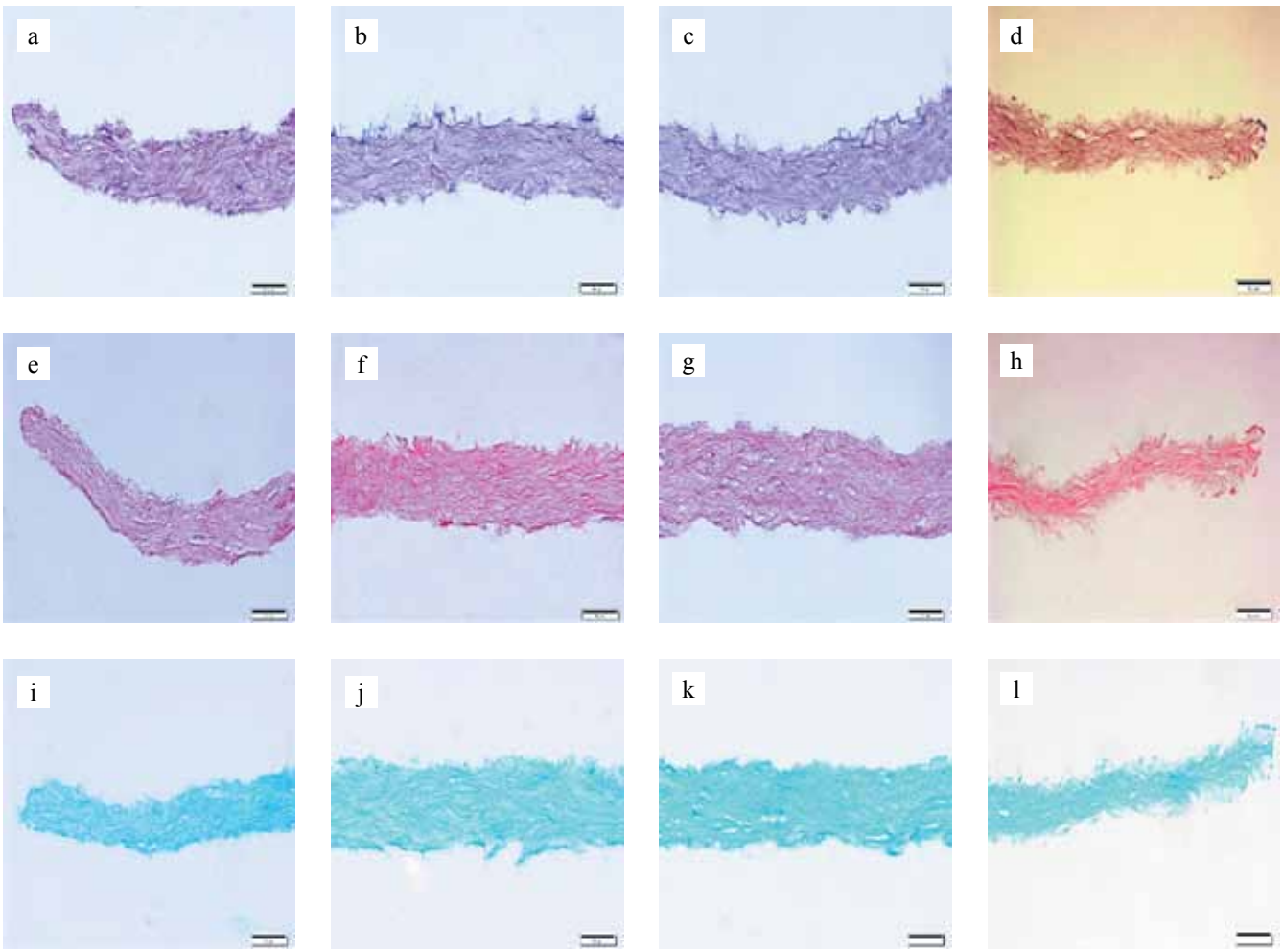

Fig. 2. Histological picture of native and decellularized lenticules. H&E stain: а – control, б – NaCl, в – SDS, г – Trypsin-EDTA; Van Gieson's stain: д – control, е – NaCl, ж – SDS, з – Trypsin-EDTA; Alcian blue stain: и – control, к – NaCl, л – SDS, м – Trypsin-EDTA; ×40

0.0001); Trypsin-EDTA/Control (13.42 ± 7.4 versus 132.18 ± 44.17; (11.45 (8.47–16.86)) versus (128.5 (102.02–154.48)); $p < 0.0001$). Comparison of experimental groups with each other revealed statistically significant differences for the NaCL/SDS groups ($p < 0.002$), for the SDS/Trypsin-EDTA groups ($p < 0.0006$), and for the NaCl/Trypsin-EDTA groups ($p < 0.0001$).

DISCUSSION

At the moment, there is an active search for an effective tissue-engineered corneal construct (TECC) as an alternative to donor corneal transplantation in conditions of severe corneal shortage.

Developing a decellularization protocol is a complex and routine technique. There are many detailed descriptions in the literature of various decellularization protocols for such organs as small intestine, pericardium, heart valves, liver, skin, bladder, and cornea [15, 16]. The effectiveness of decellularization mainly depends on the type of tissue. Unlike thicker organs or tissues, corneal tissue is thinner in thickness and has a specific structural organization, whose preservation is an extremely important condition for decellularization [17]. In this study, we used corneal lenticular tissue obtained during the ReLEX SMILE surgery. To assess decellularization, apart from the decellularization criteria suggested by Crapo et al., we additionally assessed the corneal stromal transparency, the state of collagen fiber ultrastructure and the main ECM components, such as glycosaminoglycans and total collagen [11].

In this study, the SDS and NaCl groups showed better results compared to the Trypsin-EDTA group as part of the pre- and post-treatment transparency assessment. The SDS group was statistically different from the control ($p < 0.005$), in contrast to the NaCl group ($p = 0.524$), which showed transparency results similar to the control group. On histological and immunohistochemical studies, all protocols were effective in removing nuclei. However, significant damage to the main ECM components was observed in the Trypsin-EDTA group, while these ECM components were preserved in the SDS and NaCl groups.

The thickness of the ultrastructure of collagen fibers was found to be highest in the trypsin-EDTA group and lowest in the SDS protocol. NaCl-treated lenticules showed a result closest to that of the control group.

Residual DNA content in all treatment groups was less than 50 ng/mg, which is in line with those suggested

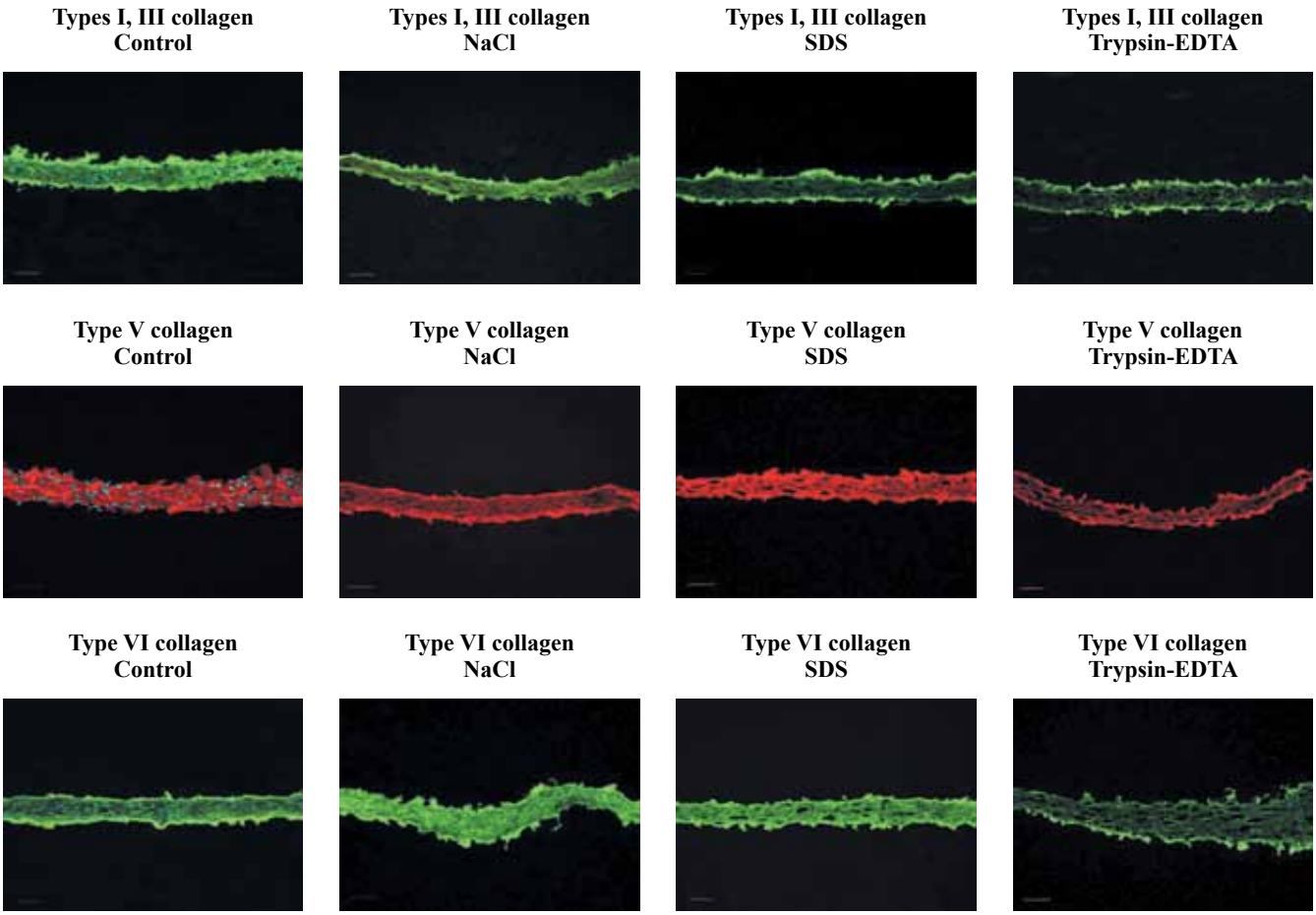

Fig. 3. Immunohistochemistry of native and decellularized lenticules

by Crapo et al. requirements [11]. The Trypsin-EDTA group had the lowest residual DNA content.

Our study did not confirm the data from Huh M.I. et al., where 0.25% and 0.5% Trypsin-EDTA were used with nucleases and double washing in hypotonic buffer solution [14]. We believe that the combined effect of the complex components of this protocol, although it leads to better results in terms of reducing the residual DNA content, nevertheless, is destructive in relation to the ultrastructure of collagen fibers and ECM components. The destructive effect of trypsin and nucleases on the collagen structure of tissues has been reported [18].

The absence of cellular material, the preservation of the collagen structure and the main ECM components are key predictors for TECC. In our study, treatment with 1.5 M NaCl with nucleases showed good results on all evaluation parameters for TECC development.

CONCLUSION

Thus, a comparative assessment of the effectiveness of different decellularization protocols for creating a corneal lenticular matrix creation has led to the creation of optimized and standardized protocols. The results presented in this work open up wide opportunities for the future use of TECC. However, the issue of TECC data storage is still unresolved and requires separate future research in this direction.

The authors declare no conflict of interest.

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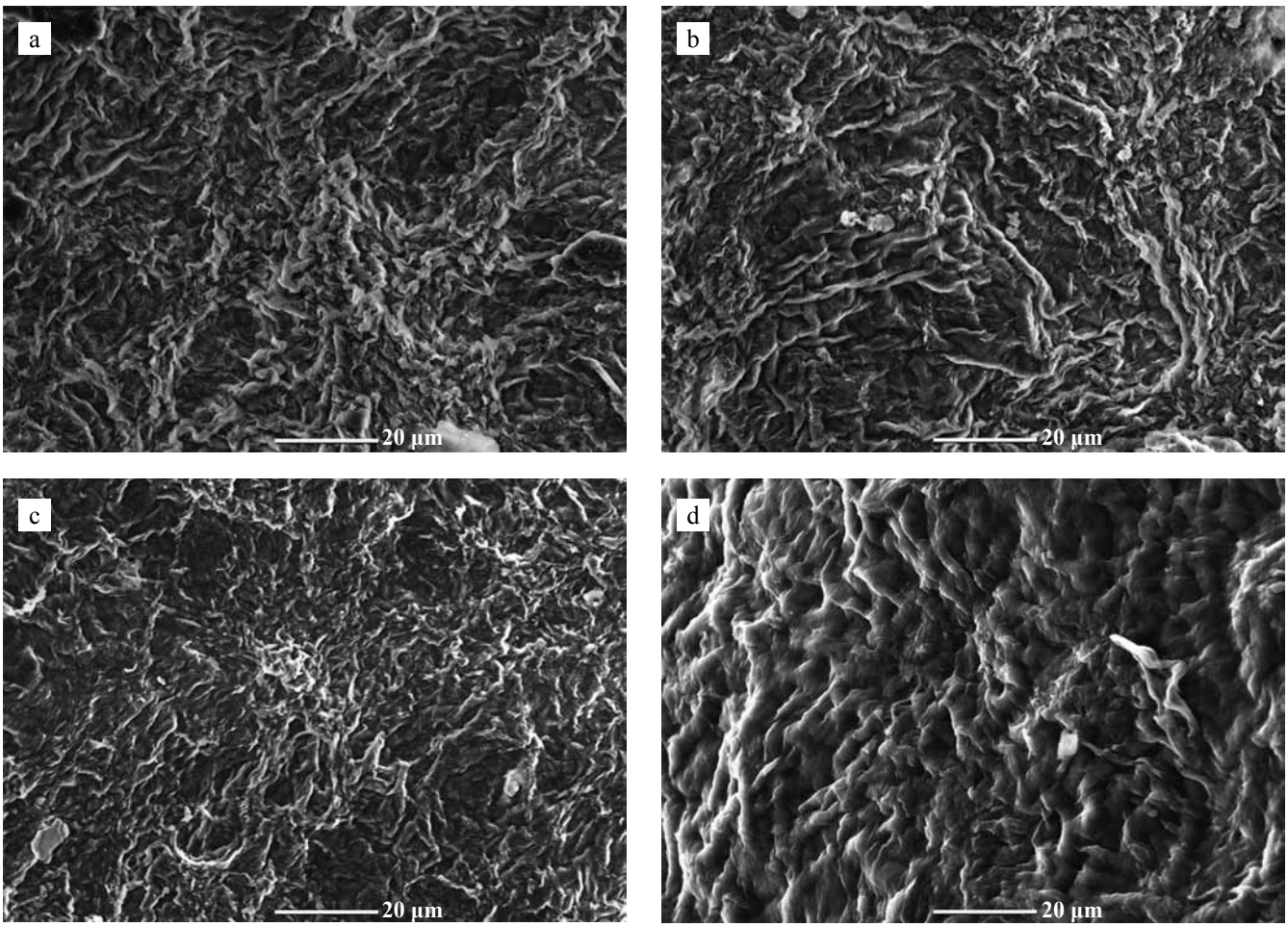

Fig. 4. Collagen ultrastructure of the ECM, where a is the Control, b is NaCl, c is SDS, and d is Trypsin-EDTA. Scanning electron microscopy 1000×

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