Cryopreservation of an artificial human oral mucosa stroma. A viability and rheological study ☆

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A R T I C L E I N F O

A B S T R A C T

The aim of this study was to evaluate the viability and biomechanical properties of artificial human oral mucosa stroma (HOMS) subjected to cryopreservation with different cryoprotectant solutions.

Artificial HOMS based on a fibrin–agarose matrix with human gingival fibroblasts cultured 7 days in vitro were cryopreserved with three cryoprotectant solutions: (A) TC-199 Medium, DMSO 15%, albumin; (B) DMEM, FCS, DMSO 10%; (C) QC Medium, glycerol. As controls, artificial HOMS not subjected to cryopreservation (CF) and HOMS cryopreserved without cryoprotectant solution (CS) were used. Histological analysis by light microscopy showed that solutions A and B preserved a pattern of porosity similar to values in CF. Based on the number of intact cells in the fibrin–agarose matrix, substitutes preserved with solution B showed the best results. Cell proliferation detected with PCNA immunochemical methods showed that the cell proliferation index was highest in substitutes cryopreserved with solution B. The reculture method and cell viability analyses with Live & Dead® revealed increased number of viable in cells preserved with solution B. Artificial stroma substitutes in CS control samples showed the greatest alterations in microstructure and cell proliferation. Analysis of the biomechanical properties showed that substitutes cryopreserved with different solutions had adequate rheological parameters (yield stress, elastic modulus and viscous modulus) and were therefore suitable for use in regenerative medicine. These results establish effective methods of cryopreservation for all experimental situations and suggest that solution B (DMEM, FCS, DMSO 10%) was the best cryoprotectant for the cryopreservation of an artificial oral human mucosa substitute based on a fibrin–agarose matrix.

Introduction

The surgical management of several clinical situations in dentistry such as gingival recession, leukoplakia with a focus of carcinoma in situ, jaw epidermoid carcinoma or salivary gland adenocarcinoma often requires regeneration or replacement of the oral mucosa [24,26]. One method of surgical management for gingival recession is the use of autologous subepithelial connective tissue [16,28], especially for cosmetic purposes, management of root hypersensitivity, shallow root caries lesions and cervical abrasions [33]. This technique has been described for areas of single or multiple root coverage, and basically consists of obtaining connective tissue from the palatal mucosa and using it as a graft to cover and repair the gingival recession. Although the incidence of these conditions is high, this solution has two disadvantages: first, it requires more than one intervention, and second, the oral mucosa wound from which connective tissue is obtained tends to heal by second intention and contract, thus reducing the oral mucosa available for future interventions [10,32]. In this context, the ex vivo preparation of artificial oral mucosa using tissue engineering techniques has shown promising experimental results and may be potentially useful in future clinical applications. Our laboratory previously generated an artificial human oral mucosa model based on fibrin and agarose biomaterials [23,1,11].

The therapeutic use of new tissues generated by tissue engineering requires compliance with biofabrication and biobanking protocols before it can be incorporated into clinical practice. Among the requirements of biobanking is the need to maintain and preserve these artificial tissues during the long term without alterations in their structure and function [13]. One of the methods

used to prevent degradation of the cellular and acellular components of tissues is cryopreservation, which aims to ensure long-term biological and therapeutic suitability [8,19]. Freezing without cryoprotection is lethal for the cells and may disrupt extracellular matrix (ECM) components; this makes it necessary to use cryoprotectant agents [21]. Typically, cryoprotectants are nontoxic water-soluble chemical agents permeable to the plasma membrane that are able to decrease the freezing point [21,20].

Numerous studies have described the use of different cryopreservation protocols for native organs and tissues [29,2,3]. In the field of tissue engineering, few reports have focused on evaluating the effects of cryopreservation on allogeneic dermis substitutes [14], and these studies are limited to bioengineered human oral mucosa models based on collagen biomaterials [27]. However, cryopreservation protocols have not been evaluated for artificial oral mucosa based on fibrin–agarose.

The aim of this study was to evaluate the viability and biomechanical properties of an artificial human oral mucosa stroma (HOMS) model subjected to cryopreservation with different cryoprotectant solutions (A, B and C) based on DMSO with or without albumin and glycerol as cryoprotective agents.

Materials and methods

Generation of artificial HOMS by tissue engineering

Ten normal human oral mucosa biopsies with an average size of $2 \times 2 \times 2$ mm were obtained from healthy donors at the School of Dental Sciences of the University of Granada. All patients gave their consent to participate in the study and the work was approved by the local research and ethics committees. To establish primary cultures of human oral fibroblasts, previously published protocols based on collagenase digestion were used [22]. Briefly, all biopsies were washed in PBS and digested in a 2-mg/mL *Clostridium histolyticum* collagenase I solution (Gibco BRL Life Technologies, Karlsruhe, Germany) for 6 h at 37 °C. Detached fibroblasts were collected by centrifugation and expanded in culture flasks containing Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and 1% antibiotic–antimycotic solution (final concentration 100 U/mL penicillin G, 0.10 mg/mL streptomycin and 0.25 μg/mL amphotericin B) (all from Sigma–Aldrich, Steinheim, Germany). These human oral mucosa fibroblast cell cultures were always used during the first 3 cell passages.

To generate the artificial HOMS, 250,000 cultured fibroblasts were resuspended in 2 mL DMEM with 10% FCS, and 21 mL human plasma was added [1,23]. Human plasma was obtained from human donors at the Tissue Bank of Granada and stored at −80 °C until the moment of use. The mixture was supplemented with 200 μL liquid tranexamic acid (Amchafibrin, Fides-Ecofarma, Valencia, Spain) to prevent degradation of the scaffold by fibrinolysis, and type VIII-agarose (Sigma–Aldrich) was added to the mixture at a final concentration of 0.1%. Finally, 2 mL of a 1% CaCl$_2$ solution was added to induce the polymerization reaction in the fibrin. The mixture was stirred briefly to ensure uniform distribution of cells, then aliquoted in Petri culture dishes (Corning® Inc., NY, USA) and allowed to jellify at 37 °C for 2 h. All HOMS had thickness of 4 mm once jellified.

Cryopreservation protocols

Once generated, artificial HOMS were cryopreserved in the presence of the three cryoprotectant solutions used in the Tissue Bank of Granada to preserve human native tissues, according to previously established protocols at this center for each case. As controls, artificial HOMS not subjected to a process of

cryopreservation (CF) and artificial HOMS cryopreserved without cryoprotectant solutions (CS) were used. The cryoprotectant solutions were:

- *Solution A*: 70 mL TC-199 Medium (Sigma–Aldrich), 15 mL DMSO (Merck, Darmstadt, Germany), 15 mL 20% albumin (Sigma–Aldrich).
- *Solution B*: 70 mL DMEM, 20 mL FCS, 10 mL DMSO.
- *Solution C*: 90 mL QC Medium (consisting of a 3:1 mixture of DMEM and Ham's F12 culture medium supplemented with 10% FCS, 24 mg/mL adenine, 0.4 mg/mL hydrocortisone, 5 mg/mL insulin, 10 ng/mL epidermal growth factor, 1.3 ng/mL triiodothyronine, all from Sigma–Aldrich), 10 mL glycerol (Merck).

Briefly, 7 days after generation, artificial HOMS were transferred to Petri culture dishes, where they were completely covered with a specific cryoprotectant solution at 4 °C. Samples were subjected to the following progressive freezing process: first, samples were maintained on ice for 30 min; then the temperature was lowered to −20 °C during the first 24 h and to −80 °C for 48 h. Finally, samples were transferred to a liquid nitrogen tank where they were stored for a period of 7 days on the vapor phase. After this period, samples were allowed to thaw at 20 °C. After thawing, samples were washed twice in PBS to remove the cryoprotectant solutions and analyzed immediately thereafter. Thirteen independent samples were analyzed per group (cryoprotectant solutions A, B, C and CF and CS controls), and a total of 65 samples were tested.

Histological analysis by light microscopy

For histological analysis by light microscopy, samples 8 mm in diameter were obtained with a sterile skin biopsy punch (Biopsy Punch, Seki, Japan), fixed in 4% buffered formaldehyde, dehydrated and embedded in paraffin. Five-micrometer-thick sections were obtained and stained with hematoxylin and eosin. To determine the preservation level of the artificial HOMS ECM, we quantified the area of the spaces existing in the fibrin–agarose fibrillar mesh, since previous work [25] demonstrated that these spaces tend to increase when ice crystals form and the ECM is damaged by cryofixation. These areas were automatically quantified with MacBiophotonic Image J software. Then the effects of the freezing and thawing protocols on cell morphology were determined by quantifying the number of intact cells per area of tissue. Previous reports noted the need to analyze both the area of the extracellular matrix spaces and the cell morphology to determine the structural integrity of cryopreserved artificial tissues [25,30]. In both cases, 10 random areas of 240 μm^2 (field obtained at 200× magnification) were selected for each sample using a Nikon Eclipse microscope (Nikon, Tokyo, Japan), and the number of intact cells per 100 μm^2 area was quantified.

Cell viability as determined by immunohistochemical study of the cell proliferation marker PCNA

To determine cell proliferation, immunohistochemical analysis of PCNA (proliferating cell nuclear antigen) was used. First, samples were covered with DMEM culture medium and incubated at 37 °C with a 5% CO$_2$ for 24 h. Then the tissues were fixed with 4% buffered formaldehyde and embedded in paraffin. Deparaffinized tissue sections were placed in pH 6 citrate buffer for 40 min at 95 °C for antigen retrieval. Nonspecific antigenic sites were blocked with horse serum (Vector, Burlingame, CA, USA) for 10 min at room temperature. Then the cells were incubated with primary anti-PCNA antibodies diluted in blocking serum (Vectastain Universal Quick Kit) at a dilution of 1:1000 for 60 min at room temperature. The secondary antibody (anti-mouse antibody Pan-specific

Universal Secondary) was incubated for 10 min. Subsequently, samples were incubated in streptavidin horseradish peroxidase. To detect the colorimetric reaction, diamino-benzidine was used, which turns dark brown when oxidized by peroxidase. After this, samples were stained with Mayer's hematoxylin and mounted using glass coverslips. The slides were examined with a Nikon Eclipse microscope with NIS-Elements software (Nikon, Japan) and a DXM 1200C digital camera (Nikon, Melville, NY, USA). To determine the percentage of PCNA positive cells, i.e., cells immuno-stained at their nuclei, we measured 10 areas at a magnification of $200\times$ for each sample.

Cell viability as determined by reculture methods

Once the artificial HOMS were thawed, we extracted a sample 8 mm in diameter from each experimental group using a sterile skin biopsy punch (Biopsy Punch, Seki, Japan). The samples were then sectioned with a scalpel to obtain small tissue explants which were cultured in 15-cm^2 flasks at 37 °C with 5% carbon dioxide for 21 days in DMEM medium with FCS and antibiotics–antimycotics. After this period the Live & Dead® fluorescence method (Invitrogen, Eugene, Oregon, USA) was used to assess cell viability This method contains calcein-AM, which is metabolically modified by living cells to a green pigment, and ethidium homodimer-1, which stains the nuclei of dead cells cells red [12]. Briefly, the culture medium was removed and cells were washed in PBS. Then we added 5 μL ethidium homodimer-1, 1.25 μL calcein-AM and 2.5 mL PBS and incubated this mixture for 5 min in the presence of cells protected from light. The cultures were then examined with a Nikon Eclipse fluorescence microscope with Nikon Ti-HGFI Intensilight C and NIS-Elements software (Nikon, Japan). For each sample 60 areas were assessed at $400\times$ magnification, corresponding to 60 mm^2 per area. Cells were counted with the MacBiophotonic Image J program. To determine the percentage of viable cells, the following formula was used: % viable cells = $100 \times$ number of green fluorescent cells/Total number of cells.

Rheological measurements

We analyzed the biomechanical properties of each sample with a Bohlin CS10 controlled-stress rheometer (Bohlin Instruments, Worcestershire, England), using a plate–plate measuring cell configuration. The plate diameter was 40 mm and the gap between the plates was between 1 and 2 mm depending on sample thickness. All measurements were recorded at 36.0 ± 0.1 °C. To avoid excessive drying of the samples, they were maintained in a water vapor-saturated atmosphere during measurement. Undesired wall-slip phenomena were prevented by using roughened plates as recommended by Barnes et al. [4].

We used two different experimental methods: steady-state and dynamic (oscillatory) measurements. In the former method, a shear stress (σ) ramp from 0.06 to 20 Pa was applied to the samples and the corresponding shear rate ($\dot{\gamma}$) was recorded. The time elapsed between two consecutive steps in the shear ramp was 3 s. The rheograms (σ vs. $\dot{\gamma}$ curves) of all the samples studied were characterized by the existence of a yield stress (σ_y), which is defined as the minimum shear stress required to induce a non-negligible shear rate in the material [4]. Note that for solid-like materials the yield stress can be identified with the stress required to cause fracture (fracture toughness).

In dynamic measurements (oscillatory measurements or simply oscillometry) a sinusoidal shear stress expressed by the equation:

$$\sigma = \sigma_0 \sin 2\pi ft \qquad (1)$$

was applied to the sample, and the corresponding (oscillatory) shear strain,

$$\gamma = \gamma_0 \sin(2\pi ft - \delta) \qquad (2)$$

was recorded. In Eqs. (1) and (2), f (Hz) is the frequency, t is the time (s), α_0 and γ_0 are the stress and strain amplitudes, respectively, and δ the lag between the phase angles of the stress and the strain. From these experiments we can obtain the so-called viscoelastic moduli, which characterize the viscoelasticity of the materials [17]: the elastic or storage modulus (G') and the viscous or loss modulus (G'').

We performed two different kinds of oscillatory tests: (i) amplitude sweep and (ii) frequency sweep. In amplitude sweeps, the frequency was fixed at $f = 1$ Hz and the amplitude of the applied stress, σ_0, was increased within a range wide enough to reach the nonlinear viscoelastic region (NLVR). The NLVR is characterized by a nonlinear response of the viscoelastic moduli (G' and G''), for σ_0 values larger than a critical shear stress amplitude (σ_c). For σ_0 values smaller than σ_c, the viscoelastic moduli are almost independent of σ_0 (a pseudoplateau is usually observed when G' and G'' are plotted as a function of σ_0). This region is commonly known as viscoelastic linear region (VLR).

Once we determined the values of σ_0 pertaining to the VLR, we carried out frequency sweeps. In these measurements the amplitude of the applied shear stress was maintained at a constant value well into the VLR, and a frequency sweep was carried out. As a result, the dependence of the viscoelastic moduli on the frequency was obtained, which represents the characteristic oscillogram of the sample. In the present study the frequency range was limited to a maximum of 7 Hz. This maximum was selected based on work by Ferry [9] in order to keep the influence of sample inertia on the measurements negligible.

Statistical analysis

The Mann–Whitney test was used to identify significant differences between two groups. The Kendall tau statistical test was used to determine the correlation between the number of intact cells per area of tissue and the percentage of proliferating cells as determined by PCNA. All tests were two-tailed and were run with SPSS 15.0 software, and all p values below 0.05 were considered statistically significant.

Results

Structural analysis of cryopreserved artificial HOMS

In this analysis we observed that CF controls showed a homogeneous structure with the presence of small interfibrillar spaces whose average area was 10.84 ± 1.66 μm^2. In contrast, in the control group where no cryoprotectant solution was used (CS), extreme microstructural alterations were observed. In this case mean interfibrillar area was 67.58 ± 11.11 μm^2, which was significantly higher ($p = 0.0001$) than in control CF. In the experimental groups subjected to cryopreservation with cryoprotective agents, a pattern of increased porosity, i.e., an increase in the ECM interfibrillar spaces, was found. In substitutes cryoprotected with solutions A and B (18.23 ± 3.66 and 11.65 ± 1.72 μm^2, respectively), nonsignificant ($p > 0.05$) differences were found compared to control CF samples. In substitutes cryoprotected with solution C, the interfibrillar space was significantly larger than in control CF (29.76 ± 3.27 μm^2, $p = 0.0005$) (Table 1 and Figs. 1 and 2A). Interestingly, statistical analysis demonstrated that the interfibrillar spaces in tissues treated with all three cryoprotectant agents (A, B and C) were significantly different to those in control CS samples with no cryoprotectant ($p = 0.0007$).

Table 1
Results of the analysis of interfibrillar spaces, quantification of cells without structural damage, PCNA expression and recultured cell quantification in controls and samples cryopreserved HOMS. CF, control tissues not subjected to cryopreservation; CS. control tissues cryopreserved without cryoprotectant solution; Sol. A, samples cryopreserved with solution A; Sol. B, samples cryopreserved with solution B; Sol. C, samples cryopreserved with solution C.

| Experimental groups	CF	CS	Sol. A	Sol. B	Sol. C
Interfibrillar spaces (μm^2)	10.84 ± 1.66	67.58 ± 11.11	18.23 ± 3.66	11.65 ± 1.72	29.76 ± 3.27
Quantification of cells without structural damage	10.30 ± 3.40	5.00 ± 5.40	7.10 ± 3.80	9.50 ± 5.60	4.50 ± 4.40
PCNA expression (percentage)	87.02 ± 10.10	0.00 ± 0.00	49.81 ± 21.48	72.41 ± 18.31	38.88 ± 27.59
Recultured cell quantification	531.28 ± 321.86	0.00 ± 0.00	24.63 ± 116.88	102.48 ± 257.38	37.83 ± 127.01
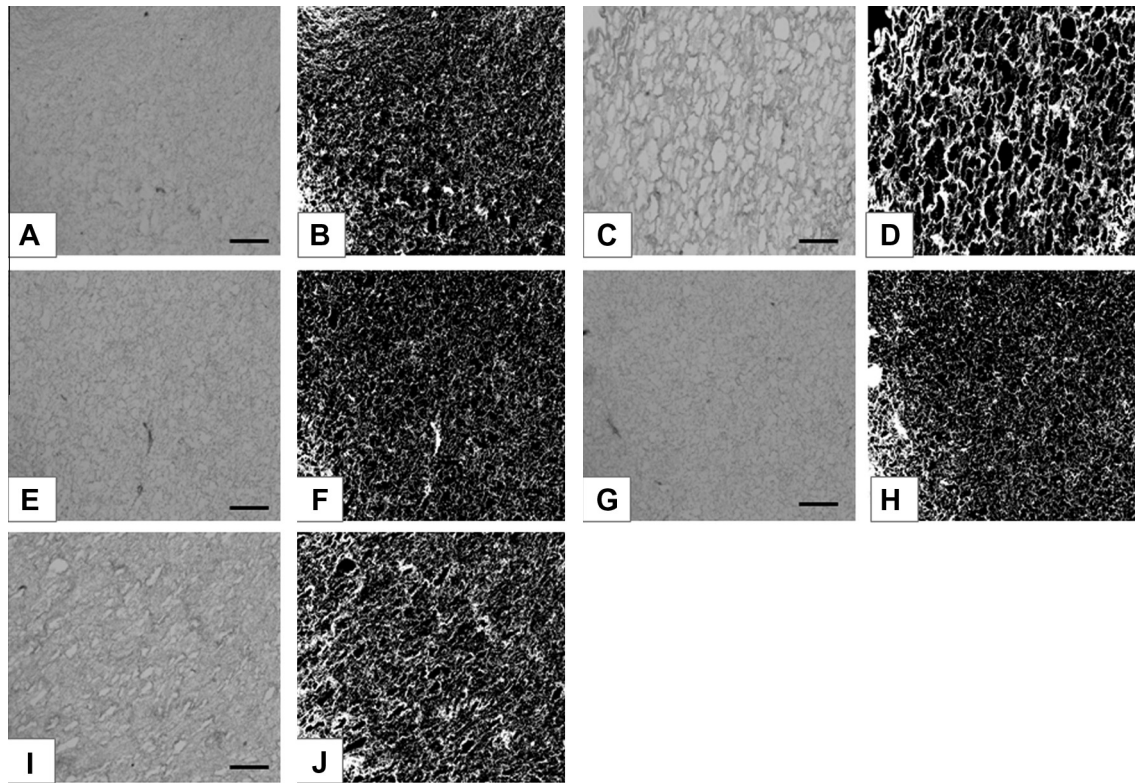
Fig. 1. Tissue structure preservation analysis as determined by the area of interfibrillar spaces in artificial HOMS. For each experimental group, tissue sections were stained with hematoxylin–eosin and transformed to binary images using the MacBiophotonic Image J program for quantification. Panels correspond to CF control tissues not subjected to cryopreservation stained with hematoxylin–eosin (A) or converted to binary images (B); CS controls cryopreserved without cryoprotectant solutions stained with hematoxylin–eosin (C) or converted to binary images (D); samples cryopreserved with solution A stained with hematoxylin–eosin (E) or converted to binary images (F); samples cryopreserved with solution B stained with hematoxylin–eosin (G) or converted to binary images (H) and samples cryopreserved with solution C stained with hematoxylin–eosin (I) or converted to binary images (J). Scale bar: 100 μm.

Quantification of cells without structural damage

As shown in Table 1 and Fig. 2B, CF control tissues showed 4.3 ± 1.5 intact cells per 100 μm^2 area, and the number of cells was uniform in all areas of the tissue. In contrast, control CS tissues showed a significant reduction in the number of cells without structural alterations (2.1 ± 2.3 cells/area). Tissues cryopreserved with solutions A and B were no different from CF controls (3.0 ± 1.6 cells/per area for solution A and 4.0 ± 2.3 cells/area for solution B), whereas tissues cryopreserved in solution C had the highest number of disrupted cells (1.9 ± 1.8 intact cells/area). The differences were statistically significant for the comparison of CF vs. CS ($p = 0.0110$, Mann–Whitney test), CF vs. solution C ($p = 0.0050$), CS vs. solution B (0.0430) and solution B vs. solution C (0.0290).

Cell viability as determined by immunohistochemical study of the cell proliferation marker PCNA

The results of the cell proliferation analysis by PCNA determination 24 h after thawing showed that 49.81 ± 21.48% of cells in substitutes cryoprotected with solution A were proliferating within the fibrin–agarose biomaterial. In solution B, 72.41 ± 18.31% of cells were proliferating, whereas in solution C, 38.88 ± 27.59% of cells were proliferating. In the CF control group, the cell proliferation index was 87.02 ± 10.1%, whereas the CS control showed no proliferative cells (0%). Statistical analysis revealed that the proliferation index of tissues cryopreserved with solutions A and C was statistically different to that of CF and CS control tissues ($p = 0.0010$). However, samples cryopreserved with solution B were different to CS but similar to CF. Strikingly, the statistical analysis revealed a significant correlation between the number of intact cells per area of tissue and the percentage of proliferating cells as determined by PCNA ($p = 0.0492$, $r = 0.8805$) (Table 1 and Figs. 2C and 3).

Cell quantification and cell viability of recultured artificial HOMS

Quantification of cells grown from recultured artificial tissue explants showed 24.63 ± 116.88 cells/per area in artificial HOMS cryoprotected with solution A, 102.48 ± 257.38 cells/per area with solution B, and 37.83 ± 127.01 cells/per area when solution C was
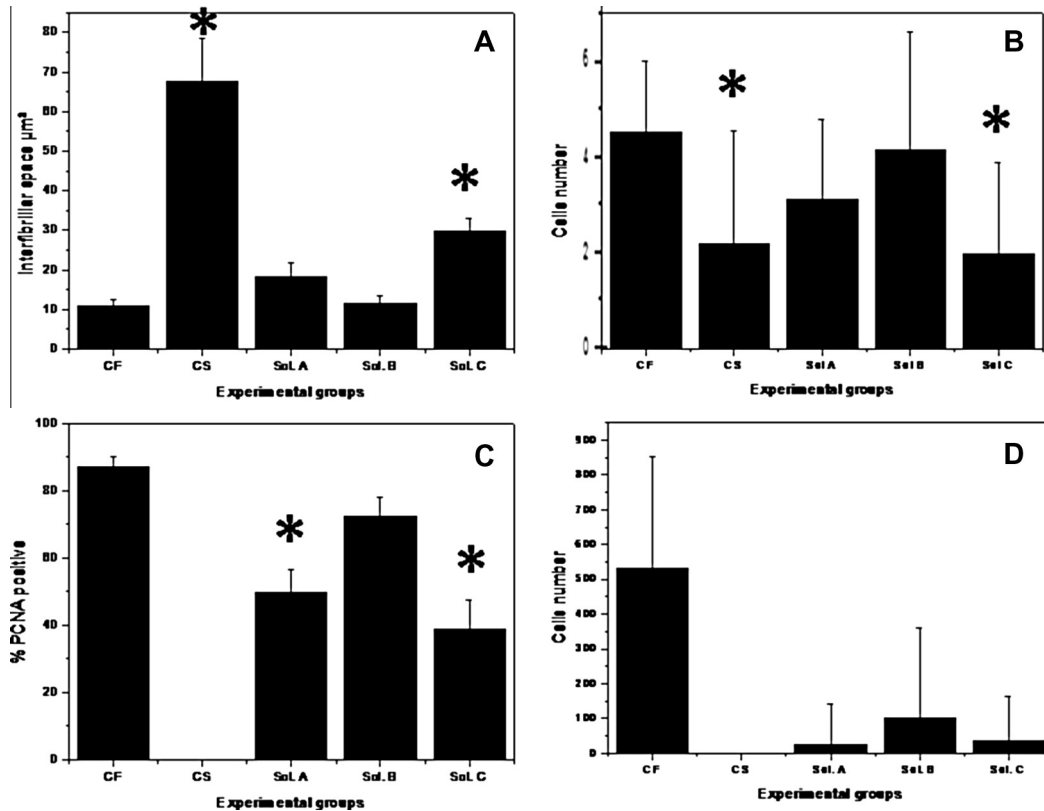
Fig. 2. Analysis of interfibrillar spaces (A), number of cells without structural damage per 100 μm² area (B), percentage of cells showing positive PCNA expression (C) and number of recultured cells per area (D) in controls and artificial HOMS subjected to cryopreservation with solutions A, B and C. Error bars correspond to standard deviations; statistically significant differences with control CF samples are labeled with asterisks (*).
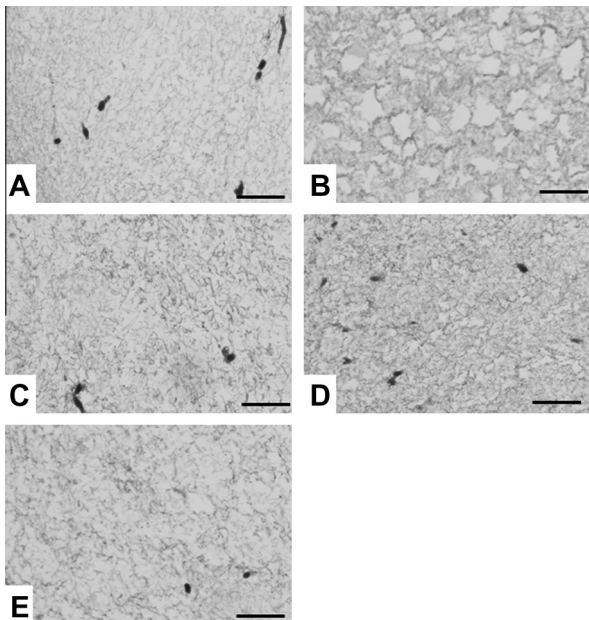

Fig. 3. Illustrative images of the immunohistochemical analysis of PCNA expression in cells immersed within the artificial HOMS. (A) Control tissue not subjected to cryopreservation; (B) control tissue cryopreserved without cryoprotectant solution; (C) samples cryopreserved with solution A; (D) samples cryopreserved with solution B; (E) samples cryopreserved with solution C. Scale bar: 100 μm.

Fig. 4. llustrative images of the cell survival analysis of recultured cells using Live & Dead®. (A) Control tissue not subjected to cryopreservation; (B) samples cryopreserved with solution A; (C) samples cryopreserved with solution B; (D) samples cryopreserved with solution C. Scale bar: 50 μm.

groups where cells proliferated, cell viability was above 99%, with 99.5% of live cells in the CF group, 99.8% for solution B and 100% for solutions A and C. The differences in cell viability were not statistically significant ($p > 0.05$).

Biomechanical properties

Steady-state measurements

From steady-state measurements, we obtained the values of yield stress (Fig. 5A). As observed, yield stress in sample cryopro-

used. In the CF control, the total number of recultured cells was 531.28 ± 321.86, whereas in CS controls did not show any cells in the culture flask (Table 1 and Figs. 2D and 4). In all experimental
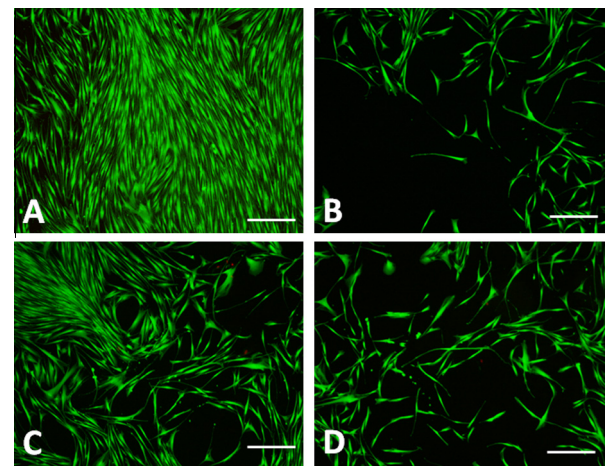
tected with solution A was similar in magnitude to sample CF. In contrast, cryoprotection with solution C and especially with solution B was associated with a substantially larger yield stress compared to sample CF. We were unable to obtain the yield stress for sample CS (or any of its characteristic rheological parameters) since this sample was disrupted at different places by the freezing–thawing process, which prevented accurate determination of its rheological properties.

Dynamic (oscillatory) measurements
Amplitude sweep. Elastic and viscous moduli. From amplitude sweeps we obtained the values of G' and G'' corresponding to the VLR (average of the low-amplitude pseudoplateau) at a frequency of 1 Hz (Fig. 5B and C). As observed, in sample CF (not subjected to cryopreservation) G' was much lower than in samples subjected to cryopreservation (Fig. 5B). In contrast to yield stress, samples cryoprotected with solution A had a G' value one order of magnitude higher than in sample CF.

All samples had similar G'' values (Fig. 5C), and we thus conclude that the viscous behavior of the samples was not altered by the cryopreservation process when cryoprotectant solutions were used.

Frequency sweep. Elastic and viscous Moduli. From frequency sweeps we obtained the values of G' and G'' pertaining to the VLR as a function of frequency in the different samples (Fig. 5D and E). As observed, the elastic modulus was higher than the viscous modulus in all samples and across the whole range of frequencies studied.

With respect to the trends in G' for different samples (Fig. 5D), we observed a common tendency for G' to increase with frequency. Note that this tendency was more pronounced at high frequencies. The behavior of G'' as a function of frequency (Fig. 5E) was remarkably different to G' (Fig. 5D). In sample CF and the sample cryoprotected with solution C, G'' was almost independent of frequency up

to a value of 2 Hz. Above this value there was a steady increase in G'' followed by a sharp decrease at higher frequencies. For samples cryoprotected with solutions A and B, G'' increased with frequency across the whole range under study. In all cases, the values of G'' were within the same order of magnitude in all samples studied.

Discussion

Cryopreservation protocols have been established for several native human tissues and organs. However, these protocols still need to be optimized for artificial hydrogel tissues, which are typically very rich in water and may therefore be affected by cryogenic conditions [25]. One of the advantages of cryopreservation of artificial tissues for clinical use as tissue grafts is the possibility of scaled production and delayed delivery to hospitals and health centers. In this connection, results published by Kuroyanagi et al. [15] demonstrated that cryopreserved artificial dermal substitutes based on hyaluronic acid and collagen could be made available to thirty hospitals around Japan, and could be transported to those hospitals in a frozen state and maintained at these locations until clinical use. However, these protocols have not been evaluated for fibrin-based tissue substitutes, whose use is increasing yearly.

For the process of cryopreservation, we selected a slow, progressive freezing method based on the recommendations of other research groups such as Kuroyanagi et al. [15], Kubo and Kuroyanagi [14], Spoerl et al. [27], Bhakta et al. [5] and Xiong et al. [34]. For native tissues, slow freezing methods allow the cells to gently dehydrate, and intracellular electrolytes will concentrate before reaching the nucleation temperature (ice formation). Therefore no residual water is frozen inside the cell, and cell damage is minimized [18]. Conversely, if the temperature descends too rapidly, the cell may not be able to dehydrate quickly enough to reach the nucleation temperature; the residual water will then freeze and intracellular ice will form, resulting in cell damage [18].
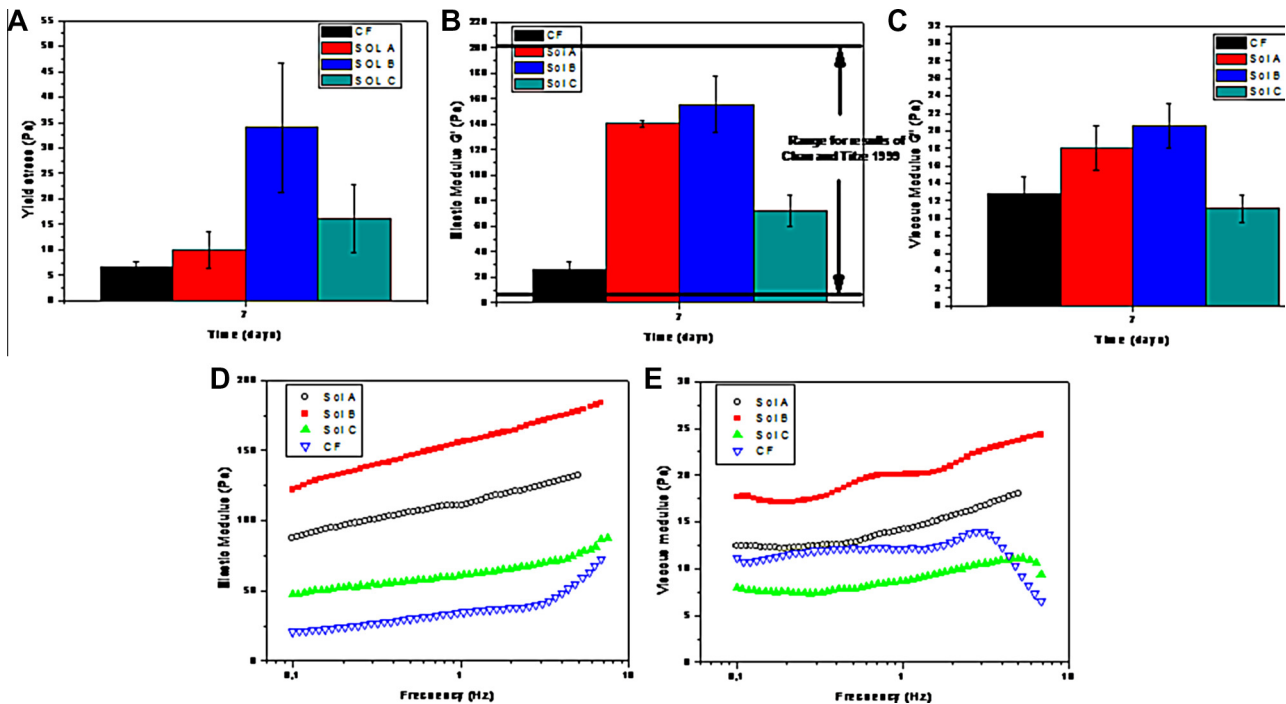

Fig. 5. Rheological analysis of control CF samples and HOMS cryopreserved with cryoprotectants A, B and C. (A) Yield stress values for each sample using a frequency of 1 Hz; (B) elastic modulus corresponding to the linear viscoelastic region obtained by oscillometry at a frequency of 1 Hz. The range reported by Chan and Titze for native soft human tissues is shown ($G' \approx 7$–200 Pa); (C) viscous modulus corresponding to the linear viscoelastic region obtained by oscillometry at a frequency of 1 Hz; (D) elastic modulus corresponding to the linear viscoelastic region for each sample using increasing oscillometry frequencies; (E) viscous modulus corresponding to the linear viscoelastic region for each sample using increasing oscillometry frequencies. Data are shown as means ± standard deviations of the means (error bars).

In the present study, histological analysis with light microscopy showed that solution B had the best cryoprotective properties among the three cryoprotectant solutions evaluated. However, no significant differences were found compared to solution A, and both solutions A and B were able to maintain the 3D structure of the tissues with no differences compared to control CF cultures. We can thus infer that the presence of DMSO in cryoprotectant solutions A and B may be responsible for this a behavior. These data agree with the findings of Kubo and Kuroyanagi [14], who demonstrated that dermal substitutes could be efficiently cryopreserved using DMSO as a cryoprotectant, as determined by scanning electron microscopy. A further consideration is that preserving the structure of cells immersed in the fibrin–agarose matrix is one of the major requirements of successful cryopreservation protocols for artificial tissues. Our results confirm that solutions A and B were the most appropriate cryopreserving agents not only for extracellular matrix preservation, but also for cell structure maintenance, especially when solution B was used (95% of preserved cells). Wang et al. [30] analyzed the effects of DMSO on the cryopreservation of dermal substitutes generated by tissue engineering with polyglycolic acid. These authors observed with scanning electron microscopy that cryoprotectant solutions based on 10% DMSO led to less cell detachment between fibroblasts and ECM compared to 15% DMSO solutions. The authors explained this finding as a consequence of the higher cell viability of fibroblasts in 10% DMSO. This might also explain the results obtained in the present study, where we observed that the number of intact cells in the fibrin–agarose matrix was higher with solution B than with solution A, although the differences were not significant.

In order to determine the proliferative capability of cells present in the artificial tissues we generated, we performed PCNA assays 24 h after thawing. Our PCNA test results showed that the highest rate of cell proliferation occurred in artificial tissues cryoprotected with solution B, and that these values were similar to those in CF controls, with no statistically significant differences between the two groups. Moreover, artificial tissues cryopreserved with solution A showed significantly lower cell proliferation than the control CF. Substitutes cryoprotected with solution C, in turn, showed the lowest cell proliferation rates of all experimental groups. These cell proliferation data correlate well with our quantitative analysis of cell viability and confirm solution B as the best cryoprotection option for artificial connective tissues. These results are supported by the fact that tissues cryopreserved with solution B showed the highest rates of cell growth after thawing and reculture with tissue explants, although no differences were found for cell viability. As shown by Kubo and Kuroyanagi [14], the possibility of obtaining viable cell cultures after freezing–thawing is proof of the usefulness of the cryopreservation method used here. According to these authors, fibroblasts recultured after thawing retain their ability to proliferate and release key cytokines (VEGF, bFGF, HGF, TGF-B1, and IL-8). These authors noted that the release of these cytokines by recultured cells plays an important role, for example, in the healing process. Studies conducted by Wang et al. [30] further support these findings by highlighting that 10% DMSO provided better cell viability than higher DMSO concentrations, because cell toxicity increases with the concentration of DMSO in the cryoprotectant. Regarding the comparatively poor results obtained with cryoprotectant solution C, Bravo et al. [6] previously documented the superior performance of DMSO compared to glycerol as a cryoprotectant agent. According to these authors, glycerol may have a lower ability to diffuse through biological membranes, and therefore less cryoprotective power. The authors noted that DMSO can establish hydrogen bonds with water molecules, thus reducing the latent heat of fusion and the cryoscopic freezing point of the medium, which in turn reduced the likelihood of ice nucleation. This process may lead to the appearance of an osmotic gradient, which would be inimical to cell viability. Despite these considerations, glycerol is an important cryopreserving agent, especially for epithelial cells, although its scope is not as broad as DMSO, probably because of its lower ability to penetrate through cell membranes. Further studies should determine if the differences observed in samples cryopreserved with different cryoprotectant agents are due to the differential cryoprotective power of each agent, or whether the cryoprotectant solutions per se are able to modify the structure and properties of the hydrogels even without freezing.

To evaluate the biomechanical behavior of the tissues, we analyzed the steady-state and dynamic properties of the artificial HOMS by rheometry. The same method has been previously used to characterize the viscoelastic properties of soft tissues [7,22,35,31]. In particular, work by Chan and Titze [7] pioneered the characterization of biomechanical properties in human vocal fold mucosa. According to our yield stress values, cryopreservation with solution A did not seem to affect fracture toughness in the artificial HOMS compared to control CF cultures. In contrast, cryopreservation with solution C and especially with solution B led to a remarkable increase in fracture toughness. This increase is likely associated with internal changes caused by the cryopreservation processes. However, in spite of this increment, in all cases the yield stress values remained below 50 Pa, as in soft human tissues. From the values of G' corresponding to the VLR, we inferred that G' increased when the substitutes were subjected to cryopreservation. In terms of physics, an increase in G' is associated with an increase in the stiffness of the material. It is important to note at this point that Chan and Titze [7] found a correlation between the value of G' of native human soft tissues and the age of individuals, with the oldest individuals showing the highest G' values. The fact that G' increased in the substitutes subjected to cryopreservation in the present study can thus be interpreted as aging triggered by these protocols. Nevertheless, samples cryoprotected with any of the solutions (A, B or C) had G' values within the range reported for native soft human tissues not subjected to cryopreservation ($G' \approx 10$–200 Pa) [7]. In contrast, cryopreservation protocols based on solutions A, B or C did not seem to affect the viscous behavior (characterized by G'') of the artificial HOMS. This is likely due to the fact that the viscous response of the sample is mainly related to water content, which must be similar before and after cryopreservation.

Regarding the tendency of G' to increase with frequency in the VLR, it is worth noting that the same trend was previously observed by Chan and Titze [7] in their study of native human vocal fold mucosa. We thus conclude that the increase in G' may be a physiological characteristic of this kind of tissue. Regarding the dependence of G'' on frequency in the VLR, we are aware of no previously published data for native human tissues. Nevertheless, Chan and Titze [7] reported values of the damping factor in this kind of tissue for the same range of frequency as in the present work. Interestingly, the trend obtained for the damping factor of native tissues correlated with the G'' results we found for CF cultures and samples cryoprotected with solution C: damping factor values were almost independent of frequency up to 2 Hz, followed by an increase and a subsequent decrease in the damping factor at higher frequencies. Although the viscous modulus and the damping factor are different quantities, from a fundamental point of view they are both related with energy loss in the material associated to its viscous response. Therefore, we may conclude that the tendency for G'' in sample CF and artificial HOMS cryoprotected with solution C is also a physiological characteristic of this kind of tissue.

In summary, cryopreservation leads to changes in artificial HOMS that can be interpreted as a process analogous to aging. In spite of these changes, cryopreserved artificial HOMS display rhe-

ological properties (yield stress and viscoelastic moduli) that make them appropriate for use in regenerative medicine, as inferred by the fact that their rheological characteristics were similar to those of the control samples and, most importantly, within the reported values for native tissues. In addition, the use of DMSO-based cryoprotectant solutions was associated with improved tissue structure and cell survival, especially with 10% DMSO. These results suggest that cryoprotectant solution B should be used preferentially and that artificial HOMS can be efficiently and safely cryopreserved and stored for delayed therapeutic use.

Acknowledgments

References

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