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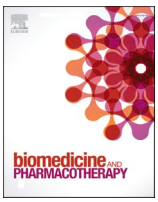

Nanostructured fibrin-agarose hydrogels loaded with allogeneic fibroblasts as bio-dressings for acute treatment of massive burns

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ABSTRACT

The prompt management of patients with massive burns is essential to maximize survival by preventing infection, hemorrhage, fluid and heat loss, and to optimally prepare the wound bed for the application of autografts or cultured tissue-engineered artificial autologous skin. Acute treatments are typically based on temporary bio-dressings, commonly cadaveric skin allografts, but supply challenges, high costs and increasingly stringent regulatory requirements preclude their widespread use. Nanostructured fibrin-agarose hydrogels (NFAH) have been proven to be safe and effective biomaterials in preclinical and clinical studies, and show good hemostatic and biomechanical properties. Here we generated and tested NFAH with embedded allogeneic dermal fibroblasts (NFAH-F) under Good Manufacturing Practice (GMP) conditions. Fibroblasts were first expanded and characterized to create a GMP bank and the NFAH-F was manufactured on demand. Three patients with major burns were treated with this product as a temporary bio-dressing under compassionate use. Our results suggest that NFAH-F product was a safe product and no adverse reactions were observed. In all cases, the patients survived until definitive treatment. Therefore, the application of NFAH-F might be a temporary bio-dressing for patients with massive burns when cadaveric skin allografts are not available.

1. Introduction

Despite advances in therapeutic strategies for the management of massive burns, patients with burns covering > 60% of the total body surface area (TBSA) remain a therapeutic challenge. Massively burned patients typically show poor outcomes and a high mortality rate [1]. Furthermore, management of a patient with a severe burn injury is a long-term process requiring an extended hospital stay as well as prolonged treatment of the systemic, psychologic and social consequences of the injury. Split-thickness skin autografts (SSA), comprising the full thickness of the epidermis and a variable thickness of the underlying dermis, are the gold standard treatment for permanent wound closure

Abbreviations: NFAH, Nanostructured fibrin-agarose hydrogels; NFAH-F, NFAH with embedded allogeneic dermal fibroblasts; GMP, Good Manufacturing Practice.

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[2,3]. However, in many patients with severe burns, the possibility of using SSA is hindered by the limited availability of skin graft donor sites [3] and by donor site-associated morbidity [4]. Recent developments in tissue-engineering applications, such as the use of cultured epithelial autografts [5], have provided new treatments for severe burn wounds, yet these strategies invariably require skin biopsies taken from the patient, which are then processed and cultured for several weeks to generate sufficient keratinocyte populations. Unfortunately, even with the improvements incorporated in modern management of burn care [6], 71.9% of patients with a burn area over 50% TBSA die before these cell cultures are established, with most of the patients who dye doing so within the first 2 months (91.3%) and usually within the first 48 h (71.0%) after hospital admission [7].

The prompt management of massive burn injuries is essential to preserve the patient's life through preventing fluid loss, protecting against infections and to stop bleeding [8], which can usually be achieved using temporary dressings. In routine clinical practice, this is usually achieved with a cadaveric skin allograft [9] supplied by tissue banks, which is used as a temporary bio-dressing prior to definitive grafting, and occasionally as a long-term graft in partial thickness burns [10]. Unfortunately, cadaveric skin is not always available, as there are many challenges with respect to its supply. Accordingly, other temporary dressings that are readily available are needed and several approaches have been developed (reviewed in [11–13]). Along this line, allogeneic mesenchymal stem cell (MSC)-based products have proven effective as wound dressings [14]. While MSC are thought to be an ideal cell source for the development of cellular therapies, they have several drawbacks and limitations, and there is increasing evidence to support the use of human fibroblasts as a robust alternative cell source. Bone marrow, which is the most common source for isolating MSCs, requires invasive extraction and provides relatively little starting material for cell expansion. By contrast, fibroblasts can be easily harvested in large numbers from several biological "waste products" (e.g., foreskin and surplus skin from abdominoplasties and mammoplasties). Fibroblasts and MSC share important surface markers, and have similar potential to differentiate and immunmodulate [15–17]. Because of this, fibroblasts (autologous and allogeneic) are now being used in several cell therapy and tissue engineering applications [5,18,19], including the treatment of facial scars [20] with autologous fibroblasts and for treatment of epidermolysis bullosa [21], diabetic foot ulcers (e.g., Dermagraft®, Organogenesis Inc., Canton, MA, USA) [22], skin ulcers due to venous insufficiency (e.g. Alpigraft, Organogenesis INC., Canton, MA, USA) [23] with allogeneic fibroblasts.

Dermis grafts (with no epidermis) can provide additional coverage in acute major burn wounds [2], and cultured fibroblasts, particularly with a dermal support, can positively contribute to wound healing processes by reducing wound contraction and supporting collagen synthesis and neovascularization [24]. Indeed, bioconstructs and scaffolds based on allogeneic fibroblasts are now commercially available for use as temporary dressings, but they are commonly derived from xenogeneic resources such as murine collagen (e.g., Stratagraft®, Stratatech Corporation, Hampton, NJ, USA) [25], equine collagen (e.g. Progenitor Biological Bandages, Lausanne Burn Center, Switzerland) [18,19] or non-biodegradable materials such as nylon mesh (e.g. Transcyte®, Advanced Tissue Sciences, La Jolla, CA) [5,26]. Therefore, overall safety, effectiveness and applicability, of the currently available dressings for full-thickness burns products remains far from perfect and could be further improved [27]. Hydrogels have been used for many years as temporary wound dressings, but an ideal patch for treating severe burns is still not available [8]. Nanostructured fibrin-agarose hydrogels (NFAH) [28] are biocompatible, biodegradable scaffolds based on human plasma and agarose, with demonstrated robust biological, biomechanical and hemostatic properties [29]. Indeed, NFAH have shown optimal performance in different pre-clinical and clinical applications, and have been used successfully without cells as a hemostatic patch in hepatic surgery [29] and also with different cell types as a substitute for the cornea [30,31], skin [28], oral mucosa [32] and peripheral nerve [33]. NFAH scaffolds have proven safe and biodegradable in different animal models [28,29,32,34], with good surgical handling properties, and they are currently being tested in clinical trials in patients with corneal trophic ulcers [30,35] and for reconstructive surgery [36,37]. The application of NFAH scaffolds containing *autologous* fibroblasts and keratinocytes (UGRSKIN model [28,38,39]) represents a promising approach to successfully treat severely burned patients, but the time required for this model to be generated [39] makes it necessary to search for alternative products that can provide immediate relief to patients with severe burns, such as the use of allogeneic fibroblasts.

In the present study, we generated and evaluated a novel model of human bioengineered dermis containing allogeneic fibroblasts immersed in NFAH (NFAH-F model). We first created and characterized a bank of human allogeneic fibroblasts. We then manufactured NFAH-F as a medicinal product under GMP conditions, and evaluated its biocompatibility and biofunctionality as a temporary graft in patients with major and severe burns affecting more than 40% of TBSA.

2. Methods

2.1. Fibroblast isolation and banking

Fibroblasts were obtained from surplus skin isolated from abdominoplasty at the Virgen del Rocio University Hospital (Seville, Spain). The local ethics committee approved the study and the skin samples were obtained after written informed consent according to the Spanish legislation on cell and tissue donation. The donor was a woman of 52 years old. She signed an informed consent where she was informed that the tissue could be used and processed for subsequent tissue or cell transplantation. Donor was not economically compensated for tissue donation. Donor serology was performed to exclude human immunodeficiency viruses (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. The skin was maintained in BASE 128 decontamination medium (Alchimia Srl, Padua, Italy) and was processed at the Unidad de Produccion y Reprogramacion Celular (UPRC, Seville). Biopsies were washed three times for 5 min with phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO) supplemented with 1% penicillin/streptomycin (P/S; Sigma-Aldrich), 20 μg/mL gentamycin and 50 μL/mL vancomycin (both from Normon, Madrid, Spain). Tissues were cut into small pieces (approximately 1 × 1 mm) using sterile scalpels, and were then distributed into tubes containing 5 mL of collagenase NB 6 GMP Grade solution (SERVA Electrophoresis GmbH, Heidelberg, Germany) (20 pieces/tube; 0.5 ± 0.1 PZ-U collagenase/mL) and incubated for 16 h at 37°C under agitation. The digestion was stopped with fibroblast medium containing Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich), 1% non-essential amino acids 100 × (NEAA; Sigma-Aldrich), 20 μg/mL gentamycin (Normon), 1% GlutaMax™ (Life Technologies, Invitrogen, Gaithesburg, MO), 5% in-house-produced human platelet lysate [40] (HPL) solution and 2 U/mL heparin (Rovi, Madrid, Spain) [40]. Cells in suspension were centrifuged, counted and seeded in 25 cm^2 flasks; one flask per each digestion tube. After digestion, cells were expanded in fibroblast medium until passage 2, when a Master Cell Bank (MCB) was generated by cryopreserving cells in CryoStor CS10™ (BioLife Solutions Inc., Bothwell, WA). One criovial from the MCB was thawed and cells were seeded at 4000 cells/cm^2. Fibroblasts were subcultured for at least three more passages to obtain a Working Cell Bank (WCB). The WCB manufacturing process was carried out three consecutive times for validation according to GMP guidelines.

2.2. NFAH-F manufacturing

WCB vials were thawed and cells were counted and embedded in fibrin-agarose hydrogels (Fig. 1). The manufacturing process for NFAH was an adaptation of methods described in previous reports [28,29]. A 110 mL mixture was prepared as follows: first, a vial (tube 1) was

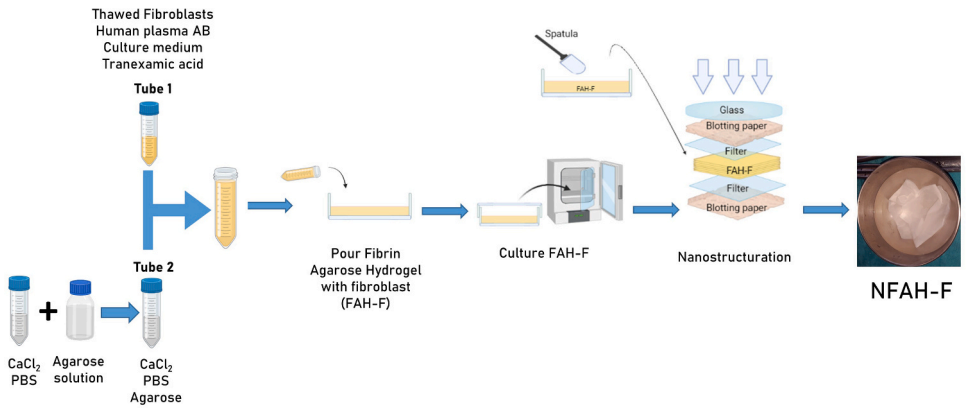

Fig. 1. Production process of nanostructured fibrin agarose hydrogels with fibroblasts (NFAH-F). Images were created with BioRender.com.

prepared by mixing 91.7 mL of AB human plasma, 7.33 mL of fibroblast medium without heparin containing 15.3 × 10^6 fibroblasts from the WCB and 1.83 mL of tranexamic acid (Amchafibrin 500 mg; Rottapharm, Milan, Italy). Tube 2 was prepared with 2.2 mL of 10% calcium chloride (B.Braun, Melsungen, Germany) and 5.5 mL of melted agarose at a final concentration of 2.2% (dissolve 0.11 g type VII-agarose [Sigma-Aldrich] in 1.5 mL of PBS). After mixing both tubes, the total volume was distributed as follows: 100 mL was added to a square 12 × 12 cm Petri dish with 144 cm^2 of surface (Corning Gosselin S.A.S. Life Sciences, Hazebrouck Cedex, France). The plates were kept at 37 °C for 2 h in a cell incubator. Once gelation was completed, the hydrogels (NFAH-F) were covered with fibroblast medium and kept at 37 °C at least for 12 h prior to nanostructuration. For nanostructuring, the hydrogels were placed between two sterilized 10 μm nylon filters (Merck-Millipore, Burlington, MA) and compressed between several layers of sterile extra-thick western blotting filter paper (ThermoFisher Scientific, Waltham, MA), using a piece of glass weighing 250 g for 1 min and 40 s. The NFAH-F (finished product) was packaged in a tissue storage pot (Medfor, Aldershot, UK) with Ringer Lactate solution (Fresenius Kabi AG, Bad Homburg, Germany) at room temperature (RT) until needed.

2.3. Microbiological assays

The presence of bacteria and fungi was checked using Gram (Biomerieux, Marcy-l'Étoile, France) and calcofluor (Becton Dickinson, Irvine, CA) staining kits, according to the manufacturers' instructions. Sterility tests were performed by inoculation of cells (MCB or WCB) or finished product (Ringer Lactate in contact with NFAH-F) in aerobic and anaerobic media (Biomerieux) that were incubated in a BACT/ALERT®3D automated microbial detection system (Biomerieux), according to the 2.6.27 Monograph of European Pharmacopoeia (Ph. Eur) [41]. This assay was performed at the Unidad de Produccion Celular e Ingenieria Tisular of the Virgen de las Nieves University Hospital (Granada, Spain). Mycoplasma tests were carried out by PCR using the Venor® Gem Mycoplasma PCR Detection kit (Minerva Biolabs, Jena, Germany) according to the Ph. Eur. [42]. Endotoxin tests were performed using the Endosafe-PTS test system (Charles River, Charleston, SC), according to the chromogenic kinetic method of Ph. Eur., [43] and following the manufacturer's instructions.

2.4. NFAH-F cell viability

Analysis of NFAH-F cell viability was performed with the LIVE/DEAD® Viability/Cytotoxicity kit (ThermoFisher Scientific) and with Hoechst 33342 (Miltenyi Biotec, Bergish Gladbach, Germany) staining. Briefly, 2 cm^2 pieces of NFAH-F were washed 3 times with PBS and incubated in 2 mL of a solution containing 4 μL of 2 mM ethidium homodimer, 0.25 μL of 4 mM Calcein and 0.5 μL of a 10 mg/mL Hoechst solution, for 30 min at RT. Samples were then washed 3 times with PBS and maintained in this buffer until analysis. Images were captured with a Nikon Eclipse Ti-S fluorescence microscope (Tokyo, Japan). Live and dead cells were counted using ImageJ software [44] from at least three random fields per preparation.

2.5. NFAH-F immunofluorescence analysis (potency)

Once nanostructured, the NFAH-F is very thin (50–60 μm). This allowed us to incubate the finished product directly with the antibodies for immunocytochemistry analysis without the need for paraffin embedding and sectioning. Immunofluorescence detection of relevant proteins was performed after fixing the tissue in 3.7–4.0% formaldehyde (PanReac AppliChem, Barcelona, Spain) for 15 min at RT. The NFAH-F was then washed 3 times with PBS and permeabilized with 0.1% Triton X-100 for 15 min at RT under agitation. This was followed by an additional 3 washes with PBS before blocking with 1% BSA in PBS for 30 min at 37ºC in a wet chamber. The NFAH-F was washed again 3 times with PBS and incubated with primary antibodies in PBS-0.1% BSA overnight at 4ºC in a wet chamber (supplementary Table S1). Subsequently, the NFAH-F was washed 3 times for 30 min with PBS and incubated with secondary antibodies, Hoechst 33342 and Phalloidin (supplementary Table S1) for 30 min at 37ºC. Finally, the NFAH-F was washed 3 times with PBS and mounted with ProLong™ antifade mounting medium (ThermoFisher Scientific). To test the background, we performed the same protocol incubating other NFAH-F samples with isotype controls and secondary antibodies only. Fluorescence was analyzed with a Leica SP5 Confocal microscope (Leica Microsystems, Wetzlar, Germany) at the Confocal Unit of the Andalusian Center of Molecular Biology and Regenerative Medicine (CABIMER).

2.6. Patient enrollment

Once NHAH-F production was validated under GMP conditions, 3 batches were manufactured and applied to 3 severely burned patients at the Burns Unit of the Virgen del Rocío University Hospital under compassionate use (cadaveric skin allografts were unavailable). The three patients were men, aged 29, 41 and 22 years. They had full-thickness flame burns and the initial burn size was 71%, 45% and 76% TBSA, respectively (Table 1).

2.7. Surgical procedure

General anesthesia was used for all surgeries. Burned tissue was surgically removed with a manual dermatome (Padgett® Electro-Dermatome) to obtain clean and viable tissue. The NFAH-F was applied and a secondary dressing based on castor oil and Peru balsam (Linitul®; AlfaSigma SL, Barcelona, Spain) was placed over the NFAH-F

Table 1
Information of patients treated with artificial dermis.

Patient/ Age/ Sex	Burnt % TBSA/ Type of burn	Previous infections	Date of admission	Date of treatment with NFAH-F	Recipient sites	Co-treatment with other tissues or products	Outcome and remarks
Patient 1 29 years Male	71% Full thickness burn from indoor gasoline explosion	In the wound: *Enterococcus casseliflavus*, *Klebsiella pneumoniae* In the eschar: *Pseudomonas aeruginosa* and *Achromobacter xylosoxidans*	26/June/ 2016	04/July/ 2016	Anterior aspect of the left foot, left patellar region and anterior region of the right leg	1st Temporary coverage with cadaveric skin allograft (28/June/ 2016 and 11/July/2016) 2nd Definitive coverage with autografts (11/July/2016) and UGRSKIN (11/August/2016)	Bronchial infection by *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Enterococcus faecalis* Death due to septic shock from a respiratory focus related to catheter infection 27/August/ 2016
Patient 2 41 years Male	45% Full-thickness flame burn with diesel	None	14/June/ 2016	12/July/ 2016	Lower limbs	28/June/2016: escharectomy and temporary coverage with homografts in the lower limbs and coverage with partial meshing skin autografts 3:1 in the left upper limb was performed (left hip donor area) 26/July/ 2016 Definitive coverage in the right upper limb with 3:1 mesh autografts (right and left thigh donor area)	-60-day biopsy histology data -Clinical improvement and discharge 02/ September/2016
Patient 3 22 years Male	76% Full thickness flame burn with gasoline	Infection in hemoculture: *Pseudomonas aeruginosa* sensitive to Meropenem	13/April/ 2019	06/August/ 2019	Back and thighs	Temporary coverage with cadaveric skin allograft at: upper limbs and back (30/April/2019); gluteus and lower limbs (21/May/2019); shoulder, cervical area, thorax and abdomen (28/May/2019); Definitive coverage with autografts at cervical area (left leg donor area) on 28/May/2019). On 20/August/ 2019, a 1:6 mesh autograft is performed and a cadaveric allograft is placed over the autograft on the back (donor area of both gluteus) Coverage with 33 scaffold of UGRSKIN and porcine collagen type I dressings (Biobrane®) over the UGRSKIN on the back, 12 UGRSKIN scaffolds on lower limbs, 3 scaffolds on abdomen, 4 scaffolds on scalp and 6 scaffolds on thorax and abdomen (02/July/2019). After scraping until bleeding was covered cranial region back (13/September/2019)	Clinical improvement and hospital discharge 22/October/2019

TBSA: total body surface area; NA: not applicable.
UGRSKIN: autologous artificial skin (cultivated skin) [38,39]

and fixed with 4 staples for each 12 × 12 cm sheet of NFAH-F (Braun Disposable Stapler with 35 staples of size 6.9 mm (width) and 3.6 mm (height)). These staples are necessary to ensure fixation during patient movements. Compresses and gauze were placed over the Linitul® dressing and covered with a bandage. Dressing changes were limited to once every 2 days, taking care not to disturb the Linitul® dressing. Staples were removed after 5–7 days.

Patients treated had a large burned skin area (>40% TSBA) and not all the necessary interventions could be performed in a single surgical act. NFAH-F was placed when cadaveric skin homograft was not available and a temporary coverage was necessary. Other surgical interventions for patients included temporary coverage with cadaveric skin homograft (when available) in other burned areas different to these treated with NFAH-F. Furthermore, definitive coverage with UGRSKIN [38,39] was performed in two of the 3 patients (see Table 1). One of the patients was not treated with UGRSKIN because significant burn improvement was achieved and patient was discharged without the need of UGRSKIN treatment.

2.8. Patient follow-up

Patients were treated according to standard clinical procedures established at the Burns Unit of Virgen del Rocio University Hospital: dressings were changed every 48 h by removing the gauze and washing the area with neutral soap and diluted chlorhexidine. After this, a new gauze and Linitul® were placed on top. At 5–7 days after the intervention placing the NFAH-F, the staples and the Linitul® dressing were removed. Dressings were changed every 2 days until patient hospital discharge or until the administration of the final dressing.

After discharge, patients received weekly treatments at the Hospital in the treatment room. After 15 days there was a monthly follow-up evaluation for the first 3 months, then once every 3–6 months. During the follow-up consultations, the attending clinician noted the stability of the coverage, and whether the patient could carry out normal activity with no limitation of movement and that no injuries are generated. Wounds were treated accordingly. The evaluation was performed by clinical observation, but was not quantified.

2.9. Statistics

Data are presented as mean ± S.E.M. Significance was determined using one-way analysis of variance (ANOVA) with Bonferroni's post-test, or with Student's t-test. All statistical analyses were performed using GraphPadPrism 5.0 software (GraphPad Software Inc., San Diego,

CA).

3. Results

3.1. Engineering an allogeneic artificial dermis (NFAH-F)

We first designed a GMP-compliant manufacturing protocol for allogeneic fibroblast isolation, expansion and cryopreservation, and their subsequent embedding in a nanostructured fibrin agarose hydrogel (NFAH-F) (Fig. 2). The donation was skin remains after an abdominoplasty. The donor signed the informed consent and serology was performed in accordance with Spanish legislation on donation. The donor was informed that the tissue could be used and processed for subsequent tissue or cell transplantation. A GMP-compliant documentary system and quality controls were also designed. Cell isolation, culture and cryopreservation were performed in a clean room under aseptic conditions, whereby particles and microorganisms were monitored according to GMP part IV [45]. Fibroblasts were isolated from residual skin derived from abdominoplasty. Cells were expanded with in-house produced GMP-compliant HPL [40] and cryopreserved after 2 passages to create a MCB. Cells at this initial stage showed a normal fibroblastic morphology, with exponential growth, expression of fibroblast markers (vimentin, CD13 and CD44) and absence of keratinocyte markers (E-cadherin and pancytokeratin) (Fig. 3**A-D**). They also showed an absence of Human Leukocyte Antigen – DR isotype (HLA-DR) expression, a requirement for allogeneic transplantation (Fig. 3**D**). Fibroblasts produced extracellular matrix (ECM) components (fibronectin and collagen I) (Fig. 3**E**). Karyotyping and mycoplasma, endotoxin, sterility and adventitious virus tests were also performed in the MCB as quality control tests for intermediate products, and results met the acceptance criteria (supplementary Fig. S1 **and** supplementary table 2). A unique MCB from one donor was produced for the GMP validation of the process and for the treatment of all three patients in the present study. A unique MCB is an appropriate strategy for manufacturers, as it can last for decades, and is a homogeneous and characterized starting material. The stability of the frozen MCB vials was tested 23 months after cryopreservation, with results showing 95.5% viability and 74.8% cell recovery after thawing (Fig. 3**F**).

A MCB vial was thawed and further expanded until passage 5. The cells were then cryopreserved to create a WCB (Fig. 2). Six WCB (thawing and expansion of six independent vials at different times) cultures were then produced in the clean room to validate the GMP process and to treat the patients. All WCB cultures maintained the expression of fibroblast markers and ECM components, exponential cell growth, and the absence of HLA-DR expression (Fig. 4**A-D**). No chromosomal abnormalities were observed in WCB cultures (supplementary Fig. S1). The acceptance criteria were met for mycoplasma, endotoxin and sterility (supplementary Table S2). We also tested the DNA fingerprint in the WCB to check for mix-ups with other cell lines during the expansion. The test checked 10 genetic loci of the donor blood and they were the same in all cases (supplementary Table S2). The stability of the frozen WCB vials was tested 58 months after cryopreservation, with results showing 84.6% viability and 55.4% cell recovery after thawing (Fig. 4**E**).

WCB vials were thawed and embedded into fibrin-agarose hydrogels (FAH). Cells were cultured inside the FAH for 4–6 days using fibroblast medium, and hydrogels were subjected to nanostructuration before grafting following previously described methods (Fig. 1) [28]. We evaluated fibroblast cell viability before and after nanostructuration to gauge the effect of the process. Results of the LIVE/DEAD viability test showed that cell viability was $\geq$ 70% limit suggested by the Food and Drug Administration (FDA) for application to patients [46], both before and after nanostructuration in all batches (Fig. 5**A**). We also tested cells in the NFAH-F for the production of collagen I and III after 24 h and 7 days. All batches produced both collagens (type I and III) at both time points after fibroblast embedding (Fig. 5**B**). Furthermore, the NFAH-F was tested for endotoxins, mycoplasma and sterility (Gram and calcofluor staining and turbidity test according to Ph.Eur. [47]), and all batches complied with the established criteria (supplementary Table S3). Finally, we designed a stability study to establish the optimal storage conditions and set the expiry date of the product. NFAH-F was stored at 25ºC and 4ºC, and viability was measured at different time points as well as the total number of cells per field over time. Higher viability rates and total cell numbers were achieved at 25ºC than at 4ºC storage (Fig. 5**C**). Cell viability was maintained above 70% (limit suggested by the FDA [46]) for up to 72 h at 25ºC, offering a broad time window for patient application. Three consecutive batches of NFAH-F were produced for the GMP validation, which consolidated the standardization of the process and product before its use in patients. We compiled all the information about the fibroblast banks and NFAH-F production and we obtained the certification from the Spanish Agency of Medicines and Medical Devices (in Spanish, AEMPS) for producing clinical grade NFAH-F.

3.2. Treatment of massively burned patients

After AEMPS certification, three additional batches were manufactured to treat patients with massive flame burns (>40% TBSA) under compassionate use. In all cases, cadaveric skin grafts were either not available or were limited in amount for the immediate treatment of the patients. Our aim was to preserve the survival of the patients while they were waiting for cultured epithelial autografts (UGRSKIN) [28,38,39], which requires several weeks of culture, or other definitive skin substitutes. All batches of NFAH-F manufactured for these patients met the safety and potency criteria established for release (supplementary Table S3).

Table 1 summarizes the characteristics of the treated patients and their outcomes. NFAH-F was applied after surgical removal of the eschar with a dermatome, covered with the Linitul® dressing and fixed to the tissue with non-absorbable surgical staples. All patients treated with NFAH-F were alive when the definitive treatment was applied. In two of the three cases (Patients 1 and 3), bacterial infection was evident before treatment with the NAFH-F (Table 1). For patient 3, *Pseudomonas aeruginosa* blood infection was treated with Meropenem. Patient 1 had colonization of several microorganisms (*Enterococcus casseliflavus, Klebsiella pneumoniae* on the wound, *Pseudomonas aeruginosa* and *Achromobacter xylosoxidans* on the eschar), which were treated locally with topical Colistin, and disappeared after treatment with NFAH-F. Unfortunately, after the treatment with the definitive coverage, Patient 1 experienced a bronchial infection with *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Enterococcus faecalis* that progressed to septic shock and death.

A skin homeostasis study was performed with Patient 2 at 44 and 64 days after treatment. It was not possible to perform this study in the other patients because of the infections. The data were collected using a system of non-invasive probes validated for clinical use (**see** supplementary methods). The homeostatic parameters analyzed were as follows: 1) pH, to assess the quality of the hydrolipidic layer of the skin [48]; 2) temperature, to observe changes in the microcirculation, although temperature measurement has been shown to be an unreliable parameter [49], and 3) transepidermal water loss (TEWL), to evaluate the barrier function of the skin. This latter parameter is the most relevant from the homeostatic perspective [50]. At day 64 the pH level decreased, reaching normal values and approaching the control value (supplementary Fig. S2A and S2B). The TEWL reference values establish normal values of 15–25 g/h/m^2, and above 25 g/h/m^2 would indicate that the skin is affected. At 44 days of treatment, the TEWL value was close to the values that indicate that the skin is affected (24 g/h/m^2), and improved to 19.2 g/h/m^2 after 64 days of treatment (supplementary Fig. S2D and S2E).

Biopsies of the skin treated with NFAH-F of Patient 2 were also taken 60 days after implantation. Histologic analysis revealed a well-

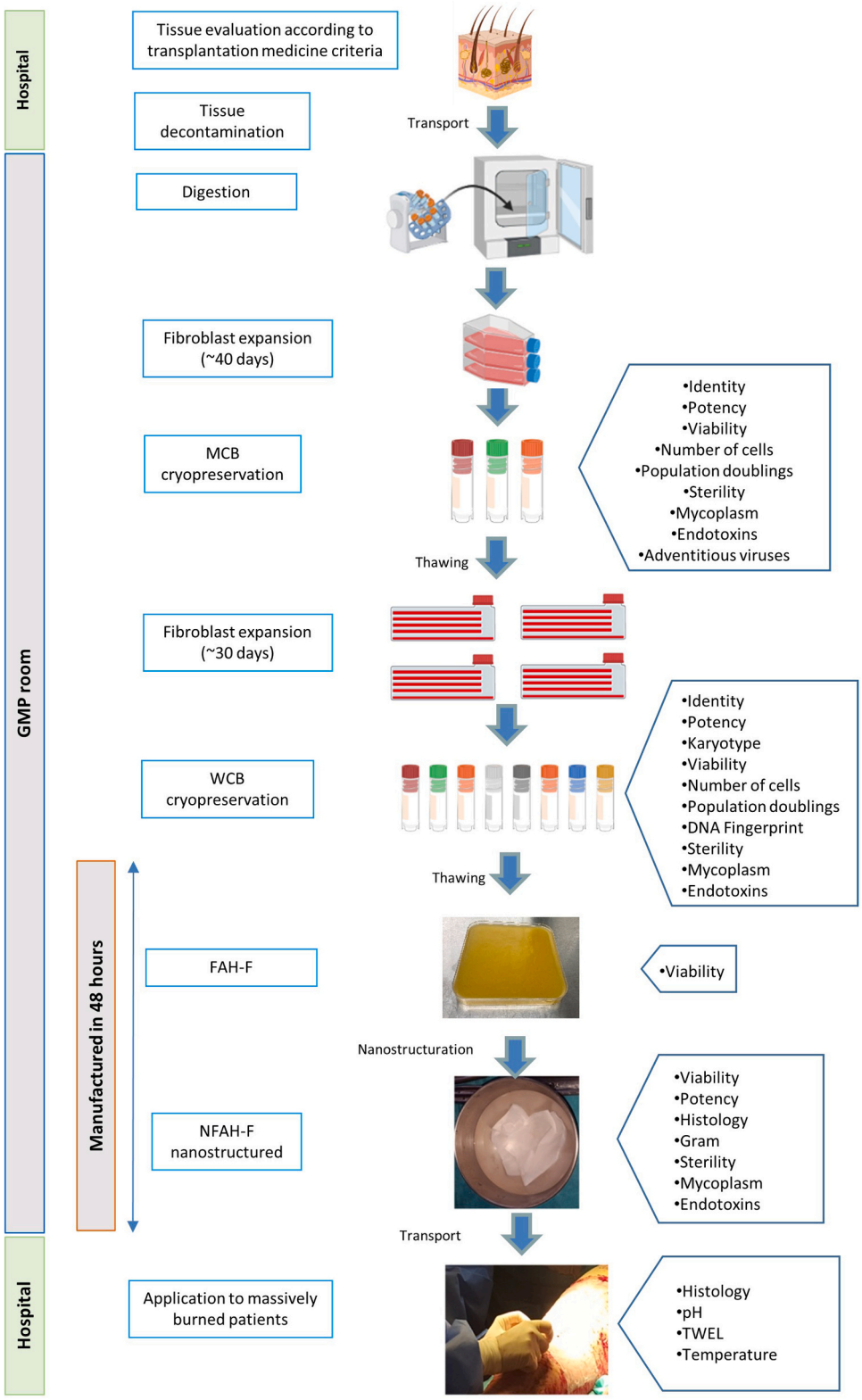

Fig. 2. Nanostructured fibrin-agarose hydrogels with embedded allogeneic dermal fibroblasts (NFAH-F) manufacturing process under GMP and aseptic conditions. Quality controls performed at each production step are indicated at the right. MCB: master cell bank; WCB: working cell bank; FAH-F: fibrin agarose hydrogel with embedded fibroblasts; NFAH-F: nanostructured fibrin agarose hydrogel with embedded fibroblasts; TWEL: transepidermal water loss. Images were created with BioRender.com.

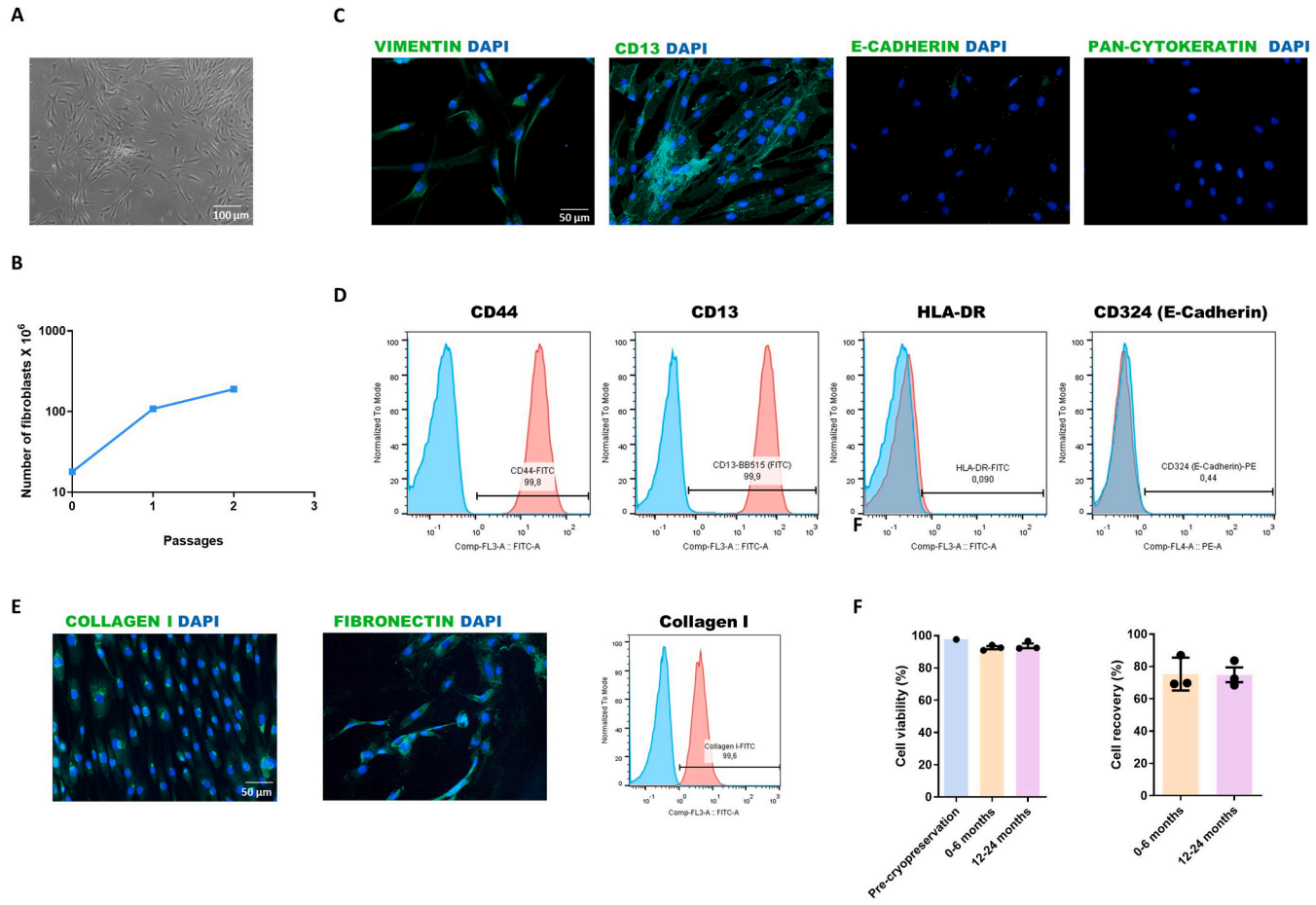

Fig. 3. Production of a clinical-grade fibroblast Master Cell Bank. A. Phase contrast micrograph of fibroblasts at passage 2. **B.** Fibroblast growth curve. **C.** Expression of fibroblast markers (vimentin, CD13) and absence of keratinocyte markers (E-cadherin and pan-cytokeratin) analyzed by immunofluorescence. **D.** Flow cytometry was used to quantify the expression of specific surface markers of fibroblasts (CD44, CD13) and keratinocytes (E-CAD) and the expression of HLA-DR. **E.** Potency of fibroblasts in terms of analysis of the extracellular matrix (ECM) components collagen I and fibronectin by immunofluorescence and collagen I by flow cytometry. **F.** Viability and percentage of recovered master cell bank (MCB) cells after thawing. Data are shown as mean ± SEM of at least 3 independent experiments.

conformed structure consisting of a stratified epithelium, a dense stroma and an underlying layer of subcutaneous adipose tissue (supplementary Fig. S3A). At the level of the epidermis, a stratified and keratinized squamous epithelium was evident, where a normal number of cell layers distributed in basal, spinous, granular and corneum strata could be distinguished, comparable to the epithelium of normal human skin. Unlike native human skin, however, the sample was devoid of specializations such as papillae-ridges, hair follicles or glands (supplementary Fig. S3B). At the stroma level, a very thick dermis was detected, and two zones clearly delimited by an interface could be distinguished. The most external zone likely corresponded to the implanted NFAH-F, and consisted of a dense ECM with abundant collagen fibers, numerous fibroblast-fibrocyte-type cells and a large number of blood vessels. The most profound zone contained fewer cells, but showed more mature and well-organized collagen bundles (supplementary Fig. S3C-**E**). The histological structure of fibroblast cells in the stroma was compatible with the presence of viable cells, as no signs of apoptosis, necrosis or other alterations were detected. The subcutaneous adipose tissue contained abundant adipocytes and blood vessels that were indistinguishable from normal tissue (supplementary Fig. S3F). There was no evidence of an inflammatory reaction around the patch, neoplastic transformation or other detectable alterations of the analyzed tissues. In summary, the histological analysis showed persistence of fibroblasts and a normal skin architecture, with vascularization and epithelialization.

Reassuringly, there were no NFAH-F-related adverse reactions.

4. Discussion

In the present study, fibroblasts were successfully isolated from surplus skin, expanded with HPL and embedded in a nanostructured fibrin-agarose hydrogel using a GMP-compliant process. A NFAH-F dressing was homogeneously produced and used to safely treat massively burned patients.

We found that the NFAH-F generated as a pharmaceutical product fulfilled all the requirements of bioartificial tissues used clinically. Indeed, the embedded fibroblasts were able to produce relevant ECM components, and the nanostructuring process applied to improve the biomechanical properties of the product did not affect the viability of the embedded cells. Finally, the NFAH-F dressing was stable for 72 h at RT, allowing a wide time window for product transport and usage. Although fibroblast isolation by the methods described here was previously validated [40], future studies will consider the use additional specific cell surface fibroblast markers for MCB and WCB flow cytometry identity test such as CD140b, CD192 or CD11A [51].

Our findings support the safety and feasibility of the NFAH-F manufacturing process to produce a temporary bio-covering for severely burned patients. In the absence of cadaveric skin allografts, application of a NFAH-F graft would contribute to protect the patient's body surface during the time required for a full-thickness bioartificial skin substitute to be generated and used as a definitive treatment [39]. Our histological analysis suggests that fibroblasts found at the stromal compartment of the NFAH-F grafted in patients were viable, and that the

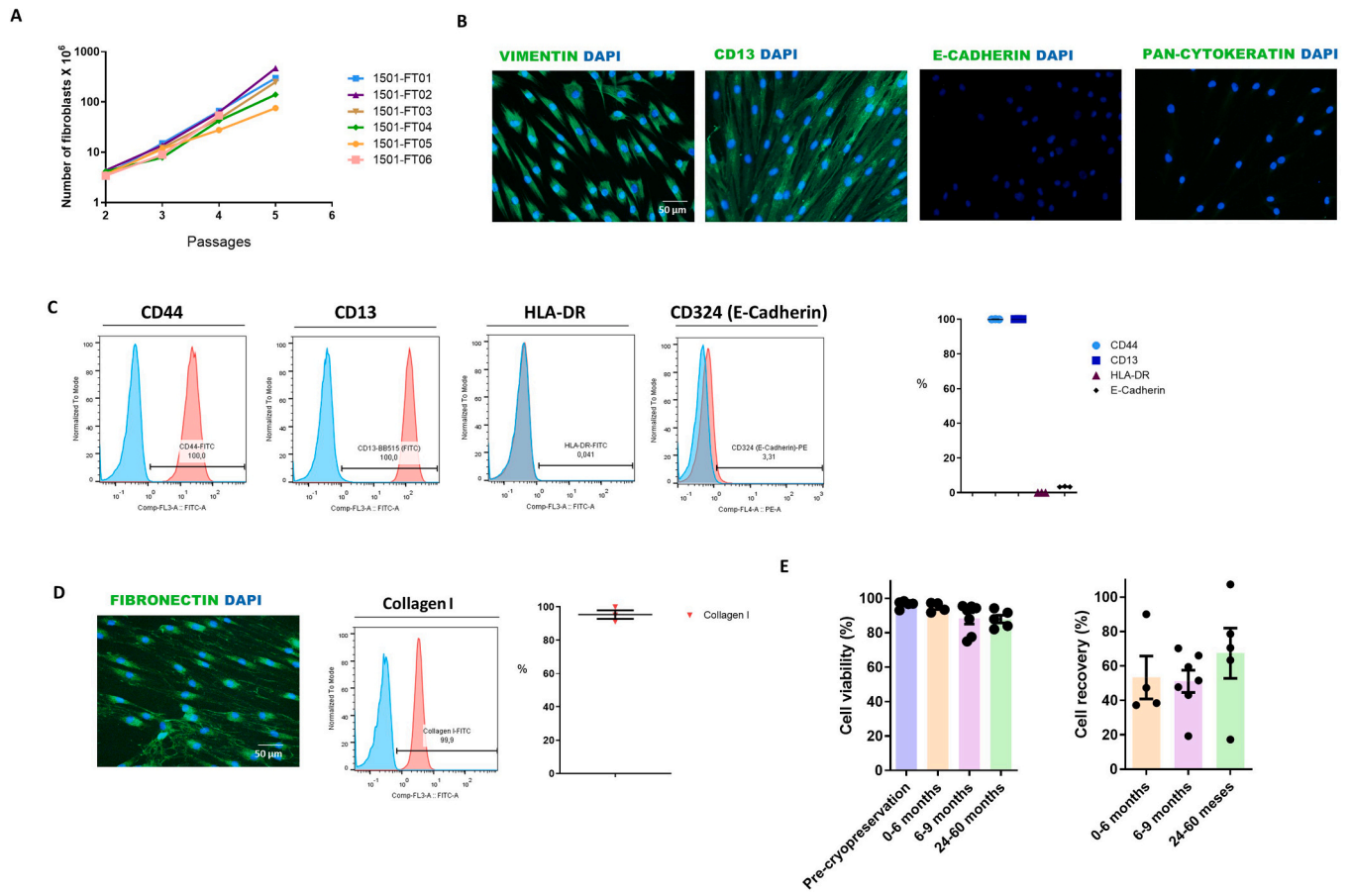

Fig. 4. Production of a clinical-grade fibroblast Working Cell bank (WCB). A. Exponential growth kinetics of fibroblasts from 6 different WCB. **B**. Expression of fibroblast markers (vimentin, CD13) and absence of keratinocyte markers (E-cadherin and pan-cytokeratin) analyzed by Immunofluorescence. **C**. Flow cytometry analysis of specific surface fibroblast markers (CD13, CD44), absence of keratinocyte markers (E-CAD) and expression of Human Leukocyte Antigen – DR isotype (HLA-DR). Data are shown as mean ± SEM of 3 independent experiments. **D.** Potency of fibroblasts in terms of analysis of the extracellular matrix (ECM) components fibronectin by immunofluorescence and collagen I by flow cytometry. **E.** Viability and percentage of recovered working cell bank (WCB) cells after thawing. Data are shown as mean ± SEM of at least 3 independent experiments.

graft progressively integrates with the host tissue, showing abundant neovascularization and re-epithelialization. There was no evidence of inflammatory reactions or immunological events around the patch, or any other side effect. However, as a specific follow-up analysis of the cells grafted in the patients was not performed, we cannot exclude the possibility that the cells implanted in the patients may have not survived the in vivo environment, and their positive effects could be associated to a stimulatory effect on host cells, as previously reported [52]. Infections are very common in burns patients and are the leading cause of death in hospitalized patients in the absence of smoke inhalation injury [53]. Infections were reported in two of the three patients before treatment with the hydrogel. One patient (Patient 3) was successfully treated for a blood infection with Meropenem after the application of the NFAH-F graft. The other patient (Patient 1) overcame the infection through local treatments, but later experienced a bronchial infection that could not be eliminated and he ultimately died of septic shock.

Measurements of skin pH and TEWL in Patient 2 improved from day 44 to day 64 after treatment with the NFAH-F, suggesting a progressive stabilization of the wound. Moreover, histological analysis indicated that the implanted allogeneic dermis integrated into the host tissues, with no significant alterations, although the interface between the grafted tissue and the recipient tissue could still be observed after this time. Our data indicate some improvement after the application of NFAH-F in terms of vascularization and re-epithelization, although an optimal situation of the skin bed, particularly in terms of absence of infection, could have likely improved the success rate of the treatment in these patients.

Although the true biosafety of this technology should be tested in a larger cohort of patients, our preliminary analysis of three patients for compassionate use treatments suggests that the treatment is safe and free from detectable adverse events related to the NFAH-F therapy. Treatment was overall well tolerated and, in all cases, the patients remained alive until definitive treatment (autologous culture skin: UGRSKIN [38,39]), which was our main objective, however one of the patient died a 54 days after the definitive treatment. Thus, NFAH-F can be safely used as a temporary dressing in situations where there is lack of cadaveric skin allografts, although the results could likely be improved by functionalizing the hydrogel with antibiotics or antimicrobials, as previously suggested [8,54]. Furthermore, a secondary impermeable dressing could decrease the evaporation rate. Possibilities for this secondary dressing include covering the NFAH-F with allograft skin or cultured keratinocytes [55,56]. Future studies should explore these options to choose the best approach for acutely treating massively burned patients. In addition, the NFAH-F graft could also be used to treat other conditions in which a connective tissue is desirable (e.g., dural effect, breast reconstruction [57]). Indeed, other uses for NFAH-F should be explored in future studies.

Finally, there are several limitations to our study that warrant mention, including the small sample size, and the fact that the patients treated represented severe stages in which improvement is difficult to achieve. In addition, further studies focused on determining the fate and survival of the cells grafted in vivo are in need. Nevertheless, our study

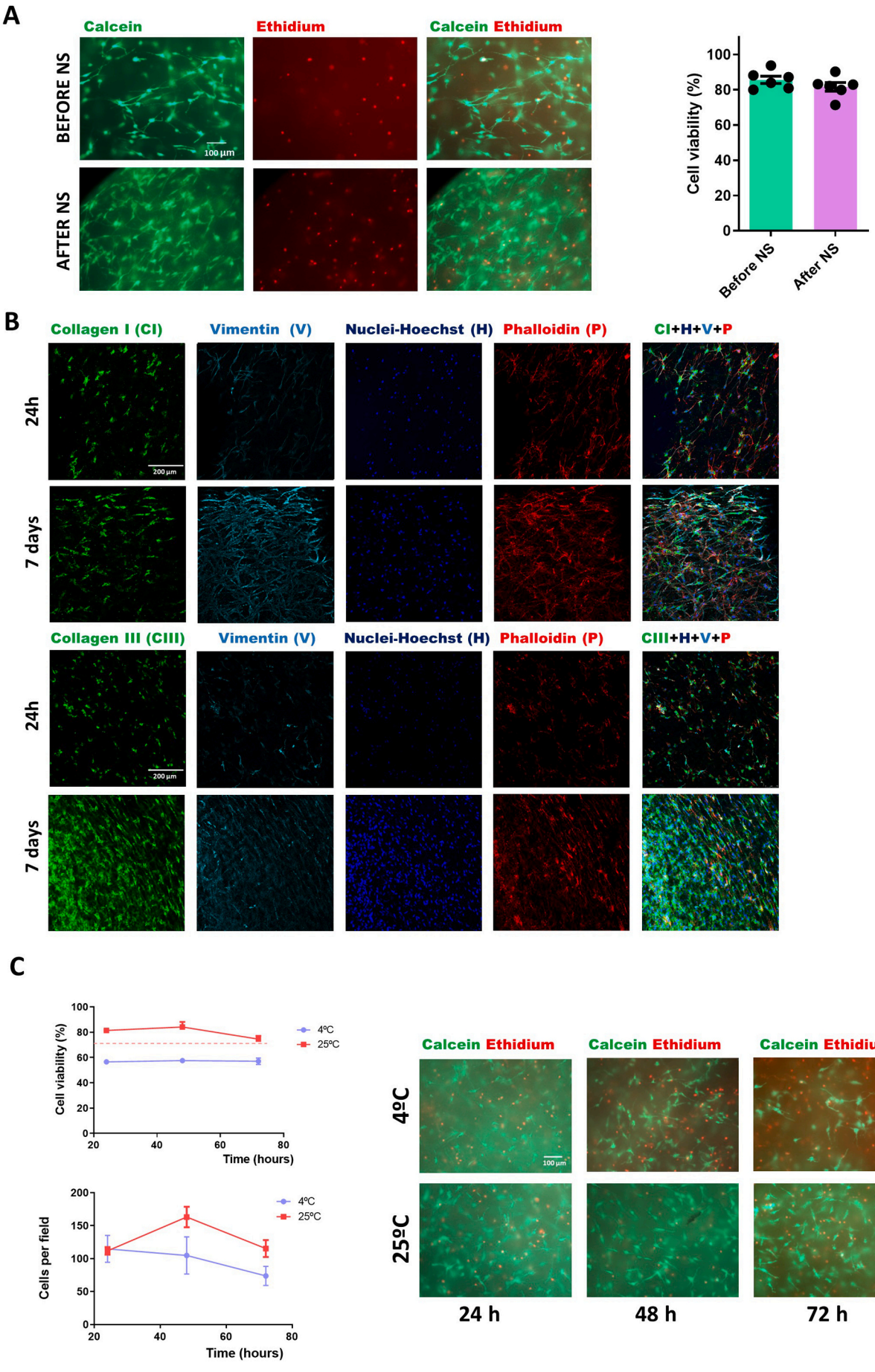

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Fig. 5. Production of nanostructured fibrin-agarose hydrogels with embedded allogeneic dermal fibroblasts (NFAH-F). A. Cell viability of fibroblasts immersed within the fibrin-agarose hydrogels. Live cells are detected in green (Calcein), and dead cells are shown in red (ethidium) before and after nanostructuration (NS). Plot data are shown as mean ± SEM of 6 independent experiments. **B.** Confocal immunofluorescence images depicting staining for extracellular matrix (ECM) markers (collagen I and III) as part of the characterization of the NFAH-F production. Fibroblasts embedded on NFAH were positive for ECM markers after 24 h and 7 days of culture in the NFAH. **C.** Viability and number of cells of the NFAH-F stored for 72 h in Ringer Lactate at 4 and 25ºC. Images of live (calcein) and dead (ethidium) cells at different time points are also shown.

provides proof-of-concept data to guide future larger prospective studies.

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CRediT authorship contribution statement

Blanca Arribas-Arribas: Conceptualization, Investigation, Data curation, Writing – original draft. **Beatriz Fernández-Muñoz**: Methodology, Investigation, Data curation, Visualization. **Rafael Campos-Cuerva:** Methodology, Investigation. **Miguel Ángel Montiel-Aguilera:** Methodology, Investigation. **María Bermejo-González:** Methodology, Investigation. **Isabel Lomas-Romero:** Methodology, Investigation. **María Martín-López:** Methodology, Investigation**. Rosario Mata Alcázar-Caballero, María del Mar Macías-Sánchez:** Resources, Data curation. **Fernando Campos**: Investigation, Resources. **Miguel Alaminos**: Conceptualization, Investigation, Resources. **Tomás Gómez-Cía:** Conceptualization, Methodology, Resources. **Purificación Gacto**: Investigation, Resources. **Gloria Carmona**: Supervision, Funding acquisition. **Mónica Santos-González**: Methodology, Investigation, Data curation, Supervision. **All authors**: Writing – review & editing.

Declaration of Competing Interest

MSG, BFM and ILR are authors of a patent application for the use of different media based on HPL [58] (nº application Spanish Patent Office: P201730713). MA is author of a patent for the use of nanostructured fibrin agarose hydrogels [59] (Patent number PCT/ES2010/070569). RCC. and BFM. are authors of a patent application for the use of NFAH as a haemostatic agent (N° application Spanish Patent Office: P201830346). The authors have no other conflicts interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115769.

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