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RESEARCH ARTICLE

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Optimizing large-scale autologous human keratinocyte sheets for major burns—Toward an animal-free production and a more accessible clinical application

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Abstract

Background and Aims: Autologous keratinocyte sheets constitute an important component of the burn wound treatment toolbox available to a surgeon and can be considered a life-saving procedure for patients with severe burns over 50% of their total body surface area. Large-scale keratinocyte sheet cultivation still fundamentally relies on the use of animal components such as inactivated murine 3T3 fibroblasts as feeders, animal-derived enzymes such as trypsin, as well as media components such as fetal bovine serum (FBS). This study was therefore aimed to optimize autologous keratinocyte sheets by comparing various alternatives to critical components in their production.

Methods: Human skin samples were retrieved from remnant operative tissues. Cell isolation efficiency and viability were investigated by comparing the efficacy of porcine-derived trypsin and animal-free enzymes (Accutase and TrypLESelect). The subsequent expansion of the cells and the keratinocyte sheet formation was analyzed, comparing various cell culture substrates (inactivated murine 3T3 fibroblasts, inactivated human fibroblasts, Collagen I or plain tissue culture plastic), as well as media containing serum or chemically defined animal-free media.

Results: The cell isolation step showed clear cell yield advantages when using porcine-derived trypsin, compared to animal-free alternatives. The keratinocyte sheets produced using animal-free serum were similar to those produced using 3T3 feeder layer and FBS-containing medium, particularly in mechanical integrity as all grafts were liftable. In addition, sheets grown on collagen in an animal-free medium showed indications of advantages in homogeneity, speed, reduced variability, and differentiation status compared to the other growth conditions investigated. Most

Laura Frese and Salim Elias Darwiche first authorship.

importantly, the procedure was compatible with the up-scaling requirements of major burn wound treatments.

Conclusion: This study demonstrated that animal-free components could be used successfully to reduce the risk profile of large-scale autologous keratinocyte sheet production, and thereby increase clinical accessibility.

KEYWORDS

autograft, epidermal skin engineering, keratinocytes, process development, xenofree

1 | INTRODUCTION

Autologous keratinocyte sheets constitute an important component of the burn wound treatment toolbox available to a surgeon. They can be used to promote wound closure and re-establish epidermal barrier protection. That is why the use of autologous keratinocyte sheets, grown from a patient's own skin cells, has been successfully applied for many years and is widely considered a life-saving procedure.[1-3] This becomes particularly important in patients with severe burns over 50% of their total body surface area (TBSA),[3] in which the use of split-thickness skin grafts is simply not possible, despite it being a gold-standard in skin reconstruction.[4,5] Indeed, any autologous skin cell therapy would require harvesting a sample of intact skin, which is in short supply in these cases.

With a reported average TBSA of 1.88 m^2 in male patients and 1.66 m^2 in female patients,[6] at least 1 m^2 of epidermal coverage would have to be restored as soon as possible in these polytraumatized major burn patients (>50% affected TBSA, with second- or third-degree burns). The challenge of upscaling epidermal coverage, starting from a 4 cm^2 skin biopsy to produce sheets covering 1 m^2 of TBSA, is significant. The technology of autologous keratinocyte sheet creation has relied on the methodology first described by J. Rheinwald and H. Green in the 1970s.[7,8] This method, while adapted over the years and used in a variety of clinical studies,[3,9-11] still fundamentally relies on the use of animal components such as inactivated murine 3T3 fibroblasts as feeders, animal-derived enzymes such as trypsin, as well as media components such as fetal bovine serum (FBS).

The murine 3T3 feeder cells, used to enhance cell proliferation,[12] must be inactivated using arresting mutagenic agents such as gamma irradiation or Mitomycin-C[13,14] and can risk introducing remnants of murine components in the transplanted human sheets.[15] Trypsin, used to isolate and dissociate cells, is extracted from the porcine pancreas, which carries the risk of contamination with adventitious agents, such as certain viruses that are widespread among pigs.[16] FBS can also be problematic, potentially carrying bovine spongiform encephalopathy (BSE) and animal viruses that could cause disease transmission.[17] The use of FBS requires extensive and expensive testing to ensure the clinical-grade serum is used and to secure against batch-to-batch variability in efficacy.[18]

Many advances in the field of cell culture have brought alternatives to the use of trypsin,[19] as well as chemically defined, serum-free media alternatives that would not require the use of murine feeder layers.[20-23] In fact, protein coatings such as Collagen IV have also been used as substrates to promote keratinocyte proliferation,[24] but the scalability of such systems has not been shown. Establishing a method of cultivating autologous keratinocyte sheets, while ensuring scalability, as well as an improved safety profile, would make autologous keratinocyte sheets much more economically accessible to burn units worldwide.

This study was therefore aimed to optimize autologous keratinocyte sheets by comparing various alternatives to critical components in their production. Specifically, cell isolation efficiency and viability (Figure 1, phase A) was investigated by comparing various enzymes (animal-derived and animal-free origins). The subsequent expansion of the cells and the keratinocyte sheet formation (Figure 1, phase B and C) was investigated, comparing various cell culture substrates, as well as media containing animal-derived components or chemically defined media. The ultimate goal of the comparative study was to reduce the risk profile of large-scale autologous keratinocyte sheet production to increase clinical accessibility.

2 | MATERIAL AND METHODS

2.1 | Source of human keratinocytes

For the present study, human keratinocytes were isolated from skin biopsies of patients undergoing plastic surgery ($n_{total} = 29$) at the Division of Plastic Surgery and Hand Surgery of the University Hospital Zürich, Switzerland. The skin tissue was obtained by full-thickness ($n = 5$) or split-thickness ($n = 24$) collection from female patients of 42.4 ± 12.1 years of age. Samples were retrieved from remnant operative tissues from the breast or abdomen region, which would otherwise be discarded. This was done following procedures approved by the local ethics committee (KEK-ZH-2014-0197). The assays described below were performed using a subpopulation of samples from the 29 total biopsies harvested for this study. For each assay, the subpopulation of biopsy samples used is specified.

2.2 | Enzymatic isolation of keratinocytes

Samples from 5 female donors, 41.7 ± 13.9 years of age, were used to test three enzymes for the isolation of keratinocytes. The skin was microdissected in equal pieces of approx. 10 × 10mm and digested with dispase solution (12 U/mL PBS) for 15–18 hours at 4°C, as previously described.[25] Thereafter, the epidermis and dermis were mechanically

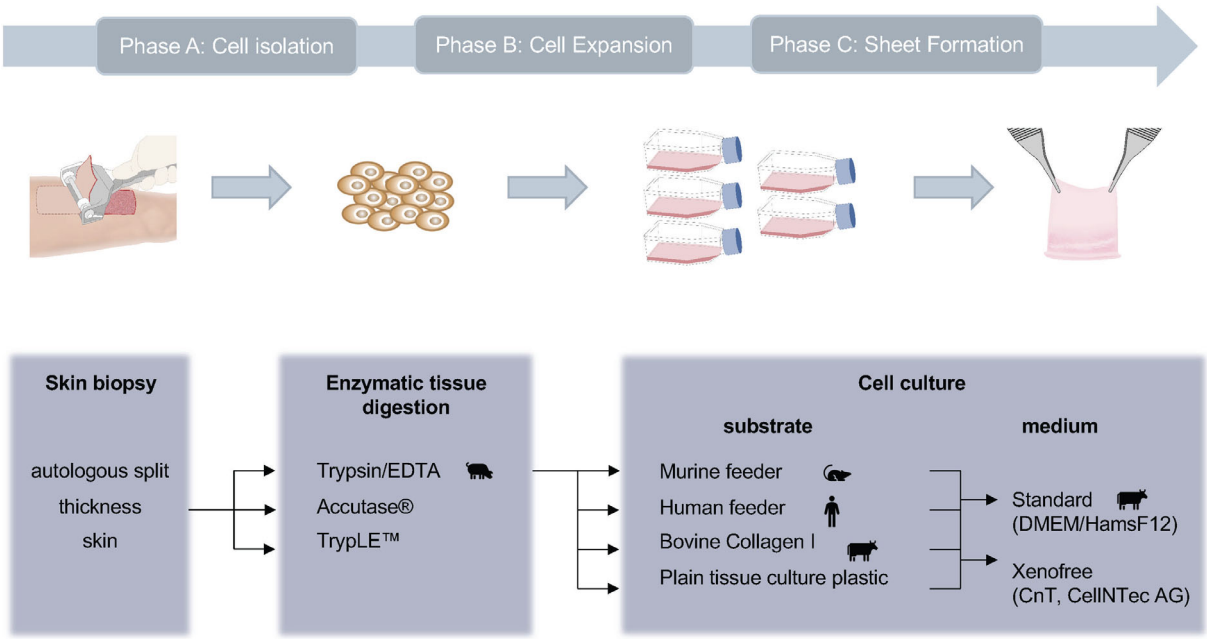

FIGURE 1 Study design. After human skin biopsy collection, various animal-derived and xenofree enzymes were investigated to obtain their best yield and the highest cell isolation efficiency (Phase A). For the expansion of the cells, as well as for the sheet formation (Phase B and C), various cell culture substrates, as well as media, were investigated to produce autologous keratinocyte sheets with mechanical and structural integrity. (Pictures C. De Simio)

separated. To investigate the use of xeno-free enzymatic alternatives compared to the commonly used porcine trypsin, StemPro Accutase 1 × (Gibco), and TrypLE Select Enzyme 1 × (Gibco) were tested in parallel to trypsin/EDTA (Gibco). Therefore, the epidermal layer was separated into three equal pieces and digested with trypsin/EDTA 5 × (0.25% Trypsin, 0.1% EDTA), Accutase, or TrypLE for 25 minutes at 37°C to get the epidermal cell fraction. With Accutase and TrypLE, the cell isolation efficiency was also tested with an extended incubation time of 60 minutes. In order to halt the digestion with trypsin/EDTA, a 3.75 mg/mL soybean trypsin inhibitor was added (1:1). In the case of Accutase or TrypLE, the enzymes were simply diluted with PBS (1:1). Acridine orange was used to stain the entire cell population, and 4′,6-Diamidin-2-phenylindol (DAPI) was used to specifically stain the DNA of dead cells. The number of isolated cells, as well as their viability, was determined using a cell counter (NucleoCounter NC-200, Chemometec).

2.3 | Keratinocyte 2D culture substrates

Murine 3T3 feeder cells (generously provided by Prof. Dr. Karl Frei, University Hospital Zurich) were inactivated with 10 μg/mL Mitomycin C (Sigma) for 2 hours at 37°C. Thereafter, the inactivated 3T3 were plated at 5,000 cells/ cm^2 in order to form a murine feeder layer for keratinocyte culture. In parallel, as allogenic human feeder cells, human foreskin fibroblasts (HFF, ATCC SCRC-1041) were tested in order to avoid the use of xenogenic feeder cells. The HFF were also inactivated using Mitomycin C, and thereafter, seeded with 5,000/cm^2, consistent with the murine ones (3T3). The use of the extracellular matrix component Collagen I was also examined instead of feeder cells as a substrate to avoid the risks associated with xenogeneic and allogeneic cell co-cultures. To that end, clinical-grade bovine Collagen I (Symatese) was used in a concentration of 6 μg/cm^2 to coat tissue culture plastic prior to keratinocyte seeding. While the source of Collagen was bovine, the processing ensures that Collagen batches are free of agents that could transmit spongiform encephalopathies. The fourth and last condition tested was plain tissue culture polystyrene without any coating or feeder cell layer.

For this assay, enzymatically isolated epidermal cells keratinocyte cells from biopsies of 18 female patients (age 42.3 ± 11.5 years) were then plated at a density of 30,000 viable cells/cm^2 on the four substrates, namely a feeder layer of inactivated murine cells (3T3), a feeder layer of inactivated human fibroblasts (HFF), Collagen I and plain tissue culture plastic (T75, EasYFlasks, Nunc). Cells of all conditions were incubated at 37°C in a humidified atmosphere (5% CO_2).

2.4 | Keratinocyte culture media

With regard to xenogenic medium supplements such as, for example, bovine serum, the culture medium has to be optimized in terms of safety and reliability. In this study, a standard keratinocyte medium, adapted from Rheinwald and Green,[8] was utilized as a benchmark. The standard medium, used in the current production facility, was composed of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 medium (3:1), supplemented with 10% fetal bovine serum (Gibco, cat.No.10270), 0.5 μg/mL hydrocortisone (Solu-Cortef, Pfizer), 9.8 ng/mL cholera enterotoxin (Sigma), 42 ng/mL epidermal growth

factor (Sigma), 7.5 μg/mL insulin (Sigma), and 1 mM glutamine (Gibco). This medium also contained antibiotic supplementation of 100 IU/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma), and 24 μg/mL gentamicin (Sigma). In contrast, a xenofree medium was also investigated, namely the chemically defined and xenofree CnT-Pr medium (CELLnTEC advanced cell systems AG). According to the manufacturer, the formulation elements for the expansion and differentiation of the human keratinocytes include 21 amino acids, 19 minerals and trace elements, including selenium, manganese, and zinc, 14 vitamins, including folate, niacinamide, and biotin, glucose, pyruvate, and buffers. Furthermore, up to six additional recombinant growth factors and cofactors, including insulin, hydrocortisone, EGF, and FGF, are supplements of the medium (two additional growth factors were considered CellNTec trade secrets and were not disclosed to the authors). The medium does not contain serum, cholera toxin, or phenol red.

Both media were changed every 2 to 3 days following keratinocyte seeding, until cells reached 80% to 90% confluency. The influence of medium and substrate regarding cell morphology and growth behavior was observed using an inverted light microscope (ZEISS Axiovert 40 CFL and ZEISS Axioplan II; Carl Zeiss AG). Additionally, the days required until confluence was reached were recorded for both media used and with all four culture substrates (3T3, HFF, Collagen, plain).

2.5 | Keratinocyte sheet formation

For this assay, cells from biopsies of 6 female patients (age 39.2 ± 9.7 years) were isolated using trypsin/EDTA digestion method and brought to confluence in a primary expansion. Then keratinocyte sheet formation was initiated after cell passaging (Passage 1) by plating the cells at a density of 5,000 viable cells/cm^2 on the four different coatings (3T3, HFF, Collagen, plain) in T115 peel off cell culture flasks (TPP), using standard and xenofree media in parallel. When the cells reached 80% to 90% confluency after 3 to 5 days, the keratinocyte differentiation was initiated by adding the appropriate differentiation medium. In the sheet production approach described by Rheinwald and Green,[7,8] the standard medium was further used for the sheet formation, with the addition of 1.2 mM $CaCl_2$. When using the xenofree medium for cell expansion, a corresponding xenofree differentiation medium was used for sheet production, namely CnT-Pr-2D (CellNTec), supplemented with 1.2 mM $CaCl_2$. After 21 days of culture, the sheets were harvested enzymatically by treating with 0.25% (w/v) dispase II (Life Technologies) at 37°C for 10 minutes. After flushing the sheets with phosphate-buffered saline (PBS), the skin grafts were carefully detached and lifted up.

2.6 | Histological characterization

In order to qualitatively evaluate the tissue organization, sheet samples were fixed with 4% formalin for 1 hour at RT, transferred into an agarose plug (Lonza, Switzerland), and carefully spread using two tweezers. Stepwise dehydration through a series of graded alcohols was performed, followed by paraffin embedding (Paraplast, Leica, Biosystems Switzerland AG, Muttenz) and slicing into 3 μm sections. For assessing the tissue composition of the keratinocyte sheets, Hematoxylin Eosin staining was performed. All sections were analyzed by a single operator using an inverted light microscope (ZEISS Axiovert 40 CFL and ZEISS Axioplan II; Carl Zeiss AG). HE stained samples were assessed for the overall architecture, including the layering, presence of a keratinized layer, as well as keratohyalin granules and qualitative cell/ECM compactivity. The thickness of the sheets was evaluated using the Image J software ($n = 3$; 4 measurements per donor).

2.7 | Immunohistochemical characterization

The expression profile of the cells within the sheets was analyzed in order to confirm keratinocyte cell identity (keratin K5/K8), as well as show the early (Desmoglein 3) and late (Filaggrin) differentiation markers. Specifically, the paraffin-embedded tissue sections (5 μm) were deparaffinized and rehydrated through a graded ethanol series. For K5/K8 staining, the deparaffinized sections were unmasked for 20 minutes at 37°C with protease (0.05% in distilled water), for Desmoglin 3 with proteinase K (DAKO) for 5 minutes. Paraffin sections for Filaggrin staining were transferred to Target Retrieval Solution High pH 9 (K8004, Dako Denmark A/S, Glostrup, Denmark) in a Dako PT Link (PT100/PT101, Dako Denmark A/S) for 20 minutes at 97°C. Anti-Keratin K5/K8 (Progen 61031, 1:10), anti-Desmoglein 3 (Clone 3G133, Abcam, ab14416, 1:100), as well as anti-Filaggrin (Abcam, ab17808, 1:100) was used following a standard immunohistochemistry staining protocol on a Dako Autostainer Link48 Instrument (Dako Denmark A/S). The visualization system consisted of a secondary Cy2 conjugated AffiniPure antibody (Jackson Immuno Research, goat antimouse) for K5/K8 detection and for Desmoglein and Filaggrin detection Dako K4001 or Dako K4008 EnVision HRP/DAB system, respectively, and Hematoxylin as a counterstain. The expression profiles of Keratin, Desmoglein 3, and Filaggrin were qualitatively assessed.

2.8 | Statistical analysis

Quantitative data are presented as mean ± SD. For statistical comparison of the differences regarding enzymatic cell isolation efficiency, culture duration on different substrates, as well as thickness of keratinocyte sheet after cultivation on different substrates and in different culture systems, one-way ANOVA combined with a posthoc TUKEY analysis was performed (SPSS 17.0, IBM, Somers, New York). Results were significantly different for a P-value <.05.

3 | RESULTS

3.1 | Enzymatic isolation of keratinocytes

The isolation of the epidermal fraction from human skin biopsies was successful using trypsin/EDTA, as well as using animal-free

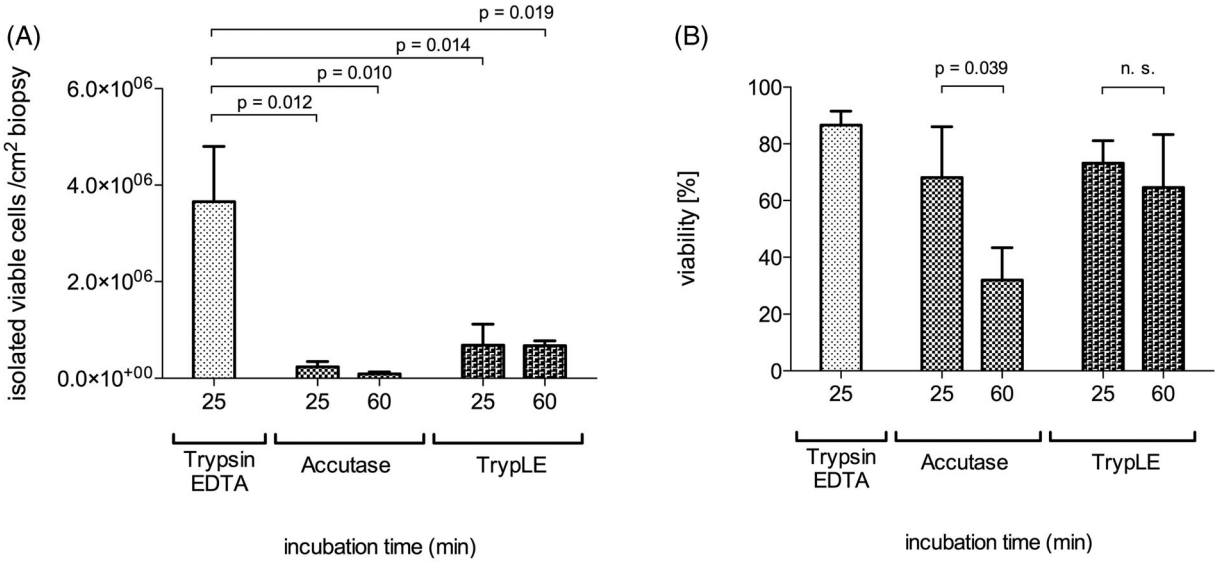

FIGURE 2 Optimization of human keratinocyte enzymatic isolation comparing Trypsin/EDTA, as well as animal-free alternatives Accutase and TrypLE Select were investigated using the standard incubation time of 25 minutes at 37°C. For increasing the isolation efficiency of Accutase, as well as TrypLE Select, the incubation time was extended up to 60 minutes. The number of isolated viable cells A, as well as the viability B, of the isolated cells, were determined after the standard incubation time of 25 minutes at 37°C or, for the animal-free alternatives, an extended incubation time of 60 minutes (Mean ± SD, n = 5)

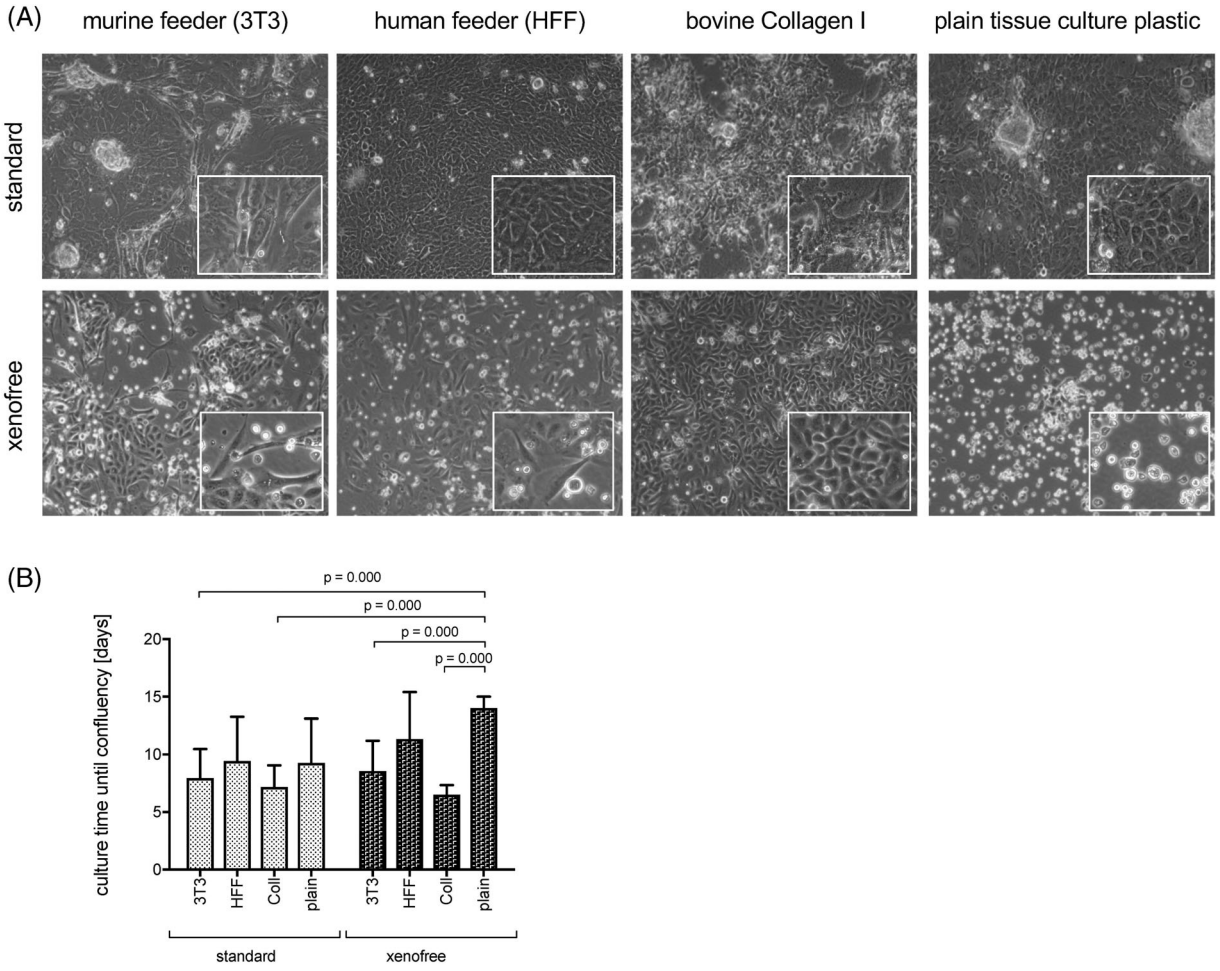

FIGURE 3 Substrates for keratinocyte cultivation. Four substrates were tested, including murine 3T3 fibroblasts (3T3), human foreskin fibroblasts (HFF), Collagen I (Coll), and tissue culture plastic (plain). Either the standard medium containing FBS or the chemically defined xenofree medium was used. The influence of medium and substrate was examined in terms of A, morphology and B, culture duration until confluency. Monolayer cultures A, are shown in 10 × magnification with an additional 2-fold zoom image section. B, Bars indicate mean ± SD

alternatives such as Accutase and TrypLE Select. However, the isolation efficiency was notably different among the three, with trypsin/EDTA outperforming TrypLE by 5 times and Accutase by 12 times (Figure 2A). Using an extended digestion time of 60 minutes, compared to the standard 25 minutes, did not lead to significant improvements in cell isolation efficiency with Accutase or TrypLE. The longer digestion time did, however, notably affect cell viability, particularly with Accutase (Figure 2B).

3.2 | Proliferation on 2D substrates

Cells seeded on the four different substrates (3T3, HFF, Collagen I, plain tissue culture plastic) and cultivated in two different media (standard medium containing FBS and chemically defined animal-free medium) attached and proliferated in all tested conditions (Figure 3A). Nevertheless, using the standard medium, the epidermal keratinocyte cultures notably had some cross-contaminating fibroblasts (Figure 3A, upper row). Using the xenofree medium, which is designed as a selection medium specifically favoring keratinocyte proliferation, the cultures showed no fibroblasts and were more homogenous (Figure 3A, lower row).

The proliferation time until cell confluency averaged 9.5 ± 3.5 days for those cultivated in standard medium and 8.1 ± 2.8 days for the cells in xenofree medium (Figure 3B). Under both medium conditions, the fastest time until confluency was recorded when cultured on Collagen I (7.2 ± 1.9 days in standard medium and 6.5 ± 0.8 days in xenofree medium). Notably, the lowest donor-to-donor variability in proliferation rate was

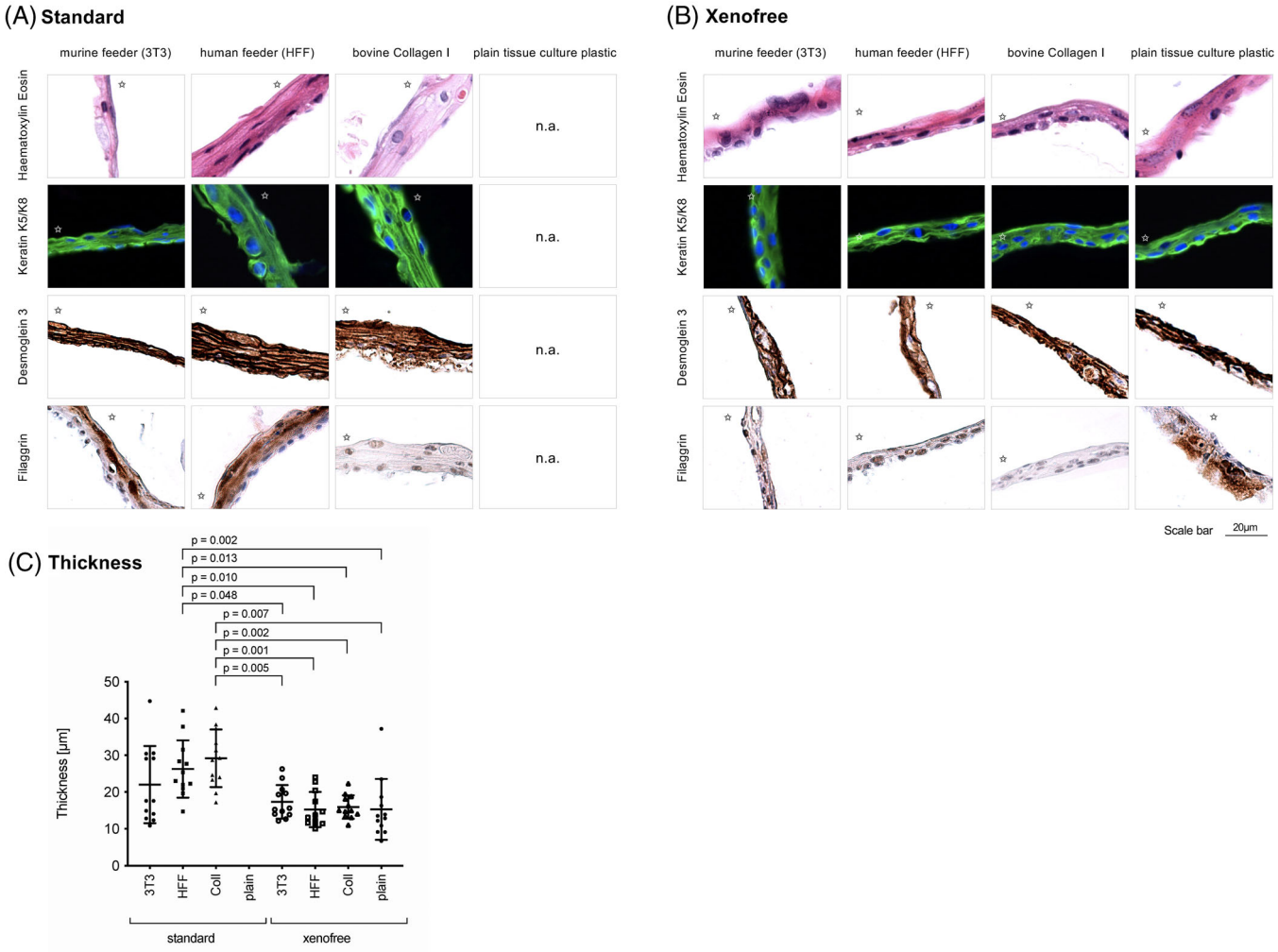

FIGURE 4 Keratinocyte sheet formation and maturation depending on culture medium and substrate. Keratinocyte sheets cultivated in either standard A, or xenofree medium B, were harvested after 21 days of culture. Hematoxylin Eosin staining reveals tissue structure (A, B first row). Expression of Keratin K5K8 (green) is shown, as well as cell nuclei (DAPI in blue) (A, B second row). The early differentiation marker Desmoglein 3 is shown in brown (A, B third row), and the late differentiation marker Filaggrin is shown in brown (A, B fourth row). Sheets could not be formed on plain tissue culture plastic using standard medium (A, fourth column; n.a. indicates not available). Keratinocyte thickness was quantified, taking two measurements per sample, with six samples per group (mean ± SD and individual sample point scatter shown in C). Scale bar for all panels indicates 20 µm, the asterisk ⋆ marks the apical side of the keratinocyte sheet

observed with cells cultured in xenofree conditions on Collagen with a SD of 0.89 days, a third of the variability using the benchmark condition (standard medium, 3T3 feeder), which had a SD of 2.55 days.

3.3 | Keratinocyte sheet formation

Keratinocyte differentiation and keratinocyte sheet formation was investigated, comparing various medium-substrate culture

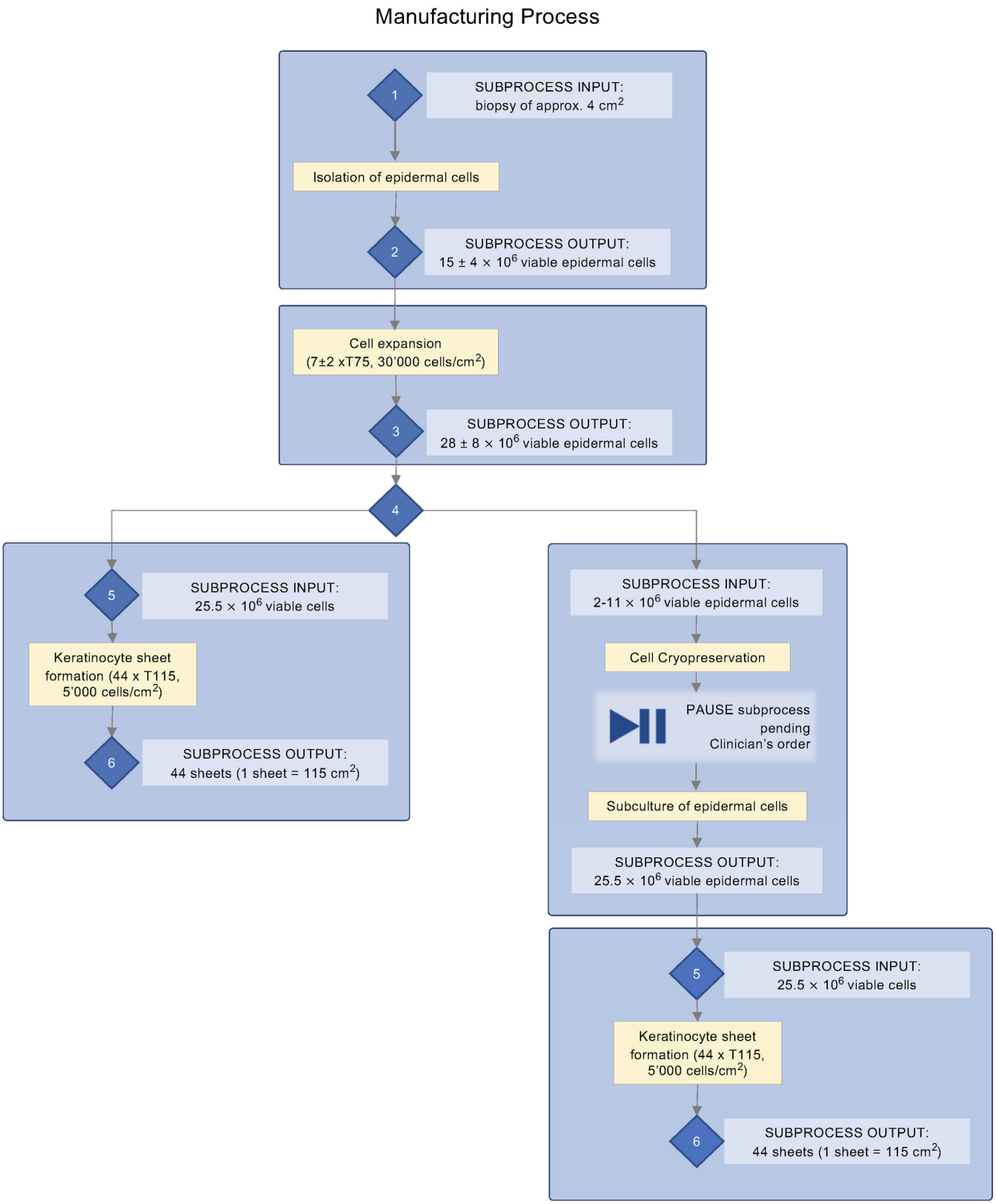

FIGURE 5 Large scale calculation for the keratinocyte sheet production procedure for major burn wound treatments, namely using a 4 cm^2 skin biopsy to produce 1 m^2 of keratinocyte sheets in a 21-day period

combination groups (Figure 4). Except when grown on plain tissue culture plastic using standard medium (Figure 4A, column "plain"), all other conditions yielded a successfully formed sheet of cells that had sufficient mechanical integrity to be lifted (Figure 4A, B).

Analyzing the keratinocytes histologically and immunohistochemically, the output with regard to the presence of keratinocytes and the early differentiation stage was comparable between the 3T3, HFF, and Collagen I conditions. The expression of K5K8 confirmed the presence of keratinocytes within all sheets. The cells in all sheets were Desmoglein 3 positive, a marker of early-stage differentiation. However, differences occurred in the late-stage differentiation marker Filaggrin. A slightly more advanced and stratified Filaggrin expression was found in the two feeder conditions (3T3, HFF) in combination with the standard medium. In contrast, a lower and more heterogeneous expression pattern was observed in the xenofree samples. The lowest Filaggrin expression was seen in Collagen groups.

Regarding structure, the sheets cultivated with the standard medium were thicker and less compacted compared to the sheets cultivated in xenofree media, which were more compacted with cells closer to each other and less interstitial ECM. This observation was also confirmed by the thickness measurements (Figure 4C), with xenofree cultured sheets about 25% to 50% thinner than those cultured in standard medium ($P < .05$ comparing standard medium HFF or Col sheet thicknesses with all xenofree sheet thicknesses). However, keratinocyte sheets cultured in standard medium showed a more heterogeneously distributed thickness with higher variances compared to xenofree cultured sheets, which exhibited an increased uniformity in thickness. Notably, even though sheets grown in the xenofree medium may have been thinner overall, they were nevertheless mechanically stable and liftable.

4 | DISCUSSION

This study successfully compared the use of alternative, animal-free components in critical steps of the autologous keratinocyte sheet production process: keratinocyte cell isolation, cell expansion, and sheet formation. On the one hand, the cell isolation step showed clear cell yield advantages when using porcine-derived trypsin, compared to animal-free alternatives. On the other hand, the medium and substrate comparative study demonstrated the feasibility of expanding keratinocytes and producing sheets using a chemically defined, animal-free medium and a Collagen coating, which would replace murine feeder layers without compromising scalability. The produced keratinocytes were largely similar to those produced using the standard method (3T3 feeder layer and FBS-containing medium), particularly in mechanical integrity, as all grafts were liftable. In addition, sheets grown on Collagen in an animal-free medium showed indications of advantages in homogeneity, speed, reduced variability, and differentiation status compared to the other growth conditions investigated. Most importantly, the procedure was compatible with the upscaling requirements of major burn wound treatments, namely using a 4 cm^2 skin biopsy to produce 1 m^2 of keratinocyte sheets in a 21-day period (Figure 5).

The knowledge about the risks of disease transmission due to the use of animal components has increased within the last decades, causing the requirements of the regulatory authorities to drastically change and become stricter.[26] Replacing as many components of animal origin as possible with xenofree ones is preferable for clinical applications nowadays, thus guaranteeing safer and more standardized therapies.[27] The move toward animal-free components would also reduce the risk profile of cell therapy production, potentially reducing the need for extensive testing, thereby increasing economic accessibility to life-saving procedures for major burn victims.

All three enzymatic candidates, the porcine-derived trypsin, as well as the two animal-free alternatives, Accutase and TrypLESelect, were able to isolate cells from the human skin biopsies. However, the yield was significantly different, which would greatly impact the scale-up potential for major burns. Notably, the precision of biopsy size measurement could be improved in this assay. However, due to skin contraction, attempting a more precise measurement of the skin biopsy size may jeopardize the integrity or the sterility of the fragile samples, and this methodological variability alone would not account for the differences observed in cell yield. Indeed, based on the cell isolation efficiency found, Accutase and TrypLE would isolate just enough cells from a 4 cm^2 skin biopsy to produce an estimated 0.05 and 0.12 m^2 of keratinocyte sheets, respectively, instead of the 1 m^2 estimated yield using trypsin. While Accutase and TrypLESelect have been described to be gentler for other applications such as cell passaging, for example, this study shows the use of trypsin efficiently isolated cells from the biopsy. The risks associated with its porcine origin, however, may be mitigated by the supplier's gamma radiation treatment, which would reduce the risk of disease transmission.[28]

Working with cell-based products, the medium component that is the most challenging to replace due to its composition is the serum. Some reports highlighted the need for serum to ensure epidermal cell stratification.[29] Serum contains numerous components and factors that influence the growth and differentiation of cells and their specific functionality. However, the composition of serum is poorly defined and has a tremendous lot-to-lot variability.[30] Because of safety concerns due to the possible transfer of animal proteins[31,32] and pathogens to patients,[33] the clinical application requires extensive testing prior to releasing a cell-based product. In recent years, various serum-free and chemically defined media were developed[34,35] to cultivate human epidermal cells. Data from this present study demonstrated that the standard serum-containing medium formulation could be substituted with the animal-free CnT-Pr medium from CELLnTEC advanced cell systems AG. In fact, using the CnT-Pr medium showed an improved homogeneity in cultured cells, as well as homogeneity in keratinocyte sheet structure and a notably reduced variability in thickness. Using an animal-free, chemically defined medium for large-scale autologous keratinocyte sheet production would have notable advantages, particularly in removing the need for serum batch validation and associated potential variability.

The use of substrates to support epidermal cell cultivation has been widely described. The use of feeders, particularly for serum-free cultivation of keratinocytes, can be a necessity in order to avoid

unwanted effects such as poor cell adhesion, slow cell proliferation, or insufficient epidermal stratification.[29,36] Concerns surrounding the use of inactivated murine 3T3 fibroblasts as feeder cells have prompted groups to investigate alternative feeder systems, some involving human cells.[27] The preparation of feeder layers requires cell inactivation using mutagenic agents such as Mitomycin-C or gamma-irradiation. However, the risks involved with trace Mitomycin-C remnants and DNA disruption,[37,38] as well as risks using gamma-irradiation and immunologic activation[31,39] drive the need for a departure from the use of feeder layers. A possible alternative to feeder layers, such as a fibroblast-embedded three-dimensional fibrin dermal matrix-like human plasma, has also been proposed.[40] However, the availability of autologous plasma from severe burn patients suffering from extensive and acute skin loss is very limited. Coating tissue culture surfaces with extracellular matrix components, such as Collagen I, have also been explored as alternatives to feeder layers.[36] This present comparative study demonstrated that human keratinocyte sheets with sufficient mechanical integrity could be grown in serum-containing and serum-free media onto a Collagen-coated surface. In fact, the serum-free Collagen substrate group required the shortest time to reach cell confluence. In the treatment of burn patients, the factor time plays a very crucial role since failure to treat patients fast enough may result in sepsis, multiple organ failure, and in the end, the death of the patient.[41] In an effort to eliminate the need for an animal-derived extracellular matrix coating, a plain tissue culture plastic group was added. However, keratinocytes could not form a sheet on plain tissue culture plastic when grown in serum-containing media and in the serum-free group, the time to the confluence was doubled compared to the Collagen group. The Collagen used in this study was of bovine origin and was manufactured following the safety guidelines and current knowledge regarding the risk associated with Transmissible Spongiform Encephalopathy (TSE) and Bovine Spongiform Encephalopathy (BSE). Due to its clear advantages in supporting keratinocyte expansion and sheet formation, the potential risk associated with its use can be mitigated by procuring certified clinical-grade products. Its risk profile would also be much lower compared to feeder layer systems.

Overall, this study presents a comparative analysis of the keratinocyte sheet forming capability of various culture systems using animal-derived or animal-free components. The data demonstrate the possibility to reduce the risk profile of large-scale human autologous keratinocyte sheet production by substituting critical components such as bovine serum and a murine feeder layer with animal-free alternatives. Furthermore, it shows that protocols using animal-free alternatives may, in fact, improve the overall quality of the keratinocyte sheets, which may, in turn, improve the graft take rate and wound healing enhancement in vivo. It would be valuable, in future studies, to further characterize keratinocyte sheets by looking at stemness markers and adhesion molecules and understand how they may enhance graft take rate and wound healing capacity.

The need for an efficient xeno-free culture system for clinically compliant applications is currently very topical, not only for the treatment of burn patients but also for all other cell-based applications. In the field of cell therapy and tissue engineering, xeno-free culture conditions are currently being intensively investigated in accordance with official requirements. There are currently major developments, for example, in cell therapies based on the use of mesenchymal stem cells (MSC) to treat Crohn's disease, multiple sclerosis, graft-vs-host disease, type 1 diabetes, bone fractures, cartilage damage, and cardiac disease.[42,43] But also tissue engineering approaches such as the replacement of tissue-engineered heart valves, cartilage, bone, ligaments, etc. are developing xeno-free alternative production protocols to meet the regulatory requirements for clinical use.[44-47]

In conclusion, optimizing autologous keratinocyte sheet cultivation using animal-free components could increase access to this life-saving technology for major burn wound victims.

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CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest. All authors have read and approved the final version of the manuscript. Dr. med. Maurizio Calcagni had full access to all of the data in this study and has taken complete responsibility for the integrity of the data and the accuracy of the data analysis.

AUTHOR CONTRIBUTIONS

Conceptualization: Laura Frese, Salim Elias Darwiche
Data Curation: Laura Frese, Salim Elias Darwiche
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TRANSPARENCY STATEMENT

Dr med. Maurizio Calcagni affirms that this manuscript is an honest, accurate, and transparent account of the study being reported with no important aspects of the study being omitted; and that any discrepancies from the study as planned have been explained.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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