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Extracellular Matrix-Derived Hydrogels to Augment Dermal Wound Healing: A Systematic Review

Linda Vriend, MD,[1,2,i] Viktor Sinkunas, BSc,[3,4] Cristina P. Camargo, MD, PhD,[4] Berend van der Lei, MD, PhD,[2,5] Martin C. Harmsen, PhD,[1] and Joris A. van Dongen, MD, PhD[6]

Chronic, non-healing, dermal wounds form a worldwide medical problem with limited and inadequate treatment options and high societal burden and costs. With the advent of regenerative therapies exploiting extracellular matrix (ECM) components, its efficacy to augment wound healing is to be explored. This systematic review was performed to assess and compare the current therapeutic efficacy of ECM hydrogels on dermal wound healing. The electronic databases of Embase, Medline Ovid, and Cochrane Central were searched for *in vivo* and clinical studies on the therapeutic effect of ECM-composed hydrogels on dermal wound healing (April 13, 2021). Two reviewers selected studies independently. Studies were assessed based on ECM content, ECM hydrogel composition, additives, and wound healing outcomes, such as wound size, angiogenesis, and complications. Of the 2102 publications, 9 rodent-based studies were included while clinical studies were not published at the time of the search. Procedures to decellularize tissue or cultured cells and subsequently generate hydrogels were highly variable and in demand of standardization. ECM hydrogels with or without additives reduced wound size and also seem to enhance angiogenesis. Serious complications were not reported. To date, preclinical studies preclude to draw firm conclusions on the efficacy and working mechanism of ECM-derived hydrogels on dermal wound healing. The use of ECM hydrogels can be considered safe. Standardization of decellularization protocols and implementation of quality and cytotoxicity controls will enable obtaining a generic and comparable ECM product.

Keywords: extracellular matrix, hydrogels, wound healing, angiogenesis

Impact Statement

Extracellular matrix (ECM)-based hydrogels are biocompatible and harbor growth factors that can instruct tissue healing. Their application is a novelty in (pre)clinical wound healing treatment. This systematic review provides an overview of the current evidence for ECM hydrogels in enhancing wound healing and an extensive overview of the decellularization procedures used. Lastly, challenges and future directions to standardize decellularization procedures and implement quality controls are proposed.

Introduction

Chronic dermal wounds have a high prevalence worldwide. In developed countries, there is an estimate of 1–2% of the general population to have a dermal wound in lifetime.[1] Over 6.5 million people suffer from chronic wounds in the United States alone.[2] According to Medicare, health care costs for all chronic wound types ranged from $28.1 to $96.8 billion in the United States of America per year.[3,4] Current wound care lacks efficacy because most therapies focus on a single process related to wound healing. For example, clinical application of *in vitro*-grown skin

Departments of [1]Pathology and Medical Biology and [2]Plastic Surgery, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands.
[3]Instituto do Coração (InCor), Hospital das Clinicas HCFMUSP, Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil.
[4]Plastic Surgery Laboratory, Department of Plastic Surgery and Microsurgery, University of São Paulo, São Paulo, Brazil.
[5]Bey Bergman Clinics, Hilversum, The Netherlands.
[6]Department of Plastic Surgery, University of Utrecht, University Medical Center Utrecht, Utrecht, The Netherlands.
[i]ORCID ID (https://orcid.org/0000-0002-9439-5810).

substitutes only re-epithelializes the skin and thus restores the mechanical barrier function of the skin. However, skin substitutes do not suppress the chronic inflammatory state of the wounds or improve angiogenesis. Therefore, new strategies simultaneously influencing multiple processes related to wound healing, for example, angiogenesis, inflammation, or re-epithelialization are warranted to improve chronic wound care.

A promising novel treatment that targets multiple wound healing-related processes is the use of hydrogels loaded with instructive factors. Hydrogels from synthetic or natural materials, that is, extracellular matrix (ECM) can act as scaffold to deliver bioactive molecules and growth factors to wound surfaces and in this way instruct wound healing.[5,6]

Hydrogels are hydrated polymers with high structural integrity[7] that harbor excellent biocompatibility and a low foreign body response after implantation.[8] Synthetic polymer hydrogels have been commonly used because of enhanced tunability and simple manufacturing.[9,10] However, due to lack of cell adhesion ligands on synthetic polymers, natural hydrogels have distinct advantages. Most importantly, natural scaffolds do not cause unwanted inflammation and provide a large diversity of integrin-binding motifs. Moreover, ECM acts as a binding and slow-release depot for instructive factors, for example, growth factors.[11,12] This way, ECM instructs tissue to regenerate, for example, forming new epidermal layers and neovessels.

Although polymer chemists realized to replicate cell adhesion and integrin-binding motifs, such as RGD (R: arginine; G: glycerine; D: aspartic acid) into polymers, natural scaffolds' benefits still outweigh those of synthetic polymers. ECM-based hydrogels might therefore better mimic natural environment and guide cell behavior to improve dermal wound healing.

This systematic review addresses the current status of the generation of ECM-based hydrogels and aims to state which has the best therapeutic use to treat dermal wounds.

Materials and Methods

Protocol and registration

This systematic review was performed according to the PRISMA protocol.[13] Search strategy was based on a PICO (population, intervention, comparison, and outcome) framework.[14] The study was not registered.

Eligibility criteria

Studies were included if native decellularized ECM (dECM) was used as a hydrogel to improve dermal wound healing in animals or humans. Studies using additives to the hydrogel, for example, biological or synthesized growth factors or cells were included as well. Studies using synthetic matrix-mimicking hydrogels were excluded as well as reviews, case studies, and *in vitro* studies. English was specified as the restricted language. Search strategy was not limited to date or publication status.

Information sources and search strategy

A systematic search was conducted in electronic medical databases: Embase, Medline Ovid, and The Cochrane Central Register of Controlled Trials until April 13, 2021. In databases where a thesaurus was available (Embase and Medline), articles were searched by thesaurus terms and by title and/or abstract. Search strategy included human and animal studies. Search terms were based on: (P) animals and humans with dermal wounds treated with (I) native dECM-based hydrogels and compared with (C) untreated wounds, placebo control, or other ECM-based hydrogels (O) to augment dermal wound healing (Supplementary Appendix S1).

Study selection and data collection process

Two authors (L.V. and V.S.) independently searched the electronic medical databases and selected studies based on eligibility criteria. Discrepancies between selected studies from both authors were discussed in a consensus meeting with the senior author (J.A.D.) giving a binding verdict.

Data items

Outcomes of interest for this systematic review were clinical improvement of dermal wound healing, histological improvement of dermal wound healing, complications as well as characteristics of decellularization procedures and characteristics of dECM. Outcomes of interest were not included in the search strategy.

Risk of bias in individual studies

It is well known that commonly used enzymatic, chemical, and physical methods to decellularize ECM affect architecture, biomechanical characteristics, and (biochemical) composition of ECM.[15] Hence, a detailed overview of enzymatic, chemical, and physical methods is given.

Data synthesis

In some studies, data were derived from reference studies or derived from graphs if actual numbers of data on outcome of interest were not given.

Verification of cellular remnants and residual chemicals as quality control of decellularization process

To date, no formal quality control of decellularization processes exists in literature. The assessment of quality in this systematic review was based on verification of cell removal and removal of residual chemicals (in particular detergents) in dECM in each study. Therefore, we designed a new scoring system, consisting of seven analyses, giving one point for each essential analysis. The first verification consisted of ECM collagen analysis with, for example, Masson's Trichrome (MT), Picrosirius or MOVAT staining (one point), followed by ECM glycosaminoglycans (GAGs) analysis with, for example, MOVAT, Alcian Blue, Fuchsin, or PAS staining (one point). Third, DNA analysis was verified through isolation plus quantification with NanoDrop technology, PicoGreen, or polymerase chain reaction (one point). Standard histological confirmation, for example, 4′,6-diamidino-2-phenylindole (DAPI), hematoxylin and eosin (H&E), MT, MOVAT, or immunohistochemical staining verified the presence of intracellular proteins, nuclei, or cell remainders (one point).

Furthermore, composition was assessed with immunodetection of ECM proteins, proteomics, GAG counting, and immunofluorescence (one point). Physical properties of ECM hydrogels, for example, porosity and stiffness, were verified with, for example, scanning electron microscopy (SEM) analysis, rheology, a low-load compression tester (LLCT), or atomic force microscope (one point). Lastly, ECM biocompatibility and cytotoxicity assessment of elutable contaminants was verified by assessing if studies indirectly measured residual chemicals with analysis of foreign body response, direct contact tests, leachable cytotoxicity, and live/dead assays (one point).

Results

Included studies

The search resulted in 3153 studies, of which 1996 remained after removing duplicates. Thirty full-text studies remained for assessment of eligibility criteria[16–45] (Fig. 1). After full-text screening, 21 studies were excluded for several reasons[16–36] and 9 studies were included.

Decellularization yielded tissue-derived ECM and cell culture-derived ECM

The included rodent studies used two types of ECM: tissue-derived ECM (tECM) and cell culture-derived ECM (cECM). The tECM is obtained after extensive decellularization by enzymatic and chemical detergents, whereas cECM is deposited by cultured cells on the culture plate and isolated after lysis of the cells. In total, two studies used cultured human lung fibroblasts to isolate cECM and one study used a mixture of human cultured lung fibroblasts and umbilical cord blood mesenchymal stem cells (Table 1).[38–40] In total, five studies used tECM of different organs and species: porcine skin and small intestinal submucosa, murine and rat dermis, and human adipose tissue.[37,41,43–45] Studies did not extensively investigate composition and ultrastructure of cECM or tECM. Thus, potential differences between cECM and tECM on these domains remain unknown, as does the influence on the regenerative potential of ECM-derived hydrogels.

Characteristics of decellularization procedures

The composition and ultrastructure of ECM is affected by detergents used for decellularization as well as the time of exposure to these detergents, for example (non)ionic and zwitterionic detergents.[46–48] However, cECM was only exposed to 10 mM ammonium hydroxide (NH_4OH) for 1–3 h, whereas tECM was obtained after a decellularization process up to 408 h. Yet, cutting and milling of tissue is included in some tECM isolation procedural times, which shortens the actual incubation time with detergents. Kuna *et al.* and Hsieh *et al.* performed the longest decellularization protocols for tECM with 408 and 234 h, respectively.[43,45] Each type of (non)ionic detergent affects tissue in a different way. For example, nonionic detergents, such as Nonidet P-40 and Triton X-100, are relatively mild, and lipids present in lipid–lipid and lipid–protein interactions, can be dissolved in these detergents. Whereas ionic detergents, such as sodium dodecyl sulfate (SDS) and sodium

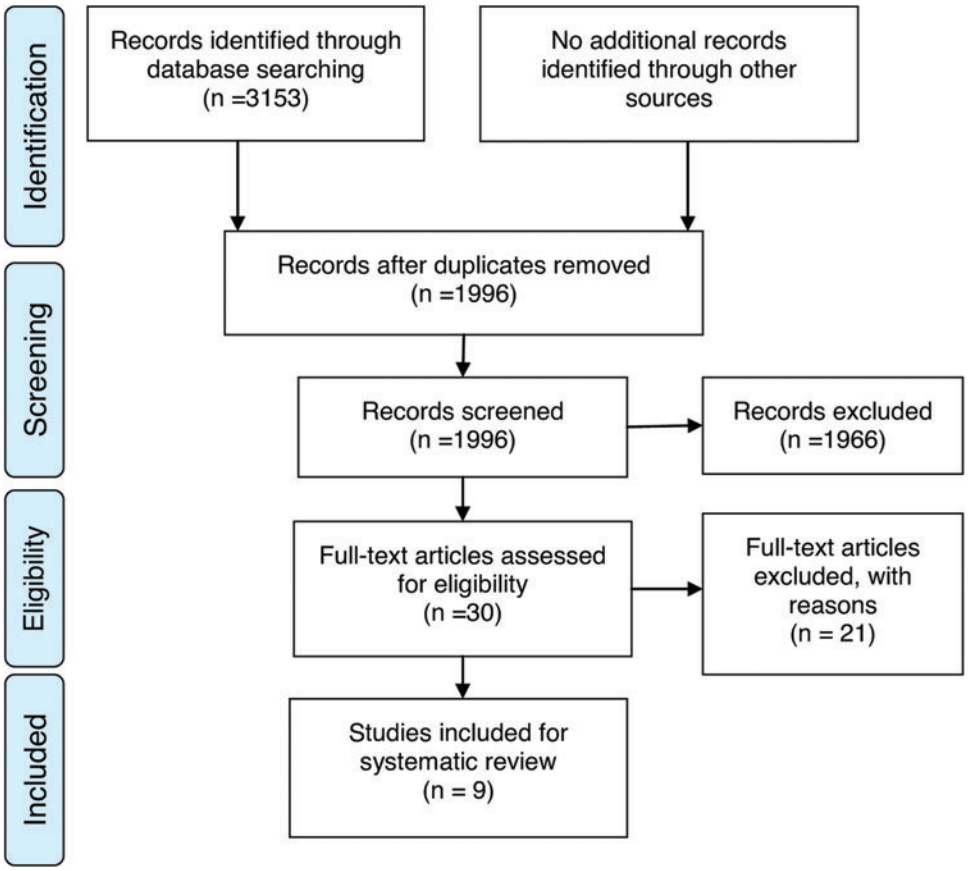

FIG. 1. Flow diagram of study selection on April 13, 2021. Color images are available online.

Table 1. Characteristics of Decellularization Procedures and the Obtained Decellularized Extracellular Matrix

Ref.	*Species (a/h) Organ*	*Process Time, h*	*Physical process*	*Enzymes*	*Extraction reagents*	*Pregel ECM Dosage*	*ECM treatment and gel assembling*	*Characterization decellularized ECM*
Lee *et al.*[37]	SIS	48	Cut in small pieces, pulverized (Precellys 24 homogenizer), stirred in pepsin solution 60 rpm (constantly for 48 h), lyophilized, ethylene oxide gas sterilized	na[a]	na[a]	30 mg/mL[b]	Pepsin 1 mg/mL in 0.01 M HCl Neutralizing pH to 7.4 (NaOH) Rising temp. to 37°C	nr[a]
Du *et al.*[38]	H Cultured lung fibroblasts	1–2	—	DNase I 50 U/mL RNase A 2.5 µL/mL	0.25% Triton X-100 50 mM ammonium hydroxide 50 µg/mL ascorbic acid 2-phosphate	250 µg/mL	Mixing with Col at 2.5 mg/mL	DNA quantification: — DAPI: no nuclei I.F. staining: Col I, fibronectin and laminin detected with fibrillar structure
Ha *et al.*[39]	H Cultured lung fibroblasts	2–3	—	DNase I 50 U/mL RNase A 2.5 µL/mL	0.25% Triton X-100 20 mM ammonium hydroxide	nr	Mixing with 8% aqueous solution of PVA+small amount of poly(ethylene glycol)	DNA quantification: — DAPI: no nuclei I.F. staining: Col I, fibronectin rich, and uniform
Savitri *et al.*[40]	H Cultured lung fibroblasts Umbilical cord blood mesenchymal stem cells	2–3	—	DNase I 50 U/mL RNase A 100 µL/mL	0.25% Triton X-100 20 mM ammonium hydroxide	nr	Mixing with 2 g Pluronics +0.15 g HA	DNA quantification: — I.F. staining: Col I and fibronectin detected
Kim *et al.*[41]	H Adipose tissue	51	ddH_2O washed, blended (3 min) 16,500 *g* (5 min), 20,000 *g* (30 min), 20,000 *g* (60 min) centrifuged, filtered, dialyzed in dialysis tubing against 30 volumes of TBS (24 h), lyophilized, ethylene oxide gas sterilized	—	3.4 M NaCl 4 M urea buffer+ protease inhibitor cocktail (12 h) 4 M guanidine+ protease inhibitor cocktail (12 h) TBS (24 h)	60 mg/mL	Rising temp. to 37°C	DNA quantification: — Col 4.572 ± 0.136 mg/mL Fibronectin 0.144 ± 0.054 mg/mL Laminin 1.038 ± 0.064 mg/mL GAGs 0.021 ± 0.004 mg/mL Elastin 16.463 ± 1.440 mg/mL
Engel *et al.*[42]	A Dermis	13–14[c]	Cut in small sections (1–2 mm), rubbed over cell sieve, 14,000 *g* centrifuged (20 min)[d]	Dispase[d]	High salt buffer solution (0.05 M Tris pH 7.4, 3.4 M NaCl, 4 mM of ethylenediaminetetraacetic	nr	Rising temp. to 37°C Reducing pH to 4.0 (acetic acid)	DNA quantification[d]: 183.7–10.2 ng/mL DAPI: limited nuclei[d]

(*continued*)

Table 1. (Continued)

Ref.	Species (a/h) Organ	Process Time, h	Physical process	Enzymes	Extraction reagents	Pregel ECM Dosage	ECM treatment and gel assembling	Characterization decellularized ECM
					acid, 2 mM of *N*-ethylmaleimide, 0.001 mg/mL pepstatin, 0.01 mg/mL aprotonin, 0.001 mg/mL leupeptin, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride). 2 M urea buffer (0.15 M NaCl, 0.05 Tris mL/g tissue) EDTA			
Kuna *et al.*[43]	A Skin	408	Freeze/thawed, agitated at 200 rpm in detergent, ddH_2O washed, lyophilized, pulverized with cryomill with 0.75 microm sieve at 14,000 rpm, temp rise to 37°C for 9 days, 25 kGy gamma irradiation sterilized	—	0.5% SDS 0.02% sodium azide and 1.86% EDTA	50 mg/mL	250 µL of HA	DNA quantification: 13.9 ng/mg H&E, MT, Verhoeff v. Gieson: No cellular remnants Col 66.6 µg/mg GAGs 4.6 µg/mg Elastin 3.5 µg/mg
Morris *et al.*[44]	A Skin	65[c]	Freeze-thawed, grounded, chrome coated (~20 nm), ddH_2O washed, shaken	0.25% Trypsin-EDTA	70% Ethanol 3% H_2O_2 1% Triton X-100 in 0.26% Tris/ 0.69% EDTA 0.1% Peracetic acid in 4% ethanol	8 mg/mL	Pepsin 1 mg/mL in 0.01 M HCl Neutralizing pH to 7.0 (NaOH) Rising temp. to 37°C	DNA quantification[e]: <50 ng/mg H&E: no cellular remnants
Hsieh *et al.*[45]	A Skin	234	Cut in small pieces, deionized water washed, agitated at 120 rpm in 30% formic acid (72 h), lyophilized	0.25% Trypsin solution	Acetone (72 h) 10% Sodium chloride (24 h) 1.92% Citrate buffer (pH 4.3) (48 h) 30% Formic acid (72 h)	25 mg/mL	Pepsin 1 mg/mL in 0.1 M HCl Neutralizing pH to 7.0 (NaOH) Rising temp. to 37°C	DNA quantification: 42.95 ± 0.73 ng/mg H&E: no cellular remnants Col 556.01 ± 5.94 µg/mg GAGs 5.50 ± 0.16 µg/mg

[a]Not applicable, SIS ECM is bought already decellularized.
[b]Based on study of Wang *et al.*[59]
[c]Overnight = 12 h.
[d]Based on former study of Uriel *et al.*[60]
[e]Based on former study of Morris *et al.*[61]

—, none/not; a, animal; cell., cellular; Col, collagen; DAPI, 4′,6-diamidino-2-phenylindole; ECM, extracellular matrix; GAG, glycosaminoglycan; h, human; HA, hyaluronic acid; H&E, hematoxylin and eosin; I.F. immunofluorescence; MEC, methylcellulose; MT, Masson's Trichrome; na, not applicable; nr, not reported; SDS, sodium dodecyl sulfate; SIS, small intestinal mucosa; TBS, Tris-buffered saline.

deoxycholate, dissolve all cellular membranes and break up protein–protein interactions. Zwitterionic detergents such as CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) possess both non- and ionic properties and may affect the molecular structure of ECM, much as ionic detergents do.

Decellularization processes of tECM generally consist of both chemical and physical treatments to remove all cellular and nuclear material while preserving the native integrity of ECM. All included studies used a combination of physical and chemical treatments. In addition to chemical compound treatment, mild proteolysis is occasionally used to loosen up ECM architecture. Three studies used trypsin to cleave peptide bonds to ECM.[42,44,45] However, trypsin can be disruptive to elastin and collagens and GAGs, which are important in binding and releasing growth factors and thus play a crucial role in the potential regenerative effect of ECM-derived hydrogels.[49] Trypsin also effectively disrupts ultrastructure, facilitating penetration of other detergents in dense tissue.[50] SDS or Triton X-100 (ionic and nonionic detergents, respectively), as well as acids, for example, peracetic acid, acetic acid, or formic acid, mainly remove cellular proteins but also contribute to solubilizing GAGs. These acids were used in five studies.[38–40,43,44]

Efficacy of decellularization and ECM quality standards

To date, standards exist to which biomaterials should comply (Fig. 2). One is that biomaterials are advised to contain maximally 50 ng/mg DNA per dry weight ECM.[50] Studies did not often comply to all standards and quality controls of decellularization processes (Table 2). Three of the nine included studies did not measure the efficiency of decellularization by analyzing DNA content, nuclei, or remaining cells.[37,40,41] Hence, it remains unknown if the decellularization processes were efficient. Six studies quantified nuclear remnants as surrogate marker for successful decellularization.[38,39,42–45] Three studies performed

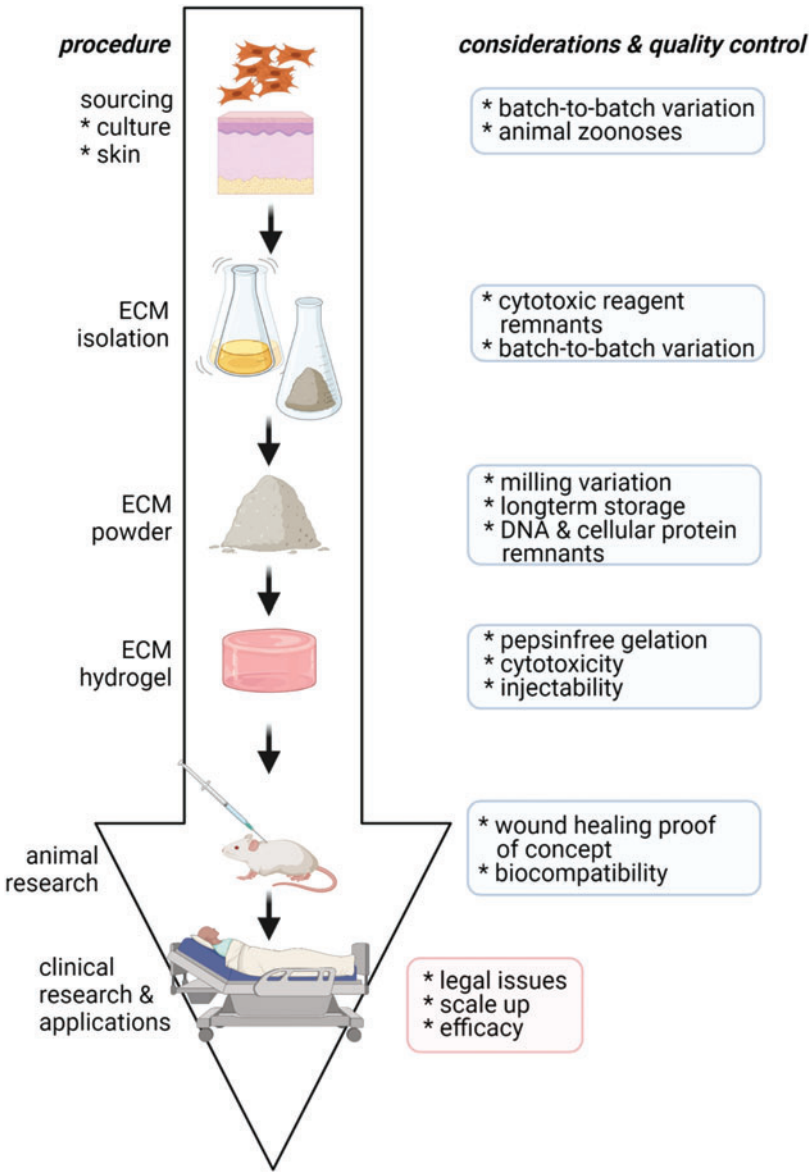

FIG. 2. ECM hydrogel procedure, procedural considerations and quality controls. ECM, extracellular matrix. Color images are available online.

Table 2. Extracellular Matrix Quality and Cytotoxicity Scoring System

Ref.	*ECM–collagen*	*ECM–GAGs*	*DNA*	*Cells/cell remainders*	*Concentration protein, GAGs*	*Physical properties*	*Biocompatibility*	*Total score*
Lee *et al.*[37]	nr	nr	nr	nr	nr	−	+	cnbd
Du *et al.*[38]	+	−	+	+	+	+/−	−	4, 5
Ha *et al.*[39]	+	−	−	−	+	+	+	4
Savitri *et al.*[40]	+	−	−	−	+	+	−	3
Kim *et al.*[41]	+	+	−	−	+	+	+	5
Engel *et al.*[42]	+	+	−[a]	−[a]	+	+	−	4
Kuna *et al.*[43]	+	+	+	+	+	+	−	6
Morris *et al.*[44]	+	−	+	+	+	+	+	6
Hsieh *et al.*[45]	+	+	+	+	+	+	+	7
Score per analysis/ total studies	8/9	4/9	4/9	4/9	8/9	8/9	5/9	

+/− Cell matrix interaction with VE-cadherin, however no other psychical property tests performed.
[a]Test performed but outcomes of ECM quality not according to advised guidelines.
cnbd, cannot be determined; VE, vascular endothelial.

a DAPI staining to determine the remaining nuclei.[38,39,42] Three other studies assessed the lack of visible nuclear material in dECM by H&E staining and detected no presence of cell remainders.[43–45] These three studies also performed DNA remnant quantity and all confirmed DNA content of <50 ng/mg.[43–45]

Ha *et al.*[39] could not detect remaining nuclei after decellularization of cECM of cultured lung fibroblasts, while Engel *et al*.[42] visualized a limited number of remaining nuclei after decellularization of tECM of dermis, which were not further counted or quantified. Engel *et al.* additionally measured a residual DNA amount of 183.7 ng/mg in dECM, which is higher than advised and compared with other studies.[42] This indicates the decellularization protocol used was not efficient while the remaining cells and DNA could jeopardize the regenerative effect of the hydrogel, for example, cause a deleterious host immune response.[51,52]

Protein and trophic factor analysis

Five studies performed additional protein analysis on dECM by, for example, SDS-PAGE (SDS–polyacrylamide gel electrophoresis) or bicinchoninic acid (BCA) protein assays and mainly demonstrated that gels consisted mostly of collagens.[39–42,44] A study by Morris *et al.* was the only one that performed proteomics on dECM to analyze residual cytoplasmic proteins.[44] Six studies evaluated the composition of dECM by immunofluorescence and histological staining, of which four studies evaluated the presence of fibronectin[38–41] and three studies determined the number of GAGs present.[41,43,45] Both fibronectin and GAGs are important proteins capable of binding and releasing growth factors and cytokines to initiate tissue regeneration. After decellularization of porcine skin, Kuna *et al.*[43] and Hsieh *et al.*[45] measured comparable concentrations of GAGs with 4.6 and 5.5 ± 0.16 μg/mg, respectively, on average. Kim *et al.*[41] decellularized human adipose tissue and measured concentrations of GAGs of 21 μg/mg. The study of Kim *et al.*[41] was the only study to quantify the concentration of fibronectin as well with a mean number of 144 ± 54 μg/mg.

A prime function of the ECM is to bind, retain, and on-demand release trophic factors such as growth factors and immunomodulatory factors such as chemokines. Since these trophic factors are much smaller than the average ECM molecules and because these bind with low affinity, these are rapidly washed out during the decellularization procedure. Two of the nine studies assessed the presence of trophic factors. Kim *et al.* performed a growth factor antibody array and mainly detected high hepatocyte growth factor (HGF), platelet-derived growth factor-BB, endothelial growth factor (EGF), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), and transforming growth factor-β1 (TGF-β1).[41] Du *et al.*, Ha *et al.*, and Savitri *et al.* identified very similar and a large number of angiogenic-related cytokines using angiogenesis array kits.[38–41] Kuna *et al.*, Morris *et al.*, and Hsieh *et al.* scored best on ECM quality controls and cytotoxicity measurements (Table 2).[43–45]

Study characteristics

In total, 45 rats and 157 mice were included in 9 studies (Table 3). One study[42] did not specify the number of rats used and, in another study,[45] the exact number of rats remained unclear. In all studies, wounds were treated directly after creation. Three studies included an intervention group with comorbidity: diabetes mellitus type 2[44,45] and irradiation.[37] All studies, except one, used ECM-only controls to assess either the influence of ECM or of supplemented ECM and neither study included additives, only controls. Six studies incorporated ECM hydrogels with additives, for example, (stem) cells: human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) (2/9), human bone marrow-derived MSC (hMSC) (1/9), adipose tissue-derived stromal cells (ASCs) (1/9), and human peripheral blood mononuclear cells (hPBMCs) (1/9) and human umbilical vein endothelial cells (HUVECs) (1/9) combined with growth factors. No meta-analysis could be performed due to small numbers and highly diverse clinical and histological outcomes.

(Pre)clinical outcome of ECM hydrogels to augment dermal wound healing

Studies measured wound sizes or areas by (i) making digital photos of wounds and analyzing and quantifying them through ImageJ or Image Analyzer, and (ii) embedding,

Table 3. Study Design, Preclinical Application of Extracellular Matrix Hydrogel, and Wound- and Histological Analysis

Study_id	*Rodent*	*Age, weeks*	*Comor-bidity*	*Interventions*	*Follow-up days*	*Follow-up diagnostics*	*Wound analysis*	*Histology*
Lee *et al.*[37]	Mice ($n=37$) Male Wound = 6 mm, injected	7	— and radiation (5 Gy)	Interv.1: ECM hydrogel Interv.2: ECM hydrogel+hUCB-MSCs Interv.3: hUCB-MSCs Control: PBS	7, 14, 21	Wound analysis CD31, vWF	Irradiated wound size decreased in ECM hydrogel+MSC vs. control** and ECM Hydrogel** (14 days) and decreased vs. control* (21 days). I-Solution-IMT	Vessel density higher in ECM hydrogel+MSC vs. other groups** in irradiated wound (21 days).
Du *et al.*[38]	Mice ($n=12$) Male 2 wounds/animal Wound = 5 mm, applied	8	—	Interv.1: HUVECs in Col+ECM Hydrogel Interv.2: HUVECs in Col+ECM Hydrogel + GFs Interv.3: HUVECs in Col Hydrogel+GFs Control: HUVECs in Col hydrogel	3, 5, 7, 14	Wound analysis CD31, aSMA	Wound size decreased in Col/ECM/GFs vs. control (3,* 5,*** 7,*** 14 days**) and decreased in Col/ECM vs. control (7*** and 14 days*) ImageJ	Vessel density higher in Col/ECM/GFs** and Col/ECM* vs. control.
Ha *et al.*[39]	Mice ($n=12$) Male 2 wounds/animal Wound = 8 mm, applied	6	—	Interv.1: PVA Interv.2: ECM/PVA Interv.3: hMSC/ECM/PVA Control: Untreated	0, 3, 7, 10, 14, 17, 21	Wound analysis H&E, MT CD31, aSMA	Wound size decreased in ECM/PVA and hMSC/ECM/PVA vs. control and PVA (3,**** 7,**** 10,**** 14,**** 17,**** 21 days**). ImageJ	Neovessel size increased in hMSC/ECM/PVA vs. control***, PVA***, and ECM/PVA** (21 days). Vessel density higher in hMSC/ECM/PVA vs. control****, PVA****, and ECM/PVA*** (21 days). Epidermal thickness in ECM/PVA, hMSC/ECM/PVA, and control nsd. PVA thickest compared with ECM/PVA and hMSC/ECM/PVA***, and control**** (21 days).

(*continued*)

Table 3. (Continued)

Study_id	*Rodent*	*Age, weeks*	*Comor-bidity*	*Interventions*	*Follow-up days*	*Follow-up diagnostics*	*Wound analysis*	*Histology*
Savitri *et al.*[40]	Mice ($n=12$) Male 2 wounds/animal Wound = 8 mm, applied	6	—	Interv.1: PH hydrogel Interv.2: PHF hydrogel Interv.3: PHU hydrogel Control: Untreated	0, 3, 7, 10, 14	Wound analysis H&E aSMA, CD206	Wound size not analyzed.	Vessel density higher in ECM hydrogels (PHF*** and PHU***) vs. control (7 days). Epidermal thickness thinner in ECM hydrogels (PHF** and PHU****) vs. PH (14 days). M2 macrophages increased in ECM hydrogels (PHF*** and PHU***) vs. PH and control (7 days).
Kim *et al.*[41]	Rat ($n=45$) Female Wound = 169 mm^2 Injected	nr	—	Interv.1: MEC ECM hydrogel Interv.2: MEC ECM hydrogel+ASCs Control: Untreated	7, 14, 21	Wound analysis CD31 (Imaging analyzer—Bio-Rad Lab, Hercules, CA)	Wound size decreased in MEC ECM hydrogel+ASCs vs control (7* and 14 days*) and vs MEC ECM Hydrogel (7 days*).	Vessel density not analyzed statistically.
Engel *et al.*[42]	Rats ($n=$ nr) Senrx: nr	nr	—	Interv.1: ECM hydrogel Control: Untreated	7, 14, 21	Wound analysis H&E, MT CD31	Re-epithelialization and wound width: nsd (7, 14, 21 days). Wound closure: 80% in ECM hydrogel vs. 100% in control (14 and 21 days).	Vessel density nsd (7, 14, 21 days). Cell influx nsd (7 days).
Kuna *et al.*[43]	Mice ($n=72$) Female Wound = 10 mm, applied	7–8	—	Interv.1: ECM hydrogel Interv.2: ECM hydrogel+hPBMC Control 1: HA Control 2: Untreated	5, 10, 15, 25	Wound analysis H&E, MT CD31 ImageJ	Wound closure: 83% for ECM hydrogel+hPBMCs* vs. 66% ECM hydrogel only* vs. 0% in untreated and HA* (15 days). Wound size decreased in ECM hydrogel* and ECM Hydrogel+ hPBMCs* (15 days).	Vessel density nsd.

(*continued*)

Table 3. (Continued)

Study_id	*Rodent*	*Age, weeks*	*Comor-bidity*	*Interventions*	*Follow-up days*	*Follow-up diagnostics*	*Wound analysis*	*Histology*
Morris *et al.*[44]	Mice ($n=24$) Sex: nr Wound = 6 mm, injected	12–14	DM 2	Interv.1: WT ECM hydrogel Interv.2: TSP-2 KO ECM hydrogel Control: Untreated	10, 21	Wound analysis H&E CD31, aSMA ImageJ	Wound size decreased in TSP-2 KO ECM Hydrogel vs control* (21 days).	Vessel density,* size,** and maturity*** higher in TSP-2 KO ECM Hydrogel vs. WT ECM Hydrogel. Epithelial gap decreased in TSP-2 KO ECM Hydrogel vs. control* and WT ECM Hydrogel* (10 days). Epithelial thickness decreased TSP-2 KO ECM Hydrogel vs. control*.
Hsieh *et al.*[45]	Rats ($n=32$–48) Male 4 wounds/animal Wound = 8 mm, applied	8	DM 2	Interv.1: ECM Interv.2: SC Interv.3: HA Interv.4: CS Interv.5: ECM/SC Interv.6: HA/SC Interv.7: CS/SC Control: HEC	0, 2, 4, 6, 8, 10, 12, 14, 16	Wound analysis CD31 ImageJ	Wound size decreased in ECM, SC, ECM/SC, and HA/SC vs. HEC (10 days*). Wound closure: 100% (14 days).	Vessel density not analyzed statistically.

*Significant $p<0.05$.
**Significant $p<0.01$.
***Significant $p<0.001$.
****Significant $p<0.0001$.
—, none/not; ASC, adipose tissue-derived stromal cell; CS, chitosan; FU, follow-up; GFs, growth factors; HA, hyaluronic acid; HEC, hydroxyethyl cellulose; hMSC, human bone marrow-derived mesenchymal stem cells; hPBMC, human peripheral blood mononuclear cells; hUCB-MSC, human umbilical cord blood-derived mesenchymal stem cell; HUVEC, human umbilical vein endothelial cell; Interv., intervention; nsd, no significant differences; PBS, phosphate-buffered saline; PH, pluronic/HA; PHF, pluronic/HA/ECM fibroblast; PHU, pluronic/HA/ECM umbilical cord blood mesenchymal stem cell; PVA, polyvinyl alcohol; SC, sacchachitin; TSP-2 KO, thrombospin-2 knockout; WT, wild type.

formalin fixing, and (H&E) staining of wound biopsies and analyzing wound width and epithelial gap through ImageJ or other software. In seven studies, a large wound size reduction occurred after application of ECM-derived hydrogels with or without additives in comparison with an untreated or placebo control group.[37,39,41–45] This difference occurred in two studies after 7 days.[39,42] In the other five studies, wound size was reduced after 14–21 days.[37,41,43–45] In one study, wound size did not reduce after treatment with ECM hydrogels in comparison with an untreated control group.[42]

This study was the only study that did not use additives. Hsieh *et al.* compared tECM-derived hydrogel with hydroxyethyl cellulose as sham control and measured a wound size reduction after ECM hydrogel treatment after 10 days ($p < 0.05$).[45] One study did not use a placebo control group and compared cECM hydrogels with HUVECs and collagen with HUVECs and collagen.[38] Wound size was decreased after cECM hydrogel treatment compared with HUVECs and collagen alone after 3 days ($p < 0.05$). No studies reported on whether complications occurred.

Histological outcome of ECM hydrogels to augment dermal wound healing

Eight out of nine studies performed histological analysis on paraffin sections of the wound area and controls.[37–44] All nine studies assessed vessel density after treatment with ECM hydrogels, of which seven tested vessel density statistically.[37–40,42–44] Two studies found no differences in angiogenesis between application of ECM hydrogel treatment and placebo controls.[42,43] In both studies, ECM hydrogel application resulted in improved wound healing (reduction of wound size), without improved angiogenesis. In one of these studies, these results were found irrespective of the use of hPBMCs[43]; in the other study, no additives were used.[42] Of the five other studies that statistically tested angiogenesis, four studies measured increased vessel density after ECM hydrogel treatment in comparison with an untreated or placebo control group.[37,39,40,44] One other study measured higher vessel density after cECM hydrogel with HUVEC treatment compared with collagen with HUVECs and collagen ($p < 0.01$).[38] The increased vessel density was combined with a decreased wound size in all five studies after both ECM hydrogel treatment and ECM hydrogel treatment with any additive.[37–40,44]

Moreover, a limited number of other histological parameters were analyzed. Three studies additionally investigated epidermal thickness after ECM hydrogel treatment based on five random images per group (one-way ANOVA with Tukey's Multiple Comparisons test).[39,40,44] Two studies measured a reduced epidermal thickness compared with untreated control groups ($p < 0.05$, $p < 0.01$, and $p < .0001$),[40,44] whereas the third study measured no differences in epidermal thickness after ECM hydrogel treatment.[39]

Risk of bias

Generally, the risk of bias in studies was moderate to high (Table 4). None of the studies reported how animals were housed (separately/together), if investigators were blinded to intervention and outcome measurements, if animals were

Table 4. Risk of Bias

	Sequence generation	*Baseline characteristics*	*Allocation concealment*	*Housing of animals*	*Blinding to interventions*	*Selective outcome assessment*	*Blinding outcome assessor*	*Incomplete data*	*Selective reporting*	*Conflict of interest*	*Overall risk of bias*
Lee *et al.*[37]	1	1	1	0	0	0	0	0	1	1	5
Du *et al.*[38]	1	1	1	0	0	0	0	0	1	1	5
Ha *et al.*[39]	0	1	1	0	0	0	0	0	1	1	4
Savitri *et al.*[40]	1	1	1	0	0	0	0	0	1	1	5
Kim *et al.*[41]	0	1	1	0	0	0	0	0	1	1	4
Engel *et al.*[42]	0	0	1	0	0	0	0	0	1	1	3
Kuna *et al.*[43]	0	1	1	0	0	0	0	0	1	0	3
Morris *et al.*[44]	0	1	1	0	0	0	0	0	1	1	4
Hsieh *et al.*[45]	0	1	1	0	0	0	0	0	1	1	4

1, Low risk of bias; 0, high risk of bias.
Overall risk of bias on a score from 0 to 10. Low risk of bias score 7–10, moderate risk of bias score 4–6, high risk of bias score 0–3.

selected randomly for outcome assessment and if all animals completed full follow-up, or data were missing. In none of the studies selective reporting was found. In most studies there were no conflicts of interest and baseline characteristics were similar between groups.

Discussion

Our systematic review shows that hydrogels based on native ECM hold promise to enhance dermal wound healing in rodents. To date, only nine studies with evaluable results have been published. Yet, these studies varied largely with respect to decellularization methodology and generation of hydrogels as well as product characterization. Therefore, no optimal reproducible and standardized procedure exists, although this would be a prerequisite for future clinical application.

All included studies, except one, demonstrated a reduced wound size after application of ECM hydrogels, irrespective of the use of additives, compared with controls.[42] In this study, administration of bare dECM hydrogels did not affect wound healing although that ECM was not fully decellularized with DNA levels well above 50 ng/mg and still a limited number of nuclei visualized in a DAPI staining.[50] The known detrimental effects this DNA contamination might cause, for example, a host immune response that may have prevented beneficial influence of bare dECM hydrogels on wound healing.[50]

An important factor for adequate wound healing is vascularization. The majority of studies reported increased angiogenesis combined with a decreased wound size after both ECM hydrogel treatment and ECM hydrogel treatment with any additive.[37–40,44] Authors of these studies ascribed the observed increased vascularization mainly to a potential synergistic effect of ECM and the used additives, that is, hUCB-MSCs, hMSCs or angiogenic growth factors.[38–40] However, these studies made no comparisons between ECM hydrogel and ECM hydrogel treatment plus additive treatment. Thus, the contribution of additives to the increased observed angiogenesis and wound size reduction remains unknown. Hence, the addition of growth factors and cells might not be necessary to stimulate angiogenesis or even wound healing. ECM has proangiogenic ability in itself, through mechanical properties as well as ECMs proteins, for example, laminins, fibronectins, and collagen IV.

However, theoretically, ECMs' ability to incorporate growth factors, for example, VEGFs, IGFs, fibroblast growth factors (FGFs), TGF-βs, and HGF may enhance angiogenic capability even more.[53] Two studies also recognized that ECM can initiate neovascularization through incorporated angiogenic factors in ECM, for example, HGF, EGF, IGF-1, VEGF, TGF-β1, basic FGF (bFGF), serpin E1, and proteolytic enzymes.[39,41] However, studies only limitedly investigated the presence of these signaling molecules in the final ECM hydrogels, which might be retained after decellularization. Moreover, these studies only concerned cECM hydrogels.[38–41] Thus, the angiogenic capability of ECM and the additional part that adding growth factors may play needs further investigation.

The role of mechanical properties, for example, viscoelasticity of ECM hydrogels has been scrutinized in the past few years. The viscoelasticity regulates basic cell processes, including growth, proliferation, apoptosis, migration, and differentiation.[54,55] For example, mesenchymal stromal cells like ASCs adipogenically differentiate under low viscoelastic circumstances, whereas ASCs osteogenically differentiate under high viscoelastic circumstances. Hence, the viscoelastic properties of ECM hydrogels are a genuine opportunity to include in further research as they might influence dermal wound healing. Four studies performed analysis of mechanical properties, all of which concerned rheological analysis,[40,41,44,45] except one that performed atomic force microsopy.[42]

Most studies mainly manufactured ECM hydrogels because these are injectable and therapeutically charged band aids that will be turned over during tissue regeneration. Therefore, ECM hydrogels seem appropriate vehicles to codeliver other therapeutic moieties, for example, (stem) cells and/or their secretomes. This is especially relevant because selective loss of small molecules, for example, growth actors and chemokines occur during isolation and processing procedures to generate ECM hydrogels. Recharging the ECM with growth factors might restore the paracrine function of native ECM, which may result in augmentation of wound healing. However, from the derived results in this study the additional effect of growth factors remains unclear until this hypothesis is further tested.[38,39,44,45]

Studies followed various decellularization methodology. Effective decellularization is dictated by efficient removal of cellular constituents, while preserving the complex and 3D ECM structure and composition, which likely preserve cell-instructing function and mechanical characteristics. Origin of tissue, specific donor characteristics, choice of cell removal agents, and exposure time to the agent, all influence the efficiency of removal of cellular content, including DNA on one hand and the preservation of ECM proteins on the other hand. Because all cell removal methods alter ECM composition, for example, due to loss of small(er) molecules, chemokines, and growth factor-binding proteins, for example, GAGs, the use of and exposure time to cell removal agents should be minimized.

However, minimization of the use of detergents might cause ineffective cell and DNA removal, risking infliction of a clinically relevant immune response once implanted repeatedly in humans. The evaluation of dECM was highly variable and often below standards across the nine included studies. Studies hardly met the minimal requirements for proper decellularization. Some studies added DNAse, aiming to remove possible DNA remnants.[38–40] Although these studies evaluated the number of nuclei with a DAPI and/or H&E staining, neither evaluated the presence of DNA remnants and thus DNA purity remains unknown. The presence of DNA remnants may potentially have caused altered wound healing.

Clearly, quality controls of dECM should be developed and implemented to maintain the balance between effective decellularization and preservation of ECM proteins. This will also enable proper comparison of different ECM decellularization protocols. First, remaining cellular host proteins should be analyzed with proper assessment, that is, a maximum tolerated amount of GAPDH, B-actin, or cellular or ribosomal protein parts per million, since the absence of cellular material is important to avoid host immune reactions. Moreover, the purity of DNA should comply

to standards below 50 ng/mg dry weight ECM for the same reason, and can be accurately evaluated by ultraviolet absorbance (NanoDrop), fluorescence dye (PicoGreen, SYBRGreen), or agarose gel or capillary electrophoresis. Second, ECM hydrogels may contain cytotoxic and pyrogenic detergents used during decellularization, which might result in cell death or unwanted immune activation. To evaluate the cytotoxicity of ECM, the half maximal inhibitory concentration (IC_{50}) is a functional assay that estimates the amount of a substance needed to induce 50% of cell death *in vitro*, thereby indicating cytotoxicity. Cell viability in ECM hydrogels can be assessed with, for example, MTT assays and histological staining with Neutral Red. MTT assay assesses metabolic cell activity through NAD(P)H enzymes and Neutral Red derives cell viability through the ability to incorporate neutral red in cells.[56,57] Third, ECM is associated with strong instructive cellular function through, among others, growth factors, ECM stiffness, and viscoelasticity that are all contributing to wound healing processes.

Therefore, biomaterials should also comply to mechanical standards. ECMs' growth factors can be measured by performing Luminex assays on bare ECM hydrogels to assess if (angiogenic) growth factors are present. To assess viscoelastic properties, ECM hydrogels can be subjected to stress relaxation testing, for example, with LLCT. Recent research has shown that stiffness of tissue can be preserved after decellularization in ECM hydrogels.[58] Lastly, further mapping of structural integrity and morphology of ECM is possible with low-vacuum SEM, for example SEM analysis.

An interesting observation is that the analyzed studies manufactured hydrogels from cECM and tECM. Certain differences between both ECM characteristics need to be addressed. Culturing of cells on stiff plastic alters their secretome, including the deposited ECM. Moreover, while *in vivo* skin fibroblasts deposit ECM all around them, cultured cells only deposit ECM basally in culture, which alters the architecture strongly. This is in contrast to tissue, where ECM embeds cells that govern its deposition and maintenance. Three studies obtained ECM from cultured cells.[38–40] The other studies obtained ECM from tissue. In case of cECM, studies used no physical agitation steps and were the only studies that used NH_4OH and DNAse/RNAse. Regardless of these differences, the rationale for selecting tissue or cells from a specific origin, in light of the goal of healing dermal wounds, was seldom made explicit in the reviewed studies. Currently, no preferred ECM source for wound healing indications is yet determined.

Limitations

Because our systematic review yielded only few and very heterogeneous preclinical studies, data pooling and a meta-analysis could not be performed. In general, study quality was low mostly due to incomplete data description, which was also reflected by the fact that the risk of bias in studies was moderate to high. This impaired specific data extraction and impeded analysis. Procedural time of decellularization procedures, for example, often had to be derived and calculated from the method section. However, sometimes methodology was poorly described and data was derived from publications that authors referred to. None of the studies reported alterations in methodology. However, if studies did alter methodology, slight unevenness's in the extracted data may have occurred. Additionally, studies did not include ECM-only hydrogels (without additives) as control groups, thus the influence of dECM hydrogels remains speculative. Lastly, the three studies that used cECM hydrogels were performed and authored by the same research group. Subsequently, the findings of this review may not reflect the breadth or scope of cECM hydrogels in this area and heterogeneity may be greater than reported.

Future research and perspectives

The high variability in decellularization procedures and end products hampers the clinical application of ECM hydrogels. Standardization of procedures and therapeutic optimization, including dozing, timing, and frequency of ECM hydrogel application, the efficacy of additions, for

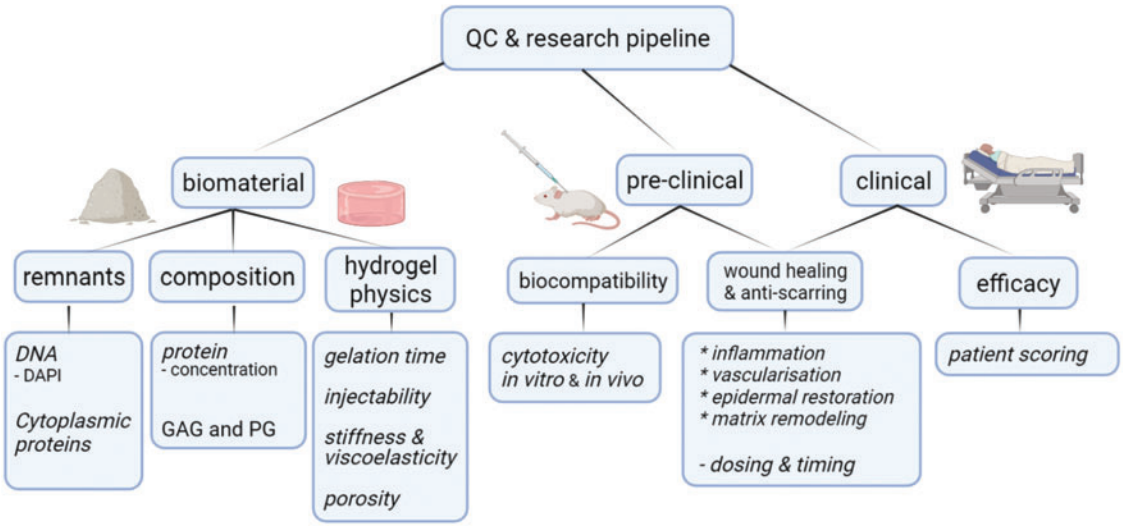

FIG. 3. Quality controls and clinical directions. GAG, glycosaminoglycan; PG, proteoglycan. Color images are available online.

example, conditioned medium, cultured cells and growth factors, and scalability are topics for future research. (Fig. 3).

To ascertain safety of use in humans, complications and adverse effects should be reported. Moreover, toxicological studies are warranted, potentially executed in bigger mammals, for example, pigs that can approximate outcomes in humans. A constraint for clinical application of autologous ECM relates to the requirements good manufacturing practice (GMP) production has for advanced therapy medicinal products. Although, decellularized tissue is already GMP compliant and chemicals used to manufacture ECM hydrogels are already available on a GMP level. These results together provide guidance and have relevant consequences for clinical application of ECM hydrogels.

Conclusion

In conclusion, prepared injectable ECM hydrogels provide new opportunities to enhance dermal wound healing in rodents. Research and (pre)clinical application of EMC-derived hydrogels are in their infancy; standardization of decellularization protocols and implementation of quality controls and cytotoxicity measurements are warranted and will enable obtaining a generic and comparable ECM product.

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Supplementary Material

Supplementary Appendix SA1

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Address correspondence to:
Martin C. Harmsen, PhD
Department of Pathology and Medical Biology
University of Groningen
University Medical Center Groningen
Hanzeplein 1
Groningen 9713 GZ

E-mail: m.c.harmsen@umcg.nl

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