Current Transplantation Reports (2021) 8:44–56
https://doi.org/10.1007/s40472-021-00317-2

CELLULAR TRANSPLANTS (G ORLANDO, SECTION EDITOR)

Engineering Vascularized Composite Tissues by Perfusion Decellularization/Recellularization: Review

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Accepted: 9 February 2021 / Published online: 3 March 2021

Abstract

Purpose of Review In the field of tissue engineering, the "perfusion decellularization and recellularization" emerged over the last decade. This technique is generating a complex and perfusable acellular scaffolds (e.g., solid organ) from discarded living tissue. The present work aims to describe all studies in which this technique was applied to complex composite tissues, or one of their elementary tissues.

Recent Findings A total of 25 experimental publications were found between 2009 and 2020. Studies interested skin/adipose, muscle and nerve flaps for elementary tissues, and larynx, ears, face, upper limbs, uterus, and penis grafts for complex tissue associations. Rat and human models were the most represented.

Summary This review showed that the current total number of studies covering the entire topic of vascularized composite tissue engineering is only 25, with a majority published within the past 5 years. This promising area of research should be investigated in the future.

Keywords Vascularized composite tissue · Tissue engineering · Perfusion decellularization · Cell culture · Recellularization · Perfusion bioreactor

Introduction

The gold standard in reconstructive surgery remains the autologous tissue transfer including skin graft (split or full thickness), local and free flap. Even routinely performed, the main limiting factor is the donor site availability and the sequalae resulting from tissue procurement. In addition, autologous surgery cannot restore anatomically the body part missing (e.g., face, hand) despite the effort to do so [1]. Vascularized composite allotransplantation (VCA) emerged as a realistic option for complex reconstruction over the last 20 years [2] that conventional surgery cannot addressed. The first human hand transplantation was performed in 1964 [3] and the first successful hand transplant [4] was performed in 1999 thanks to the advent of immunosuppressive regimen [5]. It was then followed by various transplant types like i.e. larynx [6], face [7], abdominal wall [8], uterus [9], or penis [10]. In total, more than 200 VCA were performed worldwide in highly specialized center [11]. Yet, VCA remains limited in terms of impact [12]. This is mainly due to the need for a life-long immunosuppressive regimen and the cost [13]. Therefore, VCA is still highly debated for non-vital transplants, despite some VCA are demonstrated to be considered a life-saving procedure. Moreover, similar to solid organs, chronic rejection was reported to lead to a progressive loss of transplants [14, 15]. Clinical implications are very important for the follow-up: For upper extremity VCA, a loss of the transplant allows a return to a previous state with amputation and prosthesis replacement. When loosing of a face transplant, the clinical

This article is part of the Topical Collection on *Cellular Transplant*

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scenario is dramatic: Even if the possibility to re-transplant was recently demonstrated with success [16], this causes fundamental concerns in terms of patient survival and immunological hurdle (hyper sensitization).

Tissue engineering (TE) could give new hopes for simple to complex tissue replacement. It can provide off-the-shelf constructs for clinicians avoiding long-term immunosuppression. As up today, producing adequate vascularized composite tissue extra-cellular matrix (ECM) is not possible with synthetic approaches [17] such as 3D bioprinting. Then, the decellularization techniques offer the advantage of ECM-derived over ECM-mimicking natural scaffolds. Decellularization principle is to remove cells from a living tissue with ECM structure and composition preservation. Secondarily, the tissue is repopulated by recipient cells either in vitro in a bioreactor or in vivo. Decellularization by imbibition is a reasonable option to decellularize small scaffolds without a functional vascular tree [18]. The perfusion decellularization/recellularization (P-DR) technique through the vascular pedicle, as firstly described by Ott [19] in 2009, has unlocked the production of large-scale scaffolds (whole-organ). The P-DR technique could then be applied to a different panel of vascularized tissue flaps, developing a new entire field of research described as vascularized composite engineering (VCE). Herein, we describe the first systematic review on the topic of VCA using the P-DR approach.

General Approach to Study Selection and Analysis

Definition of a Vascularized Composite Tissue Model

The term "composite" was initiated by Kleinert and Peacock [20], and defined as a "body part involving multiples tissues, at least two, derived from ectoderm and mesorderm" by Ravindra et al. [21]. In the field of VCE, we extended this definition of composite to any type of tissue flap, either isolated or associated, comprising a main perfusable and transplantable vascular pedicle: This allows to deliver decellularizing agents to any type of tissues, whatever the size or complexity is, as well as a route for in vitro seeding and culture medium perfusion, and finally a possibility for in vivo transplantation.

Study Classification

We distinguished simple tissues with no specific topographic allocation, defined as "elementary" composite tissue models, to true body parts specific to a region, defined as "complex" tissue models. Studies were then screened with the type of donor, graft model and associated tissue types, decellularization protocols, ECM and vasculature characterization, in vitro cell seeding in static conditions versus in vitro true recellularization using a perfusion bioreactor and finally in vivo samples or whole-scaffold implantation versus vascular anastomosis and transplantation. All these findings, study by study, are summarized in Table 1. The classifications following models and species involved, as well as per year of publication, are provided in Figure 1.

Elementary Vascularized Composite Tissue Models

Skin-Adipose Tissue Flap

Skin-adipose tissue flaps are the workhorse model for autologous reconstructive surgery approach, as well as a major component of many experimental VCA models. Similarly, to the epidermis/dermis layer, the adipose tissue should be considered a separate tissue compartment. We found six studies in the literature. Even if not involving any decellularization, the work from Chang et al. [22] in superficial epigastric artery (SEA) skin flaps from rats was retained for its description of ex vivo culturing in a perfusion bioreactor, the seeding of various types of cells and final in vivo transplantation, as a ground for future research. Henderson et al. [23] were first to decellularize by imbibition through a detergent-based protocol SEA skin flaps on rats. They reported a short patency of the vascular tree assessed by a perfusion machine. Qu et al. [24] compared agitation versus perfusion-decellularization efficiency, with a detergent-based deoxycholate (DXC)/sodium diazide protocol. The flaps were successfully decellularized but no venous return was observed post transplantation. The work from Zhang et al. [25] is the most successful experiment lead in the field of VCA: from acellular matrix production, recellularization, and transplantation. SEA skin flaps from athymic rats were treated with a combination of imbibition and perfusion in a non-detergent-based protocol, completed by isopropanol polar solvent agitation to treat fat. Interestingly, they showed that flaps could be frozen at −80° and thawed before P-DR application. Perfusion recellularization was achieved with human adipose-derived stem cells (ASCs) co-cultured with human umbilical vein endothelial cells (HUVECs), transplanted in vivo: The pedicle was thrombosed at postoperative day (POD) 7, but the scaffold remained vascularized by collateral branches and viable at 3 months. The limit was the small animal model and the absence of dermal regeneration analysis. Indeed, Jank et al. [26] addressed these limitations with their work on a superficial inferior epigastric (SIE) flap model in the pig. They used a sodium dodecyl sulfate (SDS)/Triton-X 100 detergent-based protocol. In vitro experiments consisted in static cell seeding with human mesenchymal stem cells (hMSCs), and keratinocytes to assess epidermis formation. They also performed allogeneic cell HUVEC seeding in a bioreactor, although no venous return was observed after in vivo transplantation. Notably, dermal patches were used to cover skin defects in rats, with a full cell's integration and a spontaneous re-

Table 1 Detailed review of publications dealing with composite tissue engineering through perfusion-decellularization/recellularization technique, from 2009 to 2020

	Tissue type/region	Model/graft	SPECIE (study reference)	Sample	Decellularization protocol	ECM Characterization
						Histology
Elementary	Skin/adipose	Skin flap (SIEA)	Rat	Unknown	No	No
			Rat	14	Agitation by 0.1% NaN3, 40U/mL DNase, and 4% SDC	Classical histology
			Rat	50	All group [5]: heparinized PBS Agitation (group 1): 1% SDS for 72h, DIW, TX-100, PBS Agitation and perfusion (P-A) with SDS 1% for 12h (group 3), for 24h (group 4), for 72h (group 5) followed by DIW, TX-100 1% for 30 min and PBS	Classical histology
			Rat	344	Perfusion + agitation: NaCl perfusion, F/T 3 cycles. 2 cycles of perfusion DIW 1d, 0.5 M NaCl and 1M NaCl for 4h each and DIW overnight, 0.25% trypsin/EDTA for 2h, DIW for 1h and isopropanol agitation overnight. Perfusion with 1% TX-100 for 2d, followed by DIW 2d and PBS for 1d	Classical histology DAPI IHC SEM
			Pig	Unknown	Perfusion: PBS, 1% SDS 10d, DIW, TX-100 1% for 24 h, 2L of DIW, 6L of PBS	IF
			Human	1	Perfusion: heparinized saline, 1% SDS for 100h, DIW for 1h, TX-100 for 1h, DIW for 1d	Classical histoloogy DAPI IHC
	Muscle	Rectus abdominis flap	Pig	Unknown	Perfusion: 0.02% trypsin/0.05% EGTA for 2h, 0.1% SDS for 12h, 1% TX-100 for 12h, 0.1% peracetic acid + 4% ethanol for 2h and 0.5h DNase. DIW washing for 7 d	Classical histology DAPI IHC SEM
		Gracilis muscle flap	Rat	40	Perfusion: heparin flush and group 1 (4.5h): Krebs Buffer, 1% Triton-X-100 for 1h, 1% SDS for 1h + DIW for 2h. Group 2 (121h): 1% SDS for 72h, DIW for 15 min, 1% TX-100 for 30 min, PBS for 48h	Classical histology SEM and TEM
	Nerve	Sciatic nerve flap	Pig	7	Perfusion: 340 mL of heparinized serum, 10L of 1% SDS, 870 mL of DIW, 1300 mL of 1% TX-100, 10 L of PBS, 1600mL DNase, and 630 mL of PBS	Classical histology DAPI, IF
Complex	Head and neck	Larynx	Rabbit	20	Perfusion: heparinized PBS for 15 min , 1% SDS for 5d, DIW for 15 min, TX-100 for 6h, PBS for 48h. RPMI-1640 culture medium perfused during decellularization	Classical histology IHC Chondrocyte viability SEM
			Rat	22	Perfusion: heparinized PBS for 15 min, 1% SDS for 12-16 h, DIW for 15 min, TX-100 for 30 min, PBS for 48h. RPMI-1640 culture medium perfused during decellularization	Classical histology IHC, DAPI SEM Chondrocyte viability
			Dog	11	Perfusion: heparinized PBS for 30 min, 1% SDS for 5d, DIW for 2h, TX-100 for 6h, PBS for 4d	Classical histology DAPI
		Ear	Pig	13	Perfusion: Heparinized serum saline 1L , SDS 1% for 88h, TX-100 for 25h, 2L of DIW, 28L of PBS and DNAse, and 2.5L of PBS	Classical histology IHC and IF SEM
			Human	13	Perfusion: 1L of eparinized serum saline, 36.5L of SDS 1%, 1200 mL of DIW, 10L of TX-100, 1200 mL of	Classical histology 3D histology Lipid droplets IHC and IF SEM

Table 1 (continued)

				DIW, isopropanol for 4h, 16L of PBS, 900 mL of DNase, and 4.5L of PBS	
	Face	Rat	7	Perfusion versus agitation Perfusion: heparinized saline for 1h, SDS 1% for 100h, PBS for 24h Agitation: heparinized saline, SDS 1% for 140h, PBS for 72h	H&E, TCM DAPI
		Human	6	Perfusion: 1.5L of heparinized saline, 70 L of SDS 1%, 6L of DIW, 9L of TX-100, 30L of PBS. agitation in isopropanol overnight then perfused for 12h and second bath of isopropanol overnight Perfusion of 1L of DIW and 26L of PBS, DNAse and 10L of PBS	Classical histology DAPI, IHC and IF
Limbs	Upper limb	Rat (illustrated with primate)	Unknown	Perfusion: PBS, 50h of SDS 1%, 1h of DIW and 1h 1% TX-100 and 124h of PBS. Initial fasciotomy and skin/adipose layer removal	Classical histology IHC
		Human	1	Perfusion: 6L of heparinized serum, 80L of DIW, 30d of SDS 1%, 15d of 1% TX-100, 15d of DIW and 50L of PBS. Initial fasciotomy	Classical histology DAPI, IF
Genitalia – reproductive	Penis	Human	Unknown	Perfusion: SDS 1% for 2 weeks (after penectomy)	Classical histology DAPI, IF SEM
	Whole uterus	Rat	27	3 perfusion protocols: 4h of DMSO 4%, 4h of TX-100, and 30 min PBS for 5 cycles, 5d Five cycles of P1 but dilution in dH2O- 0.05% NAN3) + freeze (−80°), 5 cycles, 5d 3) 2% SDC for 6h, DIW for 2h, 5 cycles, 5d	Classical histology SEM
		Rat	Unknown	Perfusion: 24h of SDS 0.01%, 24h of SDS 0.1%, 24h of SDS 1%, 15 min DIW, TX-100 for 30 min, PBS	Classical histology DAPI, IF TEM
		Pig	6	Perfusion: PBS 1h then 2 protocols: Freeze/thaw (−20°) then 0.1% SDS for 18h, DIW for 30min, 1% TX-100 for 30 min, PBS for 5h, in two cycles 2) Fresh: same protocol	Classical histology DAPI, IF SEM, TEM
		Rat	Unknown	Perfusion: two protocols F/T at −80° and thaw at room temperature, after PBS perfusion of 30 min DMSO 4% for 4h, TX-100 for 4h and DIW overnight, five cycles, 5d 2% SDC for 4h, DIW overnight, 5 cycles, 5d 1+2: peracetic acid for 30 min and PBS for 1h	No
		Sheep	24	Perfusion: 3 protocols PBS for 6h, SDS 1% for 48h, TX-100 for 24h, PBS for 72h PBS for 6h, DMSO 4% for 48h, TX-100 for 16h, PBS for 72h 3) DIW for 24h, 0.25% SDS agitation for 48h, SDS 0.5% for 24h and DIW for 24h perfusion, 4h in 10% formalin, PBS perfusion	Classical histology DAPI, IF SEM
		Sheep	121	Perfusion: 3 protocols F/T (−20°), EDTA perfusion, DIW SDS 0.5% for 26h, PBS overnight, DNase 1h, DIW for 1h. 2 cycles, DIW for 48h, frozen −20° Same but 2% SDC replace SDS SDC 2% for 4h, DIW for 6h, 1% TX-100 for 12h, DIW for 36h, 2 cycles. DNase, DIW, and frozen −20°. Sterilization with perfusion 0.1% peracetic acid	Classical histology DAPI SEM
Whole body	Whole foetus	Sheep	Unknown	Perfusion through umbilical artery: DIW for 24H, 1% TX-100 for 24h, 0.5% SDS for 96h, PBS for12h	Classical histology SEM

Table 1 (continued)

	ECM Characterization		In vitro studies		IN VIVO SUTDIES		REFERENCES
	DNA proteins	Vascular patency – mechanical test	Static sample seeding	Perfusion-bioreactor recellularization	Simple - biocompatibility	Transplantation	
Elementary	No	No	No	DMEM perfusion and MAPCs or MSCs seeding	No	Anastomosis and implantation for 7 days after DMEM perfusion or cell seeding	[22]
	DNA	Methylene blue perfusion	No	No	No	No	[23]
	DNA	Blue Evans perfusion	No	No	No	No	[24]
	DNA GAG	Microfil vascular injection Porosity	hUVEC seeding through arterial and vein pedicle associated with injected hASCs and HUVECs into the scaffold and cultured statically for 7 days	No	Non-recellularized ECM skin flap or recellularized ECM skin flap with hASCs and hUVECs implanted subcutaneously for 7ays or 3 months	Recellularized ECM with hASCs and hUVECs implanted with microanastomosis for 7days or 3 months	[25]
	DNA GAG Collagen Elastin	Biaxial mechanical test	Static cell seeding with hMSCs for 21d Keratinocytes for 7 days and then exposure to air-liquid interface	hUVECs seeded through the vascular pedicle by gravity and cultured 5 days in bioreactor	Subcutaneous implantation of acellular dermis for 14 days Full thickness skin defect model for 14d	Anastomosis of hUVECs recellularized scaffold in miniature swine	[26]
	No	Histology	No	No	No	No	[27]
	GAG Growth factor Total protein SDS	Vascular corrosion casting Biomechanical tests	C2C12 cells used for cell migration study (3h), cytocompatibility, and cell metabolism after 1, 3, or 5 days of C2C12 culture. Myogenic differentiation using digested ECM after 24h of cell incubation	No	Non-recellularized ECM patch implanted after partial thickness defect of the rat abdominal wall versus SIS-ECM. Remove after 2 and 8 weeks	No	[28]
	DNA	Evans Blue perfusion	No	No	No	No	[29]
	DNA GAG Cytokines	Fluoroscopy and angio micro-CT	No	pAECs seeded through the vascular pedicle and cultured for 7 days	No	No	[30]
Complex	No	No	Myogenesis-induced MSCs seeded by microinjection into the scaffold and then placed in DMEM for 1d	No	After 1 day of culture, recellularized larynx was implanted into the omentum for 4 weeks or 8 weeks.	No	[31]
	DNA	No	No	No	Implantation of decellularized larynx on rat omentum and	No	[32]

Table 1 (continued)

				harvested at 2, 4, 8, and 12 weeks		
DNA GAG	CT-angiography Biomechanical tests	Isolated acellular mucosa seeded with hBCs and cultured for 5d. Isolated acellular muscle seeded with hBMs and cultured for 5 d	Seeding through arteries and veins of hUVECs and cultured for 8d in bioreactor	Implantation of acellular ECM at the back of rats for 14d Implantation of recellularized laryngeal muscles between the fascia and the tibialis muscle (nude mice) for 14d	No	[33]
DNA Collagen GAG Elastin	Angio CT Mechanical tests	Cos 7 cells seeded on samples to study proliferation Induction of adipose differentiation of pBMSCs after 14d of culture	Whole ear seeding through artery with NIH-3T3 cells and pBMSCs	ECM implantation in the preperitoneum and evaluation of the antidonor IgM and IgG at 0, 10, 20, and 30d and histology at 30d	In vitro revascularization after arterial and vein anastomosis and perfused for 3h	[34]
DNA Collagen GAG Elastin Cytokine and GF	Angiography & Micro-CT Mechanical tests	rASCs seeded on artery, skin, full thickness ECM biopsies, and cultured for 14d	Whole ear seeding with hAECs through the artery and cultured for 2d	Implantation subcutaneously of ECM and removed after 15, 30, and 60d. Histology and detection Ig anti-human	No	[35]
DNA	Micro-angio CT	hASCs seeded on dermal or intern side of ECM and cultured for 7 d	No	No	No	[36]
DNA GAG Collagen Elastin Cytokines, GF	Angio CT	NIH3T3 and C2C12 cells seeded on ECM lip discs and cultured for 14d	Whole upper lip seeding through artery with C2C12 cells for 2 weeks and hAECS for 2d	No	Revascularization after arterial anastomosis in a porcine recipient for 4h	[37]
DNA GAG Proteomic	Micro-angio CT Microtomograp-hy, BMD Mechanical tests	No	Whole-limb perfusion: hUVECs seeded through the artery and C2C12 and MEF by injections, cultured for 21d. Electrical stimulation at day 6. Full thickness skin graft on ECM	No	Orthotopic transplantation with a bone intramedullary rod and electrical stimulation. Revascularization of recellularized scaffold after vascular anastomosis	[38]
DNA GAG Collagen Elastin	Angio CT Micro-CT (bone)	No	No	No	No	[39]
DNA VEGF, EGF, TGF-B1	Micro-angio CT	SVF cells seeded on transversal ECM section and cultured for 1d and 28d	No	No	No	[40]
DNA GAG Elastin Proteins Proteomic	Dye vascular perfusion Mechanical tests	No	No	No	No	[41]
No		No			No	[42]

Table 1 (continued)

	Allura Red dye perfusion		Whole-uterus perfusion: neonatal and adult uterine cells and rMSC injected with a needle into the ECM and vascular medium perfusion for 3d	Partial uterine horn replacement with a recellularized ECM horn patch for 28d and 90d. Assessment of the fertility of regenerated uterine horns and euthanized at 19 d after the presence of vaginal plug		
DNA Proteins	Vascular cast perfusion & SEM	ICE mixed stem cells seeded on ECM disc for 9d	No	No	No	[43]
No	No	No	No	No	NO	[44]
DNA	Angio CT Mechanical tests	MSC seeded on culture plate. ECM added after 7d and incubated for 72h before MTT	No	In vivo recellularization P3 ECM samples after implantation on the right uterine horn in a fertile rat	No	[45]
DNA GAG Collagen Elastin Proteins	Vascular casting Mechanical tests	Human embryonic kidney 293 cells incubated with thawed storage solution 4h before MTT assays Recellularization with isolated SF-SCs seeded by several injections through the ECM and cultured for 3 or 14d	No	No	No	[46]
Collagen	MRI and angio CT	No	No	No	No	[47]

SDS sodium dodecyl sulfate, *SDC* sodium deoxycholate, NaN_3 sodium diazide, *DIW* deionized water, *TX-100* Triton-X 100, *PBS* phosphate-buffered saline, *DMEM* Dulbecco's modified Eagle medium, *MSCs* mesenchymal stem cells, *pBMCs* porcine bone marrow mesenchymal stem cells, *MPACs* multipotent adult progenitor cells, *F/T* freeze/thaw, *hASCs* human adipose-derived stem cells, *rASCs* rodent ASCs, *HUVECs* human umbilical vein endothelial cells, *IF* immunofluorescence, *IHC* immunohistochemistry, *SEM* scanning electron microscopy, *TEM* transmission electron microscopy, *GAG* sulfated glycosaminoglans, *pAECs* porcine aortic endothelial cells, *hAECs* human AECs, *hBCs* human basal cells, *hBMs* primary human myoblasts, *BMD* bone mineral density, *MEF* mouse embryonic fibroblasts, *SVF* stromal vascular fraction, *SF-SCs* sheep fetal bone marrow stem cells

epithelialization. The last current study in this group was performed by Giatsidis et al. [27], concerning a single large volume human SIE adipose tissue flap from body contouring surgery. A detergent-based perfusion with SDS only was applied, with some ECM characterization. This paper was an interesting proof-of-concept to identify discarded allogenic tissues from plastic surgery patients, as potential flaps for tissue engineering applications.

Muscle Flap

This other vascularized tissue model is relevant both as an experimental tool in complex tissue engineering and for direct clinical applications. We found two studies in the literature. The first study was achieved by Zhang et al. [28], for skeletal muscle rectus abdominis flap in the porcine model. They were treated by the classical SDS detergent-based protocol. The ECM analysis included residual detergent quantification, precluding clinical quality control requirements. In vitro assessment was performed by static seeding of C2C12 myoblastic cell lines on scaffold samples, observing myogenic differentiation. In vivo non-seeded patches covered partial abdominal wall defects on rats, with a good outcome and remodeling, a limited retraction, and some organized neo-muscle fiber formation. All these results were better when compared to the well-established submucosa intestinal mucosa (SIS)-ECM model. There was no true perfusion recellularization performed nor investigation about functional muscle stimulation and contractility and stimulation.

The recent other study from Sabbagh et al. [29] downscaled to the gracilis flap rodent model. The essence of the study was to compare two types of decellularization protocols: with or without Krebs Henseleit Buffer, as tensioactive solution, associated with SDS/Triton-X detergent perfusion. They demonstrated that the addition of Krebs Henseleit Buffer could achieve a faster decellularization, with less damage caused to ECM.

Nerve Flap

The work from Wüthrich et al. [30] is presently the only addressing nerve flap P-DR, in order to obtain better improved nerve scaffolds. Porcine sciatic nerve flaps were perfusion decellularized with SDS/Triton-X detergents. The extensive ECM characterization insisted on cytokine analysis, highly preserved, critical to guide future axons regrowth. The correct vasculature preservation was confirmed by fluoroscopy and micro-angio CT. In vitro vascular tree recellularization was performed with porcine aortic endothelial cells, with a good distribution. This model could also serve to study nerve regeneration in complex composite scaffolds.

Complex Vascularized Composite Tissue Models

This section collects models with more complex tissue's associations, involving different functions to be restored and a specific allocated topography. The current body parts investigated are located in the head and neck, limbs, and genitalia/reproductive system. Finally, a whole total body decellularization represents an interesting feature.

Head and Neck Area

Larynx This complex composite model is based on a muscle-cartilage-mucosa tissue association. The first larynx study, in the rabbit model, was published by Hou et al. [31], with similar SDS/Triton-X detergent-based decellularization protocol than Ott et al. [19]. They added RPMI-1640 culture medium, to preserve donor chondrocyte viability: They hypothesized this would better preserve cartilage mechanical properties, without immune reaction to this avascular tissue. In vitro experiments consisted in whole-scaffold recellularization with MSCs and myogenesis-induced MSCs. After 8 weeks of culture cells, the formation of a muscle-like structure was observed. In vivo implantation was not performed. The work from Ma et al. [32]—same group as above—was performed in a rodent model, providing insights about in vivo reaction with the implantation of non-seeded and non-re-anastomosed whole scaffolds on rat omentum. The architecture was preserved and the neovascularization in the construct was observed. Less inflammation was reported than the control native implant. The absence of T cell-mediated immune response confirmed that the donor chondrocytes into the scaffolds did not elicit immunological response. Later on, Moser et al. [33] recently published a study in the canine model, with a detergent-based decellularization process which did not aim at chondrocyte viability preservation. A recellularization process was performed in a customized bioreactor, with multi-recellularization using human cells only. The use of a unique culture medium, "multi cell growth medium," allowed simultaneous culture of human basal cells for re-epithelialization, HUVECs for re-endothelialization, and primary human myoblasts for muscle culture. In vivo implantation of samples to whole-acellular scaffolds confirmed a good biocompatibility and cell repopulation from host.

Ear The ear model has always been a popular model [48] in tissue engineering for the past 20 years. No clinical application has been yet reached, due to a lack of complexity and perfusion of the constructs. This model encompasses skin, adipose tissue, and cartilage tissues.

The study from Duisit et al. [34] in a porcine model used SDS/Triton-X detergent-based decellularization protocol. Mechanical testing demonstrated a preservation of skin and cartilage properties. In vitro sample culture with porcine bone

marrow mesenchymal stem cells (BM-MSCs) demonstrated an ECM-guided adipose tissue differentiation. Whole ear recellularization was performed with porcine BM-MSCs, confirming proliferation and development of mature adipocytes. The vascular tree was functionally assessed during a short in vivo transplantation in the pig with a transient vascular patency. Implantation of samples between semi-identical MGH miniature swine did no elicit any antibody-mediated allo-immune response, with a demonstrated biocompatibility. Duisit et al. [35] applied previous findings to cadaveric human ear grafts, harvested up to 4 days after 4°C preservation. In addition to their previous protocol, a polar solvent step was added to remove residual oil cysts from adipose tissue. Numerous cytokines were retained, especially in the adipose tissue matrix. The vascular tree was patent as visualized on angiograms. After sample seeding with rat ASCs, confirming a cell-friendly ECM, partial recellularization was achieved with perfusion-bioreactor seeding of human aortic endothelial cells, repopulating vessels from large to medium diameters. In vivo experiments focused on sample implantations in rats with a fair biocompatibility, relative inflammation, and absence of circulating anti-human antibodies. This work settled human cadaveric body donation as a potential source of vascularized grafts.

Face In this study on human face from Duisit et al. [37], nose-lip grafts were selected: They involve all the tissues represented in the whole face, namely skin, adipose tissue, mucosa, cartilage, and cutaneous muscle. They were harvested from cadaveric donors deceased within 50 h, and decellularized with adapted protocols from human ears. Extensive characterization showed a well-preserved ECM and associated cytokines; the vascular tree was clearly patent on angio CT and in vivo reperfusion of acellular scaffold was successful for 4 h. Face scaffolds were divided in lip subunits, still with a vascular pedicle, and recellularized with HAEC and C2C12 cells, with a demonstrated cell survival and distribution. These findings were extended to the successful production of a complete soft face scaffold. The other work on the face model by Duisit et al. [36] was the down-scaling application to a rodent model, in order to describe a more accessible experimental model to other teams. The other purposes were to compare the SDS/Triton-X detergent-based protocol with perfusion or agitation: It was concluded that both were nearly equivalent, due to the small graft volume and the easy diffusion of decellularizing, with the exception of cartilage treatment, significantly better with perfusion.

Limb Area

All current investigations currently available are interesting the upper limb, in the small and large animal, as well as early work on human mode by the same group. These models are highly representative of complex composite tissues, with the association of skin, adipose tissue, skeletal muscle, tendons, bone, and cartilage.

The first study by Jank et al. [38] demonstrated the application of the P-DR technique to produce to the rodent forearm, with SDS/Triton-X detergent-based protocol. They described the need to perform extensive fasciotomies and skin/adipose tissue layer removal, in order to prevent compartment syndrome development, with edema formation during decellularization, that could jeopardize distal limb perfusion. ECM characterization included extensive mechanical testing. Recellularization was performed using HUVECs, C2C12 cells mixed with HUVECS, and mouse embryonic fibroblasts, cultured in a specific perfusion bioreactor allowing electrical stimulations. Isometric force and morphometric of myofiber measurements demonstrated some functional muscle tissue production. In vivo transplantations of seeded whole-limb scaffolds demonstrated arterial tree filling, with however no mention of a venous return. Electrical stimulation of recellularized grafts showed a flexion at the levels of the wrist and metacarpo-phalangeal joints. The scaling-up to a single whole primate upper limb was exposed, with same adapted protocol and fasciotomies: Decellularization success was demonstrated based upon histology. The other study, from Gerli et al. [39], is an application to an isolated human upper limb from a deceased donor. Interestingly, the graft was procured within 24 h postmortem and shipped frozen. After thawing, SDS/Triton-X detergent-based decellularization was achieved in a specifically designed chamber, under sterile conditions, and lasted an entire month. ECM was satisfyingly assessed by conventional analysis and the vascular tree was well opacified on angio CT examination.

Genitalia and Reproductive System Area

For the reproductive system and genitalia, current researches are essentially developed in the approach of whole-uterus bioengineering, a very productive area, and a recent study about human penile decellularization.

Uterus Even if located inside the body, and part of the reproductive system, the uterus is also considered a vascularized composite tissue for the epithelial aspect of the endometrium, surrounded by the myometrium layer and in line of the vagina. The first study for this model was published by Hellström et al. [41], in the rat. They compared three detergent-based protocols, involving Triton-X, dimethyl sulfoxide (DMSO), and sodium deoxycholate (SDC), with a step of freeze thaw at −80°C. ECM quality was assessed by standard tests, but not the scaffold's vasculature. SDC molecule was retained as the best candidate to treat this type of graft. In a few-week interval, another work was published by Miyazaki et al. [42] on the same model, with the increasing concentrations of SDS in

addition to Triton-X. Whole-scaffold recellularization in a bioreactor was performed with primary uterine cells and MSCs, observing endometrium-like structure formation. An in vivo vascular functional testing was achieved with eutopic transplantation. The most important finding was partial uterine horn replacement in recipient rats, achieved with non-seeded scaffold sample and able to sustain pregnancy in 75% of animals. The following work by Campo et al. [43] up-scaled to the pig model. Two SDS/Triton-X decellularization protocols were compared, and using either fresh or frozen/thawed uterus: No difference after ECM assessment and vascular casting could be observed. Some in vitro experiments showed survival of human endometrial stem cells seeded on scaffold samples.

The publication from Padma et al. [44] was a pure protocol paper on the rodent model for transplant harvest and decellularization by DMSO with Triton-X 100 or SDC. A new large model in the sheep was described by Daryabari et al. [45]. The principal aim was to compare three different decellularizing protocols, retaining SDS perfusion with formalin agitation. ECM was classically investigated, and tested in vitro with MTT for cytotoxicity seeding with rat MSCs. In vivo recellularization was completed with partial implantation on rat horn and demonstrated construct sustainment and repopulation. The latest publication in this group by Tiemann et al. [46] was reported on the same ovine model, considered superior to the cow and the pig in terms of translational approach. Three protocols were tested on an important number of grafts, retaining a SDC-based approach, as assessed by ECM quality and vascular tree patency. Notably, all scaffolds were frozen at −20°C. In vitro toxicity tests used MTT with embryonic kidney cells. Sheep fetal bone marrow stem cells were seeded on samples to preclude recellularization.

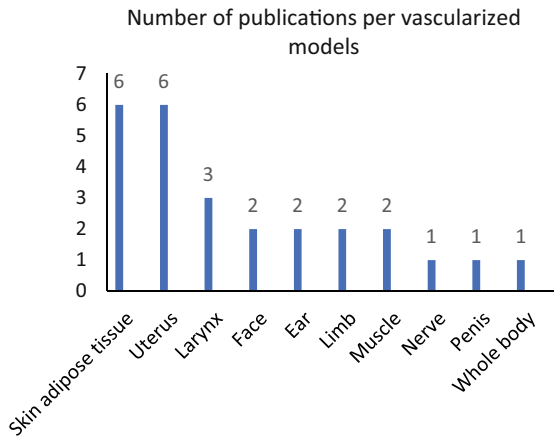
Penis The early work from Tan et al. [40] describes a new model in VCE, with human penile P-DR, pursuing the same rationale of tissue procurements from post-surgery-discarded tissues—in this case from transgender surgery—as previously described in other studies. The graft involves corpora cavernosa and corpus spongiosum, unique tissue types and new to date for experimental investigations in P-DR, but not the skin/adipose tissue envelop impossible to obtain with this type of tissue donation. Graft decellularization was SDS perfusion-based only, using both cavernosal arteries as well as the urethra. ECM preservation was satisfying and micro-CT angiography revealed the maintenance of a perfusable vascular tree. Cells from stromal vascular fraction were seeded on samples. Functional erection simulation was not investigated.

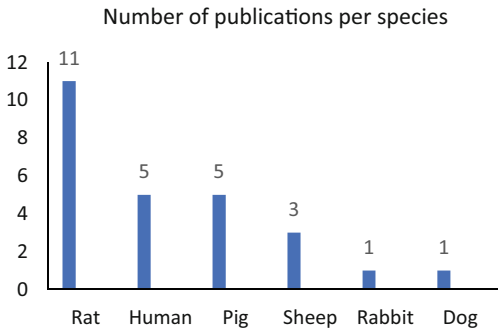
Whole Body

Very interestingly, the study from Kajbafzadeh et al. [47] already demonstrated in 2015 the possibility to perfusion decellularize a whole body, as applied to the sheep fetus: They used the umbilical artery as a route for perfusion. The protocol was SDS/TX-100 detergent-based, but perfusing first Triton-X 100 in order to destabilize cell membranes and enhance SDS penetration. ECM assessment of various organs and tissues showed that a single protocol can address nearly all tissues and components at the same time.

Discussion

As reported in Figure 1, we reported 25 publications between 2009 and 2020, all experimental, with 19 during the last 5 years, demonstrating a very recent development of the field, remaining quite low in comparison with solid organ engineering. Currently three types of elementary tissue models are described: skin/adipose tissue, the most developed model, muscle, and nerve. P-DR current research in the head and neck area is essentially focused on the larynx, the face, and its transplantation subunits; the limb is investigated on the upper limb model only due to the contra indication of the lower limb for VCA (prosthetics are providing better functional outcome [49]). For the genitalia and reproductive system, the work on uterus is particularly developed (only VCA with no long-term immunosuppressive regimen, because the uterus is removed right after the birth (can do 2 pregnancy theoretically)), whereas the penis is at of its early stage of development. Interestingly, the human model follows directly the rat, and both constitute the essential of involved species, followed by the rabbit, sheep, and dog. What can still be decellularized? Nearly all unexplored models, as long as associated with a reliable perfusable vascular territory. Indeed, the ability to perfusion decellularize any body parts and tissues was demonstrated in this review, and ultimately confirmed by the whole-fetus decellularization in the sheep. Given the location, tissue association, and size, and multiplied by the number of donor types, the number of experimental models is to be selected in gigantic: Definition on the tissue types involved, and their relative proportion, should guide this era of VCE, rather than older classifications. Notably, most of models are following previously described type of allotransplants or autologous flaps, indicating the need to pursue conventional anatomical and surgical research. On another, certain models, like lip and nose subunit transplants [50] or total eye [51], will only find an application in engineered transplantation with the absence of immunosuppression, even allogenic skin flaps. The large majority of protocols were detergent-based in a combination of SDS/Triton-X 100, with the exception of uterus protocols with SDC, making it the most versatile at this stage of experimental investigations: This is of tremendous importance to process complex and multi-tissue-associated models, as vascular perfusion cannot be selective of a specific tissue layer. The vascular tree preservation during decellularization is critical, but few studies have investigated and demonstrated a functional vascular tree at the time of





Year	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020
Number	1	1	1	0	1	2	3	3	4	4	3	3
Model /species	Skin-fat / Rat	Skin-fat / Rat	Larynx / Rabbit		Larynx / Rabbit	Uterus / Rat	Skin-fat / Rat Limb / Rat Whole-Fetus / Sheep	Skin-fat / Rat Muscle / Pig Uterus / Pig	Adipose tissue / Pig Face / Human Face / Rat Ear / Pig	Skin-fat / Human Ear / Human Limb / Human Uterus / Rat	Muscle / Rat Penis / Human Uterus / Sheep	Nerve / Pig Larynx / Human Uterus / Sheep

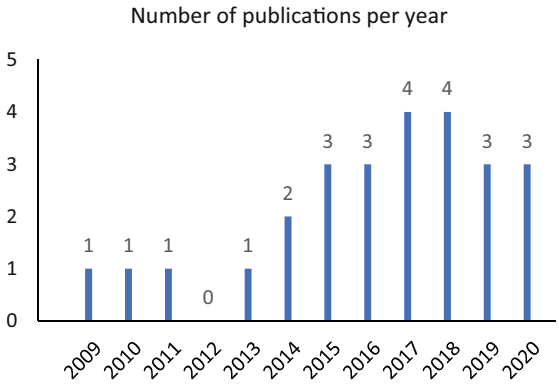


Figure 1 Type and number of models and species involved in found experimental studies. Chronology of publications during the last decade.

transplantation: This is compulsory to allow the final outcome of constructs, by reconnexion to the host vascular tree and sustain viability and long-term integration. Many studies have essentially characterized the produced ECM, being very demanding especially when dealing with models like the limb or the face involving a lot of very different tissues, but most did not push the model through the recellularization and transplantation steps: This closed loop only should validate a given protocol. For the next studies based on a same model, ECM characterization steps should be a lot simplified for the investigators, to allow targeting the next steps: This emphasized the importance of electing experimental working protocols, to be refined later on. The number of studied specimens is also very inequal in the current publications, with some work based on a single case to other interesting hundreds of flaps. Challenges ahead are mostly represented by the composite recellularization involving many types of different cells and in vivo transplantation. Restoring certain types of function, like sensitivity and motricity, is unique for this type or organs. For large flaps, like whole limbs, the number of cells will be prohibitive, even seriously questioning the cell source possibilities. The possibility to rely on the recipient as its own bioreactor to repopulate the scaffolds is a considerable advantage of composite tissue compared to organs, as they do not need to be fully functional at the time of implantation. As advocated by Badylak [52], we will have to favor a pro-regenerative aspect of the biomaterials to guide this strategy. The very important feature developed by Chang et al. [25] is an illustration of this unique approach in vascularized composite models: The

important surface of contact with recipient site also offers the possibility to consider only a transient use of native vascular pedicle, relying on the surrounding neoangiogenesis perfusion takeover.

Conclusion

The research topic of generating perfusable complex and elementary tissue matrices, by the application of perfusion-decellularization/recellularization approach, is a very promising approach: Produced scaffolds can exhibit complexity and vascularity unreachable by current ECM-mimicking technologies with synthesis, and offer new hopes to address limitations from autologous and allogenic tissue transplantation. However, the number of publications and research teams remains low in comparison with similar work on solid organ engineering, principally due to the very important number of models to be considered and the relative youngness of the corresponding allotransplantation field. This stage, in which a current review can embrace the entire publications of this whole rising field, is also the moment to clearly define the experimental references, the underlying concepts, and the challenges to be addressed. The non-vital and reversible aspects, as well as the non-absolute requirement of function at the time of implantation, make VCE models good candidates for future clinical applications. Thus, we do not doubt that promises from this new area of research will shortly increase considerably.

Acknowledgements JD is supported by Fonds de Recherche Clinique (FRC), Cliniques Universitaires Saint-Luc/UCLouvain grant. LM is a "Fonds National de La Recherche Scientifique" (FNRS, Belgium) research fellow

Declarations All reported studies/experiments with human or animal subjects performed by the authors have been previously published and complied with all applicable ethical standards (including the Helsinki declaration and its amendments, institutional/national research committee standards, and international/national/institutional guidelines).

Conflict of Interest The authors declare no competing interests.

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- • Of importance
- •• Of major importance

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