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Mesenchymal stem cells, secretome and biomaterials in *in-vivo* animal models: Regenerative medicine application in cutaneous wound healing

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Abstract: The treatment of nonhealing and chronic cutaneous wounds still needs a clinical advancement to be effective. Both mesenchymal stem cells (MSCs), obtained from different sources, and their secretome derived thereof (especially exosomes) can activate signaling pathways related to promotion of cell migration, vascularization, collagen deposition, and inflammatory response demonstrating prohealing, angiogenetic and anti-scarring capacities. On the other hand, biodegradable biomimetic scaffolds can facilitate endogenous cell attachment and proliferation as well as extracellular matrix production. In this Review, we revise the complex composites made by biomimetic scaffolds, mainly hydrogels, and MSC-derived exosomes constructed for cutaneous wound healing. Studies demonstrate that there exists a synergistic action of scaffolds with encapsulated exosomes, displaying a sustained release profiles to facilitate long-lasting healing effects. It can be envisioned that dressings made by biomimetic hydrogels and MSC-derived exosomes will be clinically applied in the near future for the effective treatment of nonhealing and chronic wounds.

Introduction

Wound healing is a complicated biological process that occurs in three distinct yet overlapping phases including inflammation, cell proliferation, and matrix remodeling, which need the support of nutrition and oxygen provided by blood vessels to cells participating in the healing process (Falanga, 2005; Arwert *et al.*, 2012; Hu *et al.*, 2014; Rodrigues *et al.*, 2019). Fig. 1 provides the main events and cues determining intercellular signaling pathways and growth factors involved in the three phases of wound healing. In a wound, damage to the skin activates platelets and the formation of a clot. Platelets and epithelial cells at the margin of the lesion release a wide range of growth factors and chemo-attractants to recruit immune cells (neutrophils and macrophages) giving rise to the inflammatory phase. In the proliferative phase, macrophages acquire a M2 phenotype and secrete growth factors to develop the granulation tissue by the activation of fibroblasts and new vessels via transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF). Keratinocytes and activated fibroblasts can also stimulate angiogenesis. Proliferating and migrating keratinocytes are engaged in the re-epithelization and reconstitution of epidermal appendages. It is the presence of epidermal stem cells in different compartments of the skin such as inter-follicular compartments and epidermal appendages (sweat glands and hair follicles with their associated sebaceous glands) that allows skin self-repair capabilities (Mathes *et al.*, 2014). In the remodeling phase, fibroblasts are stimulated by TGF-β3 to convert into myofibroblasts, which deposit extracellular matrix and determine wound contraction, reducing the surface area of the wound that must be re-epithelialized. Matrix remodelling is due to the secreton by myofibroblasts of MMPs and their respective inhibitors (tissue inhibitors of metalloproteinases, TIMPs). During time, the collagen III found in granulation tissue is gradually decreased and replaced with collagen I. In the last years, it has been elucidated that multiple signaling pathways are major players for regenerative wound healing, i.e., TGF-β, Notch, Hedgehog, and Wnt/β-catenin (Choi *et al.*, 2022). While TGF-β1 functions as a fibrosis-stimulating factor, TGF-β3 regulates anti-scarring activity (Shah *et al.*, 1995; Soo *et al.*, 2003). The Notch pathway is involved in epidermal cell differentiation, maintenance of skin homeostasis and promotion of angiogenesis (Okuyama *et al.*, 2008; Watt *et al.*, 2008; Blanpain and Fuchs, 2009; Gridley, 2010; Shi *et al.*, 2015). The Hedgehog pathway plays a

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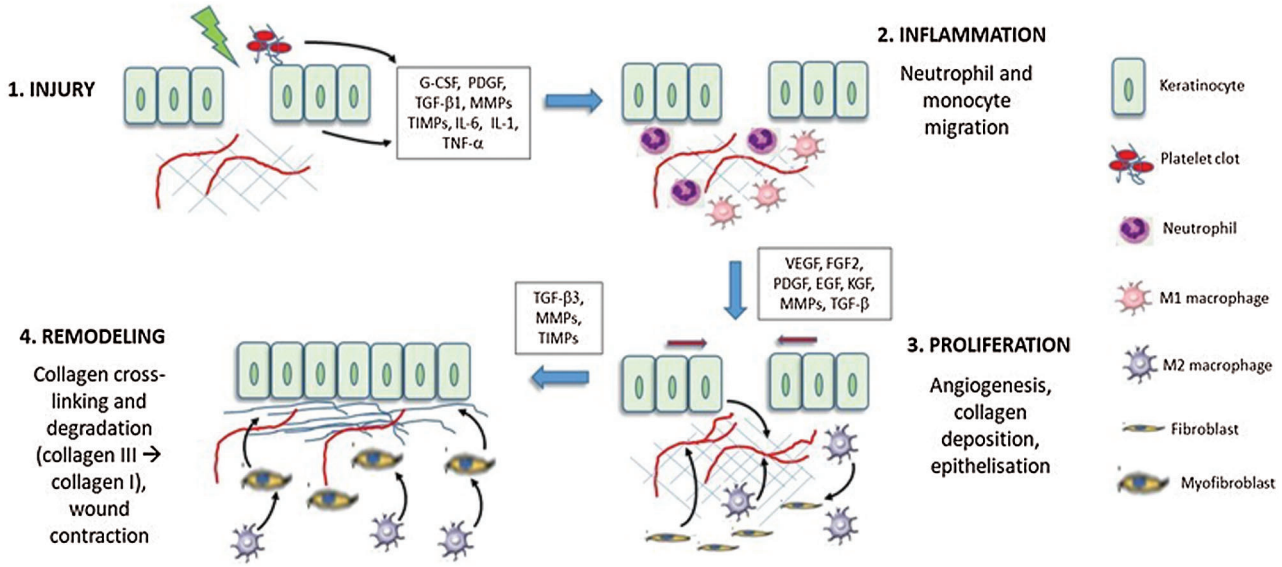

FIGURE 1. The three phases of skin wound healing after an injury. Cellular interplays are shown as black arrows. See text for details.

role in skin morphogenesis and angiogenesis, and modulates dermal repair and wound vascularization during the healing process (Asai *et al.*, 2006; Le *et al.*, 2008). Finally, the Wnt/β-catenin pathway participates in multiple steps of the wound-healing process, including the formation of skin appendages by activation of stem cells residing within skin, the differentiation and migration of keratinocytes, the migration of fibroblasts and their transformation into myofibroblasts, and angiogenesis (Houschyar *et al.*, 2015; Shi *et al.*, 2015).

Impaired wound healing, characterized by insufficient angiogenesis and easy infection, is one of the most common complications of diabetes, leading to chronic and nonhealing ulcers, with a prevalence in Europe of 5.1%. Diabetic ulcers are recalcitrant to healing due to many cellular and molecular aberrations (Gary Sibbald and Woo, 2008). In diabetes mellitus, the persistence of hyperglycemia causes peripheral nerve injury and arterial disease. Sustained hyperglycemia and induced oxidative stress impair cell migration, alter nitric oxide production at the level of endothelial cells (Forstermann and Munzel, 2006), as well as determine insufficient angiogenesis to support the collagen synthesis necessary for mature granulation and subsequent re-epithelialization. In addition, high levels of blood glucose impair leukocyte function causing an insufficient immune response, inciting infections and difficulties in the healing of foot injuries and ulcers (Gary Sibbald and Woo, 2008). Vascular and peripheral neuritis complications and abnormal collagen lead to skin wounds that are refractory and which often ulcerate. Also trauma and burns can lead to scar formation and impaired wound healing (Cerqueira *et al.*, 2016). In recent years, skin substitutes through the application of biomimetic scaffolds together with stem cells and bioactive substrates have provided an emerging therapeutic opportunity in the treatment of acute and chronic cutaneous wounds (Conese *et al.*, 2020).

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that can differentiate mainly into mesenchymal cell lineages, including adipocytes, osteoblast, chondrocytes, myoblasts, and endothelial cells, in different culture conditions and morphogens/growth factors. MSCs can be derived from diverse sources, such as bone marrow, adipose tissue, umbilical cord, fetal membranes, synovia, gingival tissue etc. (Lee *et al.*, 2016). The main types investigated in wound healing are MSCs derived from bone marrow (BM), adipose tissue (AD) and umbilical cord (UC) (Riha *et al.*, 2021; Sivaraj *et al.*, 2021). Although there exist subtle variations in MSCs from different sources and in principle they can be used equally in wound healing, AD-derived MSCs (ADSCs) are the most easily accessible, can be isolated at higher yield and in large quantities with minimal patient morbidity, thus being the most favored cell type for wound repair and regeneration (Hassan *et al.*, 2014; Bertozzi *et al.*, 2017). A great number of animal studies have purported the notion that MSCs display positive healing actions, bringing their application to clinical trials (Huang *et al.*, 2020). Especially with hard-to-heal wounds, MSC treatment results in enhanced angiogenesis, facilitated re-epithelialization, improved granulation, and accelerated wound closure. The underlying mechanisms of their therapeutic role is not completely understood, however MSCs actively respond to biological signals associated with inflammation, necrosis, and tissue injury (Prockop and Oh, 2012). MSCs can home to injured skin, operate direct differentiation into skin cells and are a reservoir of trophic factors that can be secreted and act paracrinally (Huang *et al.*, 2020). Furthermore, in the harsh inflammatory milieu of non-healing wounds, MSCs can respond to inflammatory stimuli by becoming potently immunosuppressive (Zhang *et al.*, 2015d; Cuenca *et al.*, 2018; Yu *et al.*, 2019), thus facilitating the transition from the inflammatory phase to the proliferative phase. In recent years, it has become increasingly clear that their engraftment in injury sites contribute little to their therapeutic effects. In the harsh environment of the wound, the contribution of MSC differentiation to diverse injury models have been limited, including poor postimplant cell survival, engraftment efficiency, and cell retention (Chen *et al.*, 2012). Instead, it is increasingly appreciated that their secretome is the primary mechanism exerting multifaceted functions including immunomodulation, angiogenesis, anti-apoptosis, anti-scarring, chemoattraction and modulation of local stem and

progenitor cells (Gnecchi *et al.*, 2008; Singer and Caplan, 2011; Maxson *et al.*, 2012; Khosrotehrani, 2013; Liang *et al.*, 2014). The interest in MSCs and wound healing has been pointed out by their property of sensing the environment and creating an orchestrated network of molecules to promote the tissue repair/regeneration process. MSCs can secrete pro-angiogenic factors that can promote vascularisation in the wound area and formation of granulation tissue, among which vascular endothelial growth factor (VEGF), hepatocyte growth factors (HGF), PDGF, and basic fibroblast growth factor (bFGF) are of extreme importance (Chen *et al.*, 2008; Yoon *et al.*, 2010; An *et al.*, 2015). MSCs can promote re-epithelization at the wound site via the secretion of epidermal growth factor (EGF) and keratinocyte growth factor (KGF) (Gnecchi *et al.*, 2008). MSCs are anti-inflammatory, thanks to the secretion of indoleamine-2, 3-dioxygenase (IDO), prostaglandin E2 (PGE2) and tumor necrosis factor-α (TNF-α)-stimulated gene 6 (TSG-6), thereby modulating both innate and adaptive immune responses impeding scarring in favor of regeneration (Nemeth *et al.*, 2009; Singer and Caplan, 2011; Ylostalo *et al.*, 2012).

MSC secretome

MSC secretome, represented roughly by conditioned medium (CM), is enriched in extracellular vesicles (EVs), membrane-surrounded structures released by cells that play important roles in the intercellular transmission of biological signals to regulate immunomodulatory and tissue repair processes. EVs released by MSCs are comprised of apoptotic bodies (1,000–5,000 nm), microparticles (or ectosomes, up to 1,000 nm), and exosomes (EXO, 30–150 nm) (Chen *et al.*, 2017). Exosomes are considered the main contributor to stem cells efficacy (An *et al.*, 2021). Indeed, exosomes display therapeutic effects on tissue injuries, which could be attributed to the transfer of membrane and cytosolic proteins, lipids and RNAs between cells (Raposo and Stoorvogel, 2013).

MSCs as well as their secretions (CM, extracellular vesicles, and EXO) have been shown to enhance wound healing and facilitate skin regeneration, as well as diabetic skin wound healing. MSC-conditioned medium has a potent healing effect on skin wounds (Chen *et al.*, 2008; Yew *et al.*, 2011; Shrestha *et al.*, 2013; Li *et al.*, 2017). The addition of EXO to the healing wound has been shown to promote proliferation and migration of related cells, enhance angiogenesis, re-epithelization, and regulating immune responses, highlighting exosomes as a promising approach to achieve a cell-free alternative to stem cell therapy (Zhang *et al.*, 2015a; Zhang *et al.*, 2015c; Cerqueira *et al.*, 2016; Hu *et al.*, 2016; Lee *et al.*, 2016; Liang *et al.*, 2016; Rani and Ritter, 2016; Phinney and Pittenger, 2017; Hu *et al.*, 2018; Dalirfardouei *et al.*, 2019; Ahangar *et al.*, 2020; Manchon *et al.*, 2021). Multiple studies have clarified that EXO can direct macrophage differentiation from pro-inflammatory M1 to anti-inflammatory M2 phenotype (He *et al.*, 2019), induce fibroblast proliferation and migration for the first extracellular matrix (ECM) deposition (Zhang *et al.*, 2015c; Ferreira *et al.*, 2017; Choi *et al.*, 2018), endothelial cell proliferation and migration to induce angiogenesis (Shabbir *et al.*, 2015), keratinocyte proliferation and migration for re-epithelization (Ferreira *et al.*, 2017), and to induce remodeling by ECM degradation and deposition by modulation of myofibroblasts with reduction of scar formation (Fang *et al.*, 2016; Hu *et al.*, 2016). Fig. 2 displays the main mechanisms by which MSCs and EXO determine healing at the wound injury site. The advantages of using EV-mediated cell-free therapies is of greater stability and storability, no risk of ectopic tissue formation and having a lower possibility of immune rejection as compared to MSC-based cell therapies (Merino-Gonzalez *et al.*, 2016).

Biomaterials and MSC/EXO

In order to improve survival of transplanted MSCs, a supportive microenvironment is pivotal to maximize cell viability (Kamoun *et al.*, 2017). Biomaterial-based wound dressings have been thought to accelerate cell attachment and proliferation of various cell types and interact with the released growth factors enhancing their bioavailability (Tartarini and Mele, 2015). Four main approaches that have been envisioned include: (i) sheets of cells secreting ECM (Yu *et al.*, 2018); (ii) pre-made porous scaffolds of synthetic, natural, and biodegradable biomaterial; (iii) decellularized ECM scaffolds, and (iv) cells entrapped in hydrogels (Chaudhari *et al.*, 2016). To this end, many smart "skin substitutes", made with varied combinations of synthetic and/or biologic substances, were used in order to perform many of skin's functions and to treat deep dermal and full thickness injuries of various etiologies. These skin dressings were combined with MSCs to foster skin healing and include epidermal, dermal, and dermoepidermal (composite) skin substitutes, made by collagen and hyaluronic acid, i.e., the major components of the ECM (Hu *et al.*, 2014). Other skin substitute are comprised of biocompatible and biodegradable synthetic polymers, such as polycaprolactone, polylactic acid, polyglycolic acid, poly(vinyl alcohol), poly (ethylene glycol), and polyurethanes, as well polysaccharides, such as chitosan and its derivatives (Moura *et al.*, 2013). The current available commercial tissue-engineered products for wound healing comprise acellular products mainly made of collagen, hyaluronic acid, elastin or fibrin (Ho *et al.*, 2017), among which Integra® (a bilayer made of bovine collagen and shark chondroitin sulfate with a silicone membrane, acting as a temporary barrier) was the first to be approved by U.S. Food and Drug Administration (FDA) to regenerate dermis (Savoji *et al.*, 2018). These skin substitutes may tailor tissue-engineered products to the required patient groups, except Integra® that can be applied for a wide range of treatments including full-thickness burns, chronic ulcer and full-thickness nonthermal skin wound management, among others (Bello *et al.*, 2001; Portincasa *et al.*, 2018).

A wealth of preclinical studies has demonstrated that stem cell therapy combined with biomaterials improved wound healing capacity and regeneration to skin injury by accelerating healing time, by which the correction time was shortened from 7–28 days to 7–14 days with only MSCs and MSCs combined with biomaterials, respectively (Riha *et al.*, 2021). These composites were made of nanfibrous scaffolds, gels and hydrogels, and were evaluated together with BM-MSCs, UC-MSCs, or ADSCs in mouse models representing burns, full-thickness excisional wounds, and nonhealing diabetic ulcers (Altman *et al.*, 2009; Chung *et al.*, 2016; Alapure *et al.*, 2018; Xu *et al.*, 2018; Tang *et al.*, 2019; Chen *et al.*, 2020; Lu *et al.*, 2020). Although a tremendous

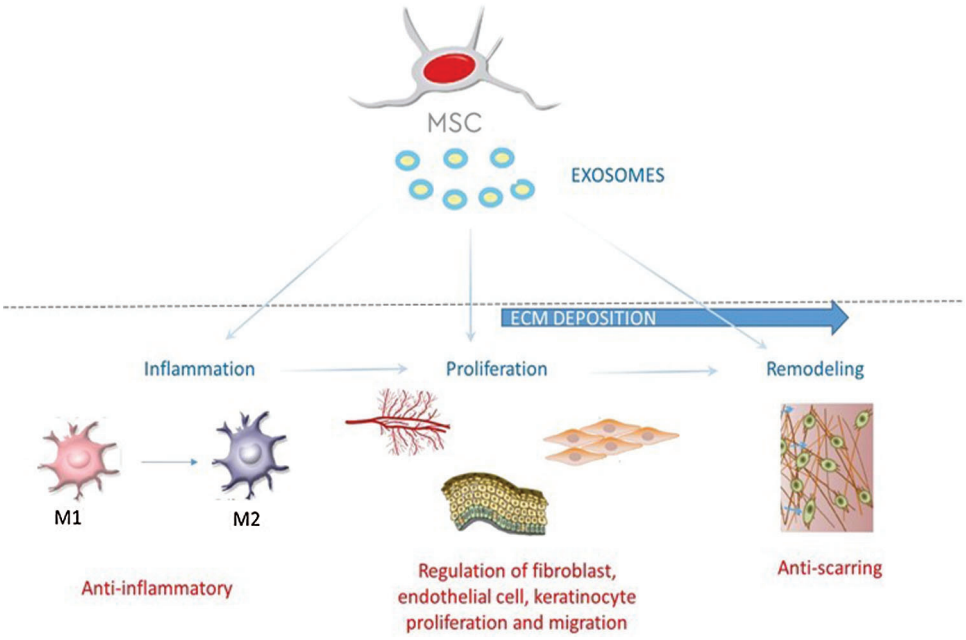

FIGURE 2. MSCs and exosomes derived thereof acting on the different stages of cutaneous wound healing, such as inflammation, proliferation and remodeling.

progress has been made over the past few decades to develop skin substitutes for the management of acute and chronic wounds, most commercially used skin substitutes are manufactured from autologous adult cells (keratinocytes and fibroblast). Indeed, there are no existing commercial skin constructs available in the market that are constructed using both MSCs and biomaterials. The main challenges to be faced ahead include characterization, optimization, and delivery of treatment of stem cells composites (Savoji *et al.*, 2018), and also unresolved drawbacks such as wound contraction and impaired vascularization should be further considered (Ho *et al.*, 2017). Moreover, it is not well known if different types of wounds would be better healed with a specific type of MSCs; in other words, which of MSCs, BM-MSCs, ADSCs or UC-MSCs, would sense properly the wound microenvironment when combined with biomaterials in the patient's setting.

The EXO incorporation into scaffolds as wound dressing for skin wound healing would make the MSCs and its secretome more realistic in clinical application, because of their direct contact with the injury site. Indeed, the common method of EXO administration is injection, which can affect their function due to the rapid clearance rate and relatively short half-life *in vivo* (Liu *et al.*, 2017). On the other hand, diabetic wound repair and regeneration require a relatively long healing time. Herein, it is necessary to develop a novel biocompatible scaffold that can serve as a sustained release carrier for EXO to maintain their bioactivity at the diabetic wound area and further accelerate wound healing.

Among biomimetic composites, hydrogels, structurally similar to the natural ECM, have been considered promising biomaterials to deliver drugs/cells for wound treatments (Sharifzadeh and Hosseinkhani, 2017; Xi *et al.*, 2018; Sivaraj *et al.*, 2021). Hydrogels are physical or chemical cross-linked three-dimensional hydrophilic polymeric networks, which possess the capacity to absorb abundant amount of water ideally for hydrating and creating a supportive environment within the wound bed that accelerates angiogenesis and removal of cell debris and alleviating pain (Sivaraj *et al.*, 2021). Hydrogels applied to wound healing should possess the following features: appropriate mechanical properties, good water retention, anti-infection capacity, injectable capacity, and excellent cell biocompatibility (Annabi *et al.*, 2017). Moreover, they should be self-healing, meaning that maintain their structural stability during the wound healing (Taylor and In Het Panhuis, 2016; Li *et al.*, 2018).

Methods

The works discussed in this Review were selected when they presented data on MSC secretome in combination with biomaterials for cutaneous wound healing in *in vivo* models. We have focused only on experimental works in animals without discussing clinical data and meta-analysis. Thus, we searched PubMed, MEDLINE, and Scopus using the keywords mesenchymal stem cell, conditioned medium, exosome, extracellular vesicle, and skin/cutaneous wound healing.

Results and Discussion

MSC-derived CM, EVs and exosomes were used in combination mostly with hydrogels, although also one study with electrospun fibers and one with decellularized amniotic membrane were found (Table 1). Diabetes chronic wounds/ulcers was the main medical problem that was considered in these studies. In general, MSC-CM/EVs/EXO scaffold application could shorten wound healing time, limit the inflammatory response, enhance re-epithelialization, promote the formation of high-quality, well vascularized granulation tissue, and attenuate the production of fibrotic or hypertrophic scar tissue, thereby improving wound healing rate and quality. In particular, therapeutic hydrogels addressed concerns such as desiccation (loss of moisture from the wound), bacterial infection, and prevention of debilitating scar formation. Notably, hydrogel-EXO treatments brought to the promotion of proper skin regeneration (growth of skin appendages, such as hair follicles, and other cutaneous glands) within the wound, indicating that epidermal stem cells were

TABLE 1

Overview of the studies using the MSC secretome in combination with biomaterials for cutaneous wound healing in *in vivo* models

Secretome	MSC source	Biomaterial	Species	Model	Results	Reference
CM	Rat ADSCs	Polycaprolactone electrospun fibers (EF)	Sprague-Dawley rat	Full-thickness excisional skin wound	The CM from MSC grown on EF determined the highest wound closure rate as compared with MSC grown onto microplates. M2 macrophage phenotype was elicited *in vivo*	(Su *et al.*, 2017)
CM	Human umbilical cord mesenchymal stem cell	Chitosan/collagen/ β-glycerophosphate thermosensitive hydrogel	C57BL/6 mice	Third-degree burn	Application of the MSC-CM/ hydrogel shortened healing time, limited the area of inflammation, enhanced reepithelialization, promoted the formation of high-quality, well-vascularized granulation tissue, and attenuated the formation of fibrotic and hypertrophic scar tissue	(Zhou *et al.*, 2019)
EVs	Human BMSCs and ADSCs	Carboxymethylcellulose	NSG mice	Diabetic full-thickness cutaneous wound	At day 10, ADSC-EVs, but not BMSC-EVs, increased the wound closure rate, reduced the scar width, increased the epithelial thickness and the percentage of re-epithelization, and increased the number of microvessels in comparison to the vehicle alone	(Pomatto *et al.*, 2021)
EXO	Human gingival mesenchymal stem cells	Chitosan/silk hydrogel	Sprague-Dawley rat	Diabetic full-thickness cutaneous wound	At 1 and 2 week post-surgery, hydrogel-loaded EXO gave the highest wound closure rate as compared with controls and hydrogel only. Higher reepithelization, collagen deposition, microvessel density, and nerve fiber density in hydrogel-EXO group	(Shi *et al.*, 2017)
EXO	miR-126- 3p-overexpressing human synovium MSCs (SMSC-126)	Chitosan (CS) hydrogel	Sprague-Dawley rat	Diabetic full-thickness cutaneous wound	EXO derived SMSC-126–loaded CS hydrogel accelerated reepithelialization, activated angiogenesis, and promotion of collagen maturity *in vivo*	(Tao *et al.*, 2017)
EXO	Rat ADSC	Hydrogel composed of Pluronic F127, oxidative hyaluronic acid, and poly-ε-L-lysine (FHE)	ICR mice	Diabetic full-thickness cutaneous wound	The FHE@EXO hydrogel significantly enhanced wound closure rates, and induced faster angiogenesis, re-epithelization and collagen deposition within the wound site. Skin appendages and less scar tissue also appeared in FHE@EXO hydrogel treated wounds	(Wang *et al.*, 2019a)
EXO	Human ADSCs	Hydrogel scaffold composed of Pluronic F127, PEI, and aldehyde pullulan (FEP)	IRC mice	Diabetic full-thickness cutaneous wound	The FEP@EXO hydrogel group showed faster healing, thicker granulation tissue and higher collagen deposition, faster reepithelization and angiogenesis. Skin appendages and less scar tissue also appeared in FEP@EXO hydrogel treated wounds	(Wang *et al.*, 2019b)

(Continued)

Table 1 (continued).

Secretome	MSC source	Biomaterial	Species	Model	Results	Reference
EXO	Human umbilical cord-mesenchymal stem cells (hUCMSC)	Pluronic F-127 hydrogel	Sprague-Dawley rat	Diabetic full-thickness cutaneous wound	hUCMSC-EXO/PF-127 hydrogel application resulted in a significantly accelerated wound closure rate, hair follicle generation, ordered collagen deposition, increased microvessel density, enhanced regeneration of granulation tissue and upregulated expression of VEGF and TGFβ-1	(Yang *et al.*, 2020)
EXO	Rat ADSCs	Alginate hydrogel	Wistar rats	Full thickness excisional wound	Alg-EXO accelerated wound closure rate, determined higher epithelial thickness, increased collagen deposition and microvessel density	(Shafei *et al.*, 2020)
EXO	Human endometrial stem cells	Chitosan (Ch)-gòycerol based hydrogel	BALB/c mice	Full-thickness excisional wound	The Ch-glycerol-EXO hydrogel accelerated wound closure rate, and determined smaller immature granulation tissue, increase epithelial thickness, formation of skin appendages (hair follicles, collagen bundles, and sebaceous gland), and higher number of microcapillaries in comparison with the control groups including Ch-glycerol and non-treated wound conditions	(Nooshabadi *et al.*, 2020)
EXO	Rat ADSCs	Polyurethane (PUAO)-based oxygen releasing antioxidant scaffolds made by incorporating calcium peroxide in PUAO cryogels (OxOBand)	Wistar rats	Diabetic full-thickness cutaneous wound	OxOBand facilitated faster wound closure, reduced the inflammation and prevented ulcer formation, enhanced collagen deposition, faster reepithelialization, hair follicle formation, increased neo-vascularization, and decreased oxidative stress within two weeks as compared to untreated diabetic control wounds. OxoBand prevented diabetic wound infections and lead to faster healing in infected chronic and diabetic wounds	(Shiekh *et al.*, 2020)
EXO	Human umbilical cord-mesenchymal stem cells	Hydrogel composed of poloxamer 407 (P407) and chitosan derivate carboxymethyl chitosan	Sprague-Dawley rats	Full-thickness dermal defect	EXO loaded hydrogel had significantly improved wound closure, reepithelialization rates, collagen deposition in the wound sites. More skin appendages were observed in EXO loaded hydrogel treated wound. Hydrogel-EXO group revealed the lowest expression quantity of TNF-α and IL-1β at 7th and 14th day compared to other groups	(Li *et al.*, 2021)
EXO	Human ADSCs	Human acellular amniotic membrane (hAAM)	BABL/C mice	Diabetic full-thickness cutaneous wound	The hAAM-EXO dressing accelerated wound closure, reduced inflammation by promoting higher recruitment of M2 macrophages, stimulated vascularization, and promoted the production of extracellular matrix	(Xiao *et al.*, 2021)

Note: ADSCs: adipose tissue-derived MSCs; BMSCs: bone marrow-derived MSCs; CM: conditioned medium; EXO: exosomes; EVs: extracellular vesicles; IL-1β: interleukin-1β; TGF-β1: transforming growth factor-β1; TNF-α: tumor necrosis factor-α; VEGF: vascular endothelial growth factor.

activated (Wang *et al.*, 2019a; Wang *et al.*, 2019b; Nooshabadi *et al.*, 2020; Shiekh *et al.*, 2020; Li *et al.*, 2021). As fewer skin appendages could be found in diabetic wounds treated by pure exosomes, these results strongly indicate that the sustained release of exosomes may facilitate complete wound healing with abundant skin appendages and scarless tissue (Wang *et al.*, 2019a).

EXO are rapidly cleared from the application site and survive *in vivo* for only a short time (Liu *et al.*, 2017), and specifically EXO rapidly degrades at body temperature particularly in the chronic wound sites, which may decrease their therapeutic efficacy. On the other hand, chronic wound repair and regeneration, particularly in diabetic patients, require a long healing time (more than 3 months) during which many proteases are released by concurring cells (Zeng and Liu, 2021). The biomaterials used in the combination with MSC secretome are of paramount importance to stabilize and allow EXO biological activities. In the study by Yang *et al.* (2020), a hydrogel based on Pluronic 127 (PF-127), that provide a moist environment for wound healing and act as a barrier against harmful substances, was used with human UC (hUC)-MSC-EXO in the treatment of diabetic wounds, demonstrating that both hUC-MSC-EXO and hUC-MSC-EXO/PF-127 reduced the wound area in the first three days after treatment *in vivo*. Subsequently, hUC-MSC-EXO/PF-127 evoked a faster healing rate than the other treatments at 7, 10, and 14 days. A study with self-healing polypeptide-based hydrogel, made of PF-127, oxidative hyaluronic acid (OHA), and Poly-ε-L-lysine (EPL), showed a long-term exosomes release (up to 21 days) and an enhanced proliferation of human umbilical vein endothelial cells (HUVECs) than one-time treatment of exosomes, as well as a higher diabetic wound healing rate at 14 days as compared with the EXO-only group (Wang *et al.*, 2019a). These results are compatible with the notion that, besides in the first phases of wound healing, the biological activity of hUC-MSC-EXO was prolonged by the protection of the PF-127 gel, and we can assume that these exosomes were continuously released, leading to increased, sustained, and rapid wound healing. In summary, from these studies we have learnt that developing a biocompatible scaffold that can maintain the function of EXO and sustained release would be critical for exosomes-based therapeutics for cutaneous wound healing. Moreover, it can be noticed a synergistic action of scaffolds with exosomes. In particular, the sustained release of bioactive factors in scaffold dressing could efficiently enhance the early angiogenesis in the diabetic wound and accelerate the healing. The main primeval action of EXO released by scaffolds is to increase cell proliferation and migration underlying the first stages during wound healing, i.e., re-epithelization and neoangiogenesis, giving rise to granulation tissue and subsequent matrix deposition and remodeling. It remains to understand the specific functional component of EXO and mechanism by which exosomes released by scaffolds operate and accelerate wound healing. Non-excluding mechanisms include the modulation of signaling pathways (Chen *et al.*, 2017) and the delivery of anti-inflammatory and anti-scarring miRNAs (Golchin *et al.*, 2018). Activation of AKT, ERK, and STAT3, and the induction of the expression of cell cycle genes and growth factors as well (including HGF, insulin-like growth factor (IGF)-1, nerve growth factor (NGF), and stromal cell-derived factor (SDF)-1) by MSC-derived EVs/EXO play a role in inhibiting stress-induced skin cell apoptosis, and in promoting their migration and proliferation (Shabbir *et al.*, 2015; Kim *et al.*, 2018; Ren *et al.*, 2019). HUC-MSCs-EXO could promote wound healing in the rat model of skin deep second-degree burn injury through activation of Wnt/β-catenin to enhance proliferation and migration of skin cells and AKT signaling to reduce heat stress-induced apoptosis (Zhang *et al.*, 2015a). The same group showed that the administration of hUC-MSCs-EXO in a deep second-degree burn injury skin model promoted wound healing and angiogenesis by delivering Wnt4 and activating Wnt/β-catenin signaling in endothelial cells (Zhang *et al.*, 2015b).

MiR-181c in UC-MSC-EXO was demonstrated to reduce burn-induced excessive inflammation by downregulating the TLR4 signaling pathway (Li *et al.*, 2016). In a rat deep second-degree burn injury model, hUC-MSC-derived EXO promoted activation of β-catenin and skin stem cell proliferation in the early stages of tissue repair and restricted excessive cell expansion by inhibiting Wnt signaling via transfer of the 14–3-3ζ protein, inducing cytoplasmic retention of the YAP protein (Zhang *et al.*, 2016). hUC-MSC-Exo could promote wound healing and reduce scarring by delivering a group of specific microRNAs (miR-21, miR-23a, miR-125b, and miR-145) that were found to suppress myofibroblast formation by inhibiting excess α-smooth muscle actin and collagen deposition associated with activity of the TGF-β/SMAD2 signaling pathway (Fang *et al.*, 2016).

As regarding the techniques used in the studies procuring biocomposites with MSC-EXO, electrospun biomaterials, which mimics ECM structure, have been shown to give rise to homogenous mixtures made of nanofibres with high tensile strength (Riha *et al.*, 2021), however they are derived from a complicated process that produces ECM matrix structure with an unsatisfactory strain (Sadeghi-Avalshahr *et al.*, 2017). Moreover, the elecrospinning process depends on many variables, and it is problematic to obtain 3D structures with the required pore size needed for biomedical application (Law *et al.*, 2017; Keirouz *et al.*, 2020). Decellularised scaffolds retain native ECM thus maintaining normal atomical features, as well as present less inflammatory and immune response with higher mechanical strength (Chaudhari *et al.*, 2016). Although the human amniotic membrane (AM) presents many advantages, including anti-bacterial, anti-inflammatory, and non-immunogenic properties, promotes reduced pain and dehydration, and favors the reepithelialization process, disadvantages of AM include poor mechanical properties and a high biodegradability rate, which complicate its extensive use in clinic (Dussoyer *et al.*, 2020). Due to the limited use of decellularized AM in the skin regeneration field combining composites and EXO, and unknown mechanisms of action, further studies are needed to comprehend its usefulness as compared with other decellularized sources and other composites, either made of natural or synthetic polymers.

Hydrogels, that were found the most used composites in the above outlined studies, however show on their own some limitation when used in conjunction with MSCs. First, MSCs pre-encapsulated within hydrogels may slowly alter hydrogel stability and mechanical properties due to secretion of

proteases. Thereby, seeding premade hydrogels with MSCs should be operated at the point of care, implying that the un-seeded hydrogel must be easy to handle and encourage rapid cell seeding (Garg *et al.*, 2014). The integration of MSC secretome, and significantly EXO, should overcome these limitations. Another critical issue is linked to already moist wounds, such as venous leg ulcers, as hydrogels may cause a high amount of output drainage and exudate from the site that further impedes healing by slowing down cell growth, degrading the tissue matrix structure, promoting inflammation or bacterial contamination (Murakami *et al.*, 2010). Finally, these hydrogels are usually changed every 3 days, so production of these scaffolds must be simple, quick, and inexpensive to be commercially appealing for physicians (Sivaraj *et al.*, 2021). All these issues will be the focus of future studies on hydrogels applied to the MSC secretome delivery, in particular EXO, to wound-healing settings in animal models first and in patients hereafter.

Although a direct comparison among CM, EVs and EXO has not been conducted in the setting of cutaneous wound repair in combination with biomaterials, it has been shown that MSC-EXO's role is not static during the entire cutaneous tissue regeneration process and they exert distinct effects on skin cell proliferation at various cell densities (Zhang *et al.*, 2016). However, further studies are deemed to definitively understand which MSC secretome would have better results in terms of application to different pathological skin wound healing processes.

Finally, from a logistic point of view, clinic application of MSC-derived exosomes in wound healing needs that the standard procedures for purification, storage, and administration of therapeutic exosomes with low cost ought to be developed (Hettich *et al.*, 2020).

Conclusion

Nonhealing and chronic wounds (mainly diabetic) deserve more efficient treatment options that accelerate wound healing, favor neoangiogenesis, reduce scarring, and allow optimal epidermal reconstitution. Biomimetic materials and MSC-derived exosomes possess all these properties and therefore have great potential in achieving satisfactory healing in recalcitrant wounds. Due to their versatility, different fabrication techniques, and numerous biological properties, hydrogels represent a promising approach to advance the combination of EXO with tissue engineering scaffolds to the clinic.

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