Journal of Controlled Release 353 (2023) 1096–1106

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

The therapeutic and commercial landscape of stem cell vesicles in regenerative dermatology

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ARTICLE INFO

Keywords:
Extracellular vesicle
Exosome
Dermatology
Cosmetics
Regenerative

ABSTRACT

Extracellular vesicles (EVs) are lipid enveloped nanoparticles that are naturally produced by cells and function in the intercellular transfer of biological material such as proteins, RNAs and metabolites. They have been shown to act in an autocrine and paracrine manner to alter the functions of local and distant recipient cells, with a growing body of evidence highlighting their wide-ranging functions in regenerative processes such as stem cell maintenance, tissue repair and immune modulation. EVs offer several potential advantages over stem cell therapies such as improved safety profiles, scalability, and enhanced storage and quality control of the final product. In fact, many of the pro-regenerative outcomes of stem cell therapies have been attributed to the release of mesenchymal stem cell-derived EVs (MSC-EVs) and their potent effects on extracellular matrix turnover, local cell recruitment, proliferation and angiogenesis is now well described. These positive outcomes have led to clinical trials assessing the safety of MSC-EVs for applications in wound healing and the treatment of cutaneous ulcers, as well as the emergence of multiple commercial MSC-EV sources marketed for topical application in cosmetic medicine. However, regenerative EV therapeutics remain in their infancy and pertinent questions regarding product standardisation, potency and the regulatory landscape surrounding the development of these promising nanotherapeutics must be addressed to ensure safe and effective clinical adoption. In this article we provide an overview of the emerging landscape of MSC-EVs in regenerative dermatology and cosmetic science, highlighting the underlying biological mechanisms pertinent to their application and providing a perspective on current safety considerations, regulation and future directions in the field.

1. Introduction

Regenerative medicine is an interdisciplinary field that aims to repair, replace or restore function in instances of disease, trauma or ageing. In dermatological medicine, regenerative approaches have diverse potential applications in the treatment of debilitating soft tissue injuries such as scars, burns and chronic ulcers, as well as in the aligned practice of cosmetic and aesthetic medicine. To date, regenerative approaches have typically centred around three strategic pillars – cells, biological cues (biocues) and scaffolds [1]. Of these three approaches, the application of biocues has been widely applied in regenerative dermatology. Existing biocues include platelet rich plasma (PRP), platelet rich fibrin (PRF), stromal vascular fraction (SVF) and a variety of isolated growth factors. These approaches generally act to stimulate processes integral to soft tissue rejuvenation, such as local cell proliferation and differentiation, matrix deposition and turnover, angiogenesis and cell migration [2]. The autologous nature of treatment modalities such as PRP has been an advantage in gaining widespread clinical acceptance. However, the efficacy of these treatments can be negatively impacted by increased donor age and lifestyle choices (e.g. smoking, poor diet). Lastly, these autologous treatments are highly heterogeneous and present challenges in their scale up and standardisation, with therapeutic efficacy varying depending on the method of preparation and a limited understanding of the precise mechanism(s) of biological action [3]. As such, there is a need to develop novel regenerative strategies that can be applied in regenerative dermatology which offer improved reproducibility, scalability and efficacy when compared with current approaches.

Recently, cell-derived nanoparticles termed extracellular vesicles (EVs) have generated considerable interest in the field of regenerative dermatology. EVs offer a potent, scalable and stable source of biocues that can positively modulate the local tissue microenvironment and

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https://doi.org/10.1016/j.jconrel.2022.12.025
Received 28 September 2022; Received in revised form 7 December 2022; Accepted 10 December 2022
Available online 23 December 2022

provide a stand-alone clinical procedure or an adjuvant to existing treatment modalities. As such, they offer a novel therapeutic strategy for improving soft tissue quality and reducing patient morbidity associated with soft tissue injuries such as scars and burns, or simply improving cosmetic outcomes related to tissue ageing in the field of regenerative aesthetics. EVs are lipid enveloped nanoparticles that are naturally produced by cells and function in the intercellular transfer of biological material such as proteins, RNAs and metabolites. These ubiquitous nanoparticles are shed by practically every cell type in the human body, acting in an autocrine, paracrine and endocrine manner to alter the functions of local and distant recipient cells [4]. A continually growing body of evidence now exists highlighting the complex and wide-ranging functions of EVs in many physiological and regenerative processes including stem cell maintenance, tissue repair and immune modulation [5]. These natural functions, in addition to their biocompatibility, enhanced retention and recognised ability to cross biological barriers make them appealing candidates for a variety of healthcare applications [6].

Within the field of dermatology, studies have indicated EVs play important immunomodulatory roles in a range of inflammatory skin disorders including psoriasis, atopic dermatitis, lichen planus, bullous pemphigoid, systemic lupus erythematosus and chronic wound healing [7]. Due to the often complex and refractory nature of these conditions, the majority of recent studies have sought better understand the function(s) of EVs in these pathologies and illustrate their potential as future biomarkers that can be applied non-invasively to predict the onset of inflammatory skin disorders, as well as relapses or reactions to drugs [8–11]. However, the purpose of the current review is to outline opportunities to deploy EVs therapeutically and provide a current overview of the developing commercial landscape in regenerative dermatology. To this end, EVs have been shown to exert immunomodulatory effects, modulate cell senescence and induce angiogenesis and de novo collagen synthesis [12,13]. Their size and deformability make them amenable to topical application in line with current regulatory standards [14]. There is also considerable scope to further enhance the natural pro-regenerative effects of EVs derived from MSCs and other compatible cell types by priming the parental cell source via manipulation of the cell culture environment. This could be simply achieved through hypoxic conditioning, acidosis or incubation with inflammatory cytokines [15,16]. Additionally, EVs can act as carriers for a wide range of chemical and biological drugs, enhancing cellular targeting and uptake. While the combined delivery of EVs with adjunct therapies such as microneedling or through association with biocompatible scaffolds offers the opportunity to further enhance delivery across the skin barrier and modulate their release kinetics to enhance clinical outcomes [17]. To date, the majority of published outcomes have been observed through the delivery of mesenchymal stem cell-derived EVs (MSC-EVs), with this cell source offering benefits in the translation of EV therapies due to their immunoregulatory effects and ability to be sourced allogeneically. However, it should be noted that positive outcomes have also been observed for autologous cell sources such as keratinocytes and plasma EVs, with commercial research and development projects utilising these cell sources ongoing and outlined in the present review.

The aim of this paper is to provide a critical commentary on the rapid emergence of MSC-EVs in regenerative dermatology and cosmetic medicine. We provide an up-to-date overview of the current scientific and commercial landscape of MSC-EVs in dermatological medicine, with an emphasis on aligning observed developments in the field with good practice in EV therapeutics and current regulatory standards. Lastly, we provide a balanced opinion on future directions necessary for the development of reproducible and clinically effective EV therapies.

2. Advantages of EVs

To date, many studies have utilised mesenchymal stem cell (MSC) sources for the manufacture of therapeutic EV preparations. However, there are also examples of EVs being obtained from a variety of other mammalian cell sources (e.g. dendritic cells), biofluids (e.g. milk) and even plant-based sources [18,19]. Interestingly, some of these plant-based EVs could be readily utilised in dermatological medicine due to their availability, stability and acknowledged bioactivity on keratinocytes and potential anti-melanogenic effects [20]. EVs offer several potential advantages over cellular therapies, with improved safety profiles and inherent biocompatibility. Furthermore, many EVs are <200 nm in size and are amenable to sterilisation by filtration. Unlike cells, EVs are not dynamic, with no ability to proliferate or differentiate. Instead, EVs contain a defined and stable biological cargo, providing optimal storage and handling protocols are applied. These features also improve the reproducibility of the product, with the opportunity to implement rigorous quality control procedures and reduce batch-to-batch variation. This might eventually open avenues for scaled-up EV production using immortalised cell lines [21]. Further, EVs do not require specialised cryostorage facilities and could be amenable to lyophilisation, reducing economic and logistical considerations and permitting off the shelf applications [22,23]. However, to the best of our knowledge, no optimal lyophilisation protocol has been published and in-depth studies on the effects of the freeze-drying process are required to ensure EV integrity, organisation and bioactivity is fully retained following this procedure. Lastly, the size and charge of these nanoparticles also positively influences their migration, retention and uptake within physiological environments. While the expression of surface proteins such as CD47 have been proposed to reduce clearance by the mononuclear phagocyte system [24]. These multiple features have made EVs particularly appealing candidates for a range of therapeutic approaches, including recent applications in regenerative dermatology, as we shall discuss.

3. Applications in regenerative dermatology

Multiple animal studies have applied stem cell derived EVs for cutaneous wound healing, scar removal, skin rejuvenation, pigment regulation and hair loss within the last several years. Four studies applying adipose MSC-derived EVs (ADMSC-EVs) in murine skin wound models highlighted their regulatory and temporal effects on collagen synthesis during the wound repair process that could be important in mitigating fibroproliferation and scar formation [25–28]. However, it should be noted that some of these studies isolated EV preparations using a combination of ultrafiltration (MWCO 100 kDa) and a commercial precipitation kit [25], while one study lacked relevant controls to accurately validate EV uptake [27]. As such, it is perhaps more appropriate to regard the resulting preparations as EV-enriched fractions, with possible contributions from co-isolated nanoparticles such as lipoproteins, larger free proteins and EV-associated proteins such as those that have been shown to form a corona around the outer surface. While it is a matter of contention whether the protein corona can be regarded as true representation of the EV proper, it has nonetheless been shown to exert a positive effect on processes such as skin regeneration and immunomodulation [29]. For example, the pleiotropic effects of topically administered high density lipoproteins (HDLs) have been shown to exert positive effects on wound healing in apolipoprotein E deficient mice and in diabetic wound healing models [30,31]. While the functions of large proteins such as collagens and the mechanosensory proteoglycan agrin that are likely not observed within the EV lumen are also highly relevant to wound healing [32]. The size and organisation of this corona will be dependent on the EV source and isolation method applied. For example, EVs derived from blood plasma can have a comparatively rich corona due to the complexity of proteins available in this biofluid [33]. While certain isolation methods such as size-exclusion chromatography (SEC) and ultracentrifugation (UC) can result in loss of many functional proteins within the corona [29]. These variations can mean that applying different protocols could result in the isolation of EVs with differential biochemical profiles and inconsistent functional effects that could impact downstream regenerative applications if not

stringently controlled. Nonetheless, the immunoregulatory properties of MSC-EVs has been extensively documented throughout the literature, with ADMSC-EVs shown to downregulate the production of the proinflammatory mediator IFN-α in $CD4^+$ T cells [34]. Similar observations have also been recorded for bone marrow MSC-EVs (BMSC-EVs), which were observed to inhibit T lymphocyte proliferation, boost regulatory T cell numbers, and increase the secretion of anti-inflammatory cytokines such as TGF-β1 and IL-10 [35]. These observations have clear implications in dermal wound healing, since the potent antifibrotic and pro-regenerative effects of IL-10 have been well described [36,37]. However, wound healing models in which there is an abolition of T cells (athymic mouse models and antibody-mediated depletion) have frequently provided variable results, with examples of regulatory $CD4^+$ and cytotoxic $CD8^+$ T cell neutralisation or knockout generating unreproducible and often contradictory effects on parameters such as collagen deposition or wound breaking strength [38,39]. As such, questions remain regarding the origin (e.g. dermal or lymph node) and precise functions of T cell subsets in dermal wound healing that require further investigation [40]. In addition to their immunomodulatory effects, ADMSC-EVs have been shown to promote angiogenesis through the transfer of miR-125a and miR-31 to vascular endothelial cells and possibly inhibit the action of Delta-like ligand 4 (DLL4) to promote angiogenic sprouting in vascular endothelial cells [41–43]. Enhanced keratinocyte migration and proliferation has also been observed both in vitro and in vivo in the presence of ADMSC-EVs through regulation of the AKT/HIF-1α signalling pathway [44]. While hydrogel-coated umbilical cord tissue-derived MSC (hUC-MSC) EVs enriched in miR-21, −23a, −125b and − 145 targeted the same signalling pathway to supress myofibroblast formation in murine models of full-thickness skin defects, which has implications for the treatment of scarring [45]. Similar findings were also observed in a study that combined hUC-MSC-EVs with a thermoresponsive PF-127 hydrogel in the treatment of full thickness wounds in a diabetic rat model, with accelerated wound closure and upregulated expression of angiogenic growth factors observed [46]. Umbilical cord blood-derived MSC-EVs (UCMSC-EVs) enriched in miR-21-3p encouraged fibroblast proliferation and migration, while accelerating angiogenesis and re-epithelialisation [47]. Similar observations were recorded when the effects of human menstrual blood-derived MSC-EVs (MenSC-EVs) on wound healing were studied in a diabetic mouse model. In this study the authors observed that 10 μg intradermally injected MenSC-EV preparations resolved inflammation through M1-M2 macrophage polarisation and enhanced the rate of wound closure even when compared with MenSCs [48]. Macrophages represent resident inflammatory cells that have important functions in wound healing through the promotion of collagen deposition and angiogenesis. During the wound healing cycle, these cells are polarised to a proinflammatory M1 phenotype or anti-inflammatory reparative M2 phenotype. The ability to regulate macrophage phenotype to achieve a time-dependent transition from M1 to M2 is considered a promising approach to accelerate wound healing, with aberrations in this transition linked with chronic inflammation and reduced wound closure [49]. The ratio of collagen type-1 to −3 expression was also modified in response to MenSC-EV administration in this study, supporting outcomes reported for ADMSC-EV studies and highlighting their potential applications in the treatment of scarring [50,51]. Human induced pluripotent stem cell (iPSC) MSC-derived EVs have also been applied in the context of wound healing. For example, Zhang et al. (2015) demonstrated that when 40 μg of EV preparation was subcutaneously injected in a rat skin wound model, reductions in scar width were observed [52]. While EVs isolated from BMSCs were observed to accelerate wound healing by promoting keratinocyte and human dermal fibroblast proliferation via TGF-β/Smad signalling. [53] Several research studies have demonstrated that the topical application of EVs lead to improvements in chronic inflammatory skin diseases such as psoriasis by alleviating IL-17 release from neutrophils that accumulate within the region of the stratum corneum, which has been implicated in psoriasis [54]. Similar improvements have also been observed in research studies looking at the effects of the topical application of ADMSC-EVs for the treatment of atopic dermatitis [55]. These are important developments since current treatments for inflammatory conditions such as atopic dermatitis are not curative, with the application of over-the-counter treatments (moisturisers) and prescription medications (e.g. steroids) only able to reduce itching and inflammation, respectively. While skin substitutes (e.g. acellular dermal substitutes) applied for chronic wound management are costly and can lead to scarring [56]. Unlike these existing treatment modalities, EVs offer the possibility to target multiple signalling pathways using just a single package, allowing them to act on many facets of the chronic wound environment, including local inflammation, ECM synthesis, cell proliferation and angiogenesis. Furthermore, there is some evidence to suggest that these potent packages of biological information are safe, therapeutically scalable and stable. However, challenges remain in the understanding of storage-mediated changes in EVs that will need to be overcome before the feasibility of creating an off-the-shelf therapeutic can be determined, as we shall discuss in the latter portion of the review.

In addition to naturally synthesised EVs, it is possible to artificially derive plasma membrane vesicles by passing cells through pores of decreasing diameters in a process known as extrusion [57]. Exosome-mimetic nanovesicles derived from the extrusion of human iPSCs have been shown to recover senescence-induced alterations in human dermal fibroblasts [58]. However, while the derivation of nanovesicles using the cell extrusion method can provide comparatively high yields, this method is likely to lead to the inclusion of nuclear material absent from naturally derived EVs. The incorporation of nuclear material is likely to impact the safety and clinical translation of such an approach, perhaps limiting its clinical translation. While studies concerning MSC-EV safety and toxicity are limited, there is evidence to suggest that EVs derived from ADMSCs are non-sensitisers when applied topically with no adverse effects reported [59]. What is clear from these studies is that many of the positive outcomes resulting from the application of MSC-EVs appear to be due to their effects on fibroblasts and their capacity to modulate the local inflammatory environment. It is also clear that although stem cells isolated from a variety of neonatal and adult sources have demonstrated potential therapeutic utility, the parental cell from which EVs are obtained has a considerable influence on their biological content and therapeutic activity. This has been demonstrated in models of wound healing where BMSC-EVs and ADMSC-EVs were predicted to target distinct pathways related to cellular proliferation and angiogenesis, respectively [60]. Similar findings have also been demonstrated in models of endochondral ossification [61]. While basic proteomic comparisons between ADMSCs and ADMSC-EVs have provided evidence to suggest that EVs could be selectively enriched in pro-angiogenic factors, inflammatory regulators and proteins involved in extracellular matrix remodelling when compared to the parental cell source [62]. Much like the stem cells from which they are derived, the therapeutic efficacy of MSC-EVs also appears to be inversely related to the developmental maturity of the donor from which they are obtained [63]. Similarly, reduction in the pro-vascularising activity of MSC-EVs was also evident with increased passage [64]. As such, it is important that basic parameters including parental cell type, culture environment (e.g. 2D or 3D), seeding density, passage number, frequency of EV collection and culture conditions are carefully considered when utilising EVs therapeutically. While the impact of donor maturity on MSC-EV potency could prove to be a limiting factor if these therapies are to be applied autologously (Fig. 1).

4. Registered clinical trials

To date, eight clinical trials have been completed and over twenty-five are currently registered or in progress according to Clinicaltrials.gov. Most of these trials are assessing the safety and efficacy of EVs for treating COVID-19 (NCT04969172, NCT04747574, NCT04902183,

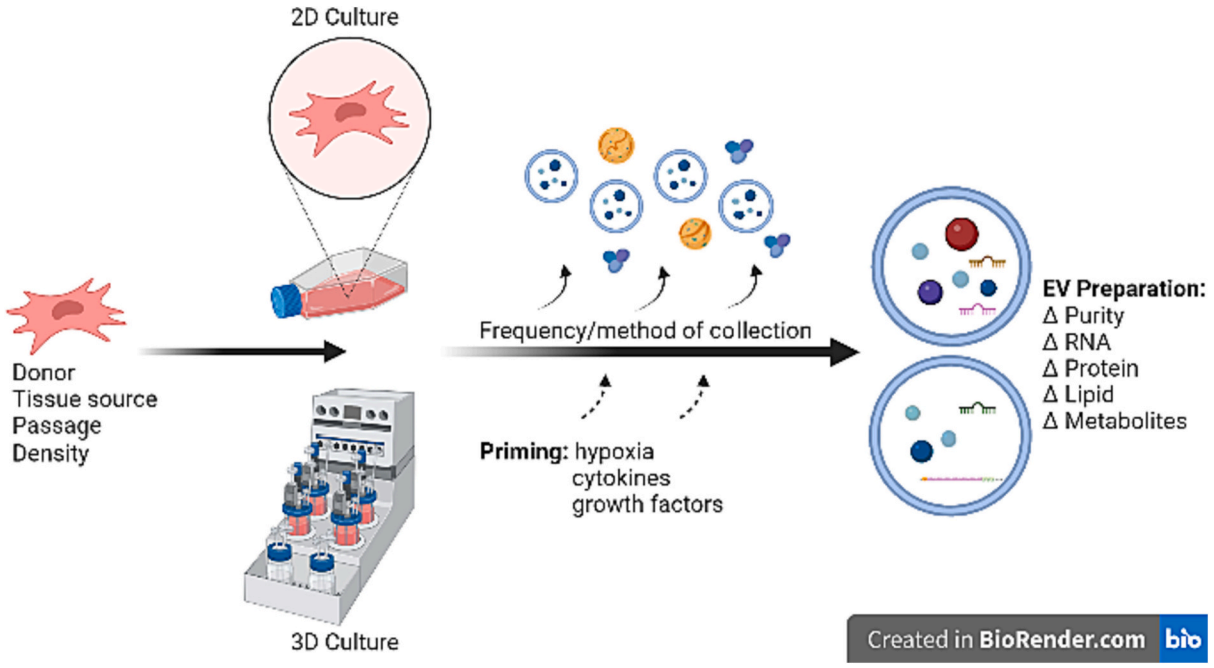

Fig. 1. Overview of EV production parameters and their potential impact on the resulting preparation. Parameters are related to the cell source, the method of culture (e.g. 2D vs. 3D), frequency and timing of EV collection, the method of EV isolation applied (e.g. ultracentrifugation, size-exclusion chromatography, tangential flow filtration) and the addition of exogenous priming agents to cell cultures (e.g. hypoxia or stimulation with inflammatory cytokines). Variations in these parameters will ultimately impact batch to batch variability and product regulation.

NCT04276987, NCT04798716, NCT04389385, NCT04602442, NCT04491240, NCT04493242) and cancer (NCT01159288, NCT01294072, NCT01668849, NCT03608631, NCT01854866, NCT02657460). However, there are also a growing number of studies applying EVs for tissue repair and regeneration, with trials registered to assess the application of EVs in conditions including periodontitis (NCT04270006), post-surgical bone cavity chronic inflammation (NCT04281901), macular holes (NCT03437759), chronic middle ear infections (NCT04761562), type-I diabetes (NCT02138331) and ischaemic stroke (NCT03384433), to name a few. These trials have utilised EVs from a diverse range of cell types including allogeneic MSCs and autologous tumour cells, autologous serum and platelets, dendritic cells and ascites [65]. For more comprehensive information on the status and design of these trials we recommend the following papers by Herrmann et al. (2021), Perocheau et al. (2021) and Nagelkerke et al. (2021), as they lie outside the scope of our review [66–68]. To date, only four trials have sought to establish the safety and clinical efficacy of EV treatments for dermatological applications (Table 1). This includes a phase I safety trial evaluating the efficacy of 0.3 mg/mL intradermally injected autologous platelet derived EVs (PLEXOVAL, Exopharm Limited) for wound healing in up to 20 healthy participants following a punch biopsy. Outcomes from the trial focused on adverse reactions to PLEXOVAL, time to wound closure, presence of hypertrophic scarring and wound characteristics (e.g. granulation, pus, odour). However, the trial was concluded early due to issues with recruitment and no outcomes published. Other trials include the application of EVs or the broader MSC secretome for the treatment of the rare inherited skin disorder dystrophic epidermolysis bullosa (EB, NCT04173650), and cutaneous (NCT02565264) and chronic ulcers (NCT04134676). Of these trials, NCT04173650 is yet to recruit, with an estimated study completion date of January 2025. This study has been designed to test the toxicity and efficacy of an allogeneic donor MSC-EV preparation (AGLE-102) on lesions in subjects with chronic EB. AGLE-102 will be administered up to 6 times, with wound closure evaluated monthly over a period of 4 months. In this trial, AGLE-102 will be applied in two ascending doses. However, no information was provided on the doses

Table 1
Registered clinical trials assessing the safety and efficacy of EV treatments for dermatological applications. *estimated/target numbers.

Identifier	Condition	Participants	Inclusion Criteria	Intervention	Model	Status	Location
NCT04134676	Chronic ulcer wounds	38	18–80 yrs., mixed sex, chronic skin ulcers (<10x15cm) unresponsive to therapy after 1 month	Wharton's jelly stem cell conditioned medium	Experimental, open label, single group assessment	Phase 1, completed 06/2020	Indonesia
NCT04173650	Dystrophic epidermolysis bullosa (DEB)	10*	>6 yrs., mixed sex, mild to severe DEB, ≥1 active wounds between 10 and 50 cm^2	Allogeneic MSC-EVs	Non-randomised, multicentre, ascending dose, single group assessment	Phase 1/2, not yet recruiting	Undisclosed
NCT02565264	Cutaneous ulcers	5	No age criteria, mixed sex, Intractable cutaneous ulcers	Autologous Plasma-derived EVs	Single group assessment, open label	Early phase 1, unknown	Japan
ACTRN12619001378112	Wound healing	20*	18–45 yrs., mixed sex, healthy volunteers with skin punch biopsies	Autologous platelet derived EVs	Non-randomised, prospective, open label, matched placebo control	Phase 1, stopped early, participant recruitment issues	Australia

applied or the purity of EV preparations. However, an accompanying publication demonstrated the presence of CD63 MSC-EVs isolated by differential centrifugation with ultracentrifugation into a 30% sucrose cushion. Outcomes from this paper suggest that the MSC-EVs courier collagen type VII (Col7A1) mRNA and protein that can stimulate recessive dystrophic epidermolysis bullosa fibroblasts to synthesise their own collagen VII, which could improve dermal-epidermal adhesion in this pathology [69]. However, the copy number of Col7A1 was not determined in this study. Consequently, it is unclear how many MSC-EVs will be required to achieve a similar outcome in the clinical trial. Additionally, the Col7A1 protein was co-isolated with MSC-EV fractions, with no conclusive evidence to demonstrate this high molecular weight protein (~170 kDa) was associated with EVs. Notably, the company conducting the trial (Aegle Therapeutics) also has an EV product development pipeline that also targets burns and scarring (Table 2). However, to the best of the authors' knowledge, no clinical trial has been established for these applications. Finally, the recruitment status of NCT02565264 remains unknown. This trail began in 2015 and aims to evaluate the effects of autologous plasma derived EVs applied every day for a period of 28 days on wound healing in patients with intractable cutaneous ulcers. However, plasma EVs applied in this trial appear to be isolated by passing autologous plasma through a series of filters (0.45, 0.20 and 0.02 μm), with no further purification steps identified. As such, the resulting EV preparations are likely to be highly heterogeneous and littered with co-isolated particles of similar sizes such as lipoproteins, which outnumber EVs in the blood by a factor of a billion [70]. As such, any positive outcomes to emerge from this trial cannot conclusively be claimed to be EV mediated. Furthermore, the reproducibility of such an approach is likely to be an issue in any subsequent trials. These issues are also likely to be prevalent when using a less defined conditioned MSC medium, which will contain a host of EV associated and free regenerative factors.

5. Commercial landscape

The significant commercial potential of EV therapeutics is reflected by the fact that over 30 international companies currently have EV products in the research and development phase. Products being developed by these companies include naturally secreted EVs (typically of stem cell origin), engineered EVs (where the parental cell source has been genetically manipulated to overexpress a therapeutic cargo of interest) or hybrid systems in which natural EVs are fused with synthetic lipid nanoparticles (e.g. liposomes) that carry a defined therapeutic cargo. Products being developed aim to target a wide range of indications including neurologic diseases, neuromuscular diseases, inflammatory and autoimmune diseases, respiratory diseases, osteoarthritis, cancer, severe burns and general drug delivery. For a comprehensive list of companies, we recommend the comprehensive review by Nagelkerke et al. (2021), since they lie outside the scope of the current review [68]. Several companies are currently conducting research into the development of EV products for the treatment of chronic wounds and inflammatory skins diseases (Table 2). For example, the Portuguese company Exogenus Therapeutics is developing Exo-Wound, which combines EVs derived from human umbilical cord blood mononuclear cells (Exo-101) with a slow-release hydrogel to tailor the release kinetics. Exo-101 EVs utilised in this technology are isolated using a combination of ultrafiltration (UF) and SEC, which was able to provide a 3-fold greater EV yield when compared with conventional UC. Topical administration of Exo-101 (2.5×10^8 particles) resulted in accelerated wound closure with evidence of increased cell proliferation, a reduced inflammatory profile and reduced fibrinous matrix in a diabetic mouse model [71,72]. Regeneus is also developing an allogenic EV product (Sygenus) that is delivered topically in combination with a hydrogel. The Sygenus product is being developed for the treatment of acne and incorporates the ADMSC secretome rather than purified EVs. Research publications that are suggested to be linked to Sygenus have demonstrated through the application of targeted ELISA that the ADMSC secretome was predominantly enriched in FGF, which appeared to decline in ADMSC cultures after passage 7 [73]. However, this observation was not validated using methods complimentary to ELISA, such as mass spectrometry or western blotting and it is unclear whether publications listed on the Regeneus webpage are directly related to the Sygenus product. Finally, Exopharm Ltd. are utilising autologous platelets as a source of EVs for applications in wound healing. The resulting product is being marketed as Plexaris™, which was recently withdrawn from phase 1 clinical trials due to issues with participant enrolment (ACTRN12619001378112). An overview this clinical trial in addition to MSC-EV products being developed by Aegle Therapeutics can be found in the subsection on clinical trials.

Commercially, there appears to be a growing trend in combining EVs with moisturisers and serums as over the counter beauty products. Available products are summarised in Table 3, with EV content principally derived from adult human stem cells. Although it is difficult to find freely available information on how EVs were isolated and characterised in these products, we can comment on dose. In the Zen [3] product developed by Exoskin Simple, 15 million EVs are combined in a 10 oz. skincare formulation. Therefore, even if we assumed 100% purity, this would roughly equate to a concentration of only 500,000 EVs per mL, which is markedly lower than products being marketed for topical clinical application (Table 4). Further, everyday skincare examples do also exist where stem cell EVs are delivered in combination with fibroblast conditioned medium (e.g. S^2RM®). However, these products are not the focus of this review owning to their non-clinical applications. Table 4 presents a list of companies that currently manufacture commercially formulated topical EV products for aesthetic, cosmetic

Table 2
Dermatological EV products in the research and development phase.

Company	Product	EV Source	Indication
Regeneus, Australia	Sygenus (topical, in development, pre-clinical)	Human adipose MSCs (secretome)	Age spots, acne, wound healing
Exogenus Therapeutics, Portugal	Exo-101 and ExoWound (pre-clinical)	Human umbilical cord blood EVs	Inflammatory skins diseases, chronic wounds
Aegle Therapeutics, USA	Not specified (Phase 1 and 2 trials approved)	Human bone marrow MSCs	Severe dermatological conditions
Exopharm Limited, Australia	PLEXOVAL	Human platelets	Wound healing

Table 3
Commercially available everyday cosmetic skincare products containing EV material. Information provided in reference to EV dosage and application are based on information obtained from the supplier websites and have not been independently verified by the authors.

Company	Product	EV Source	Dosage
Exoskin Simple, USA	Bio.digital Perfection Moisturiser, Cream and Lotion	Adipose MSC-derived (Zen [3])	>150 million per bottle
BioRegenerative Science Inc., USA	NeoGenesis stem cell-released molecules (S^2RM®)-based product formulations	Human MSC and fibroblast conditioned media	Unknown
hMSC Skincare, USA	hMSC Serum and Amplify formulations	Human adipose MSC and fibroblast conditioned media	Unknown
Invitrx Therapeutics, USA	Invitra EX™	Allogeneic Wharton's jelly MSC	Unknown
Avalon Globocare Corp	Avalon Clinical Grade Tissue-Specific Exosome (ACTEX™)	MSC-derived	Unknown

Table 4
Commercially available EV products for dermatological and cosmetic applications. Information provided in reference to EV dosage and application are based on information obtained from the supplier websites and have not been independently verified by the authors. n.r: not reported.

Company	Product	EV Source	Dosage	Application(s)
ExoCoBio, South Korea	ASCE + Derma Signal SRLV1	Human adipose MSC-derived, lyophilised	5 billion	Skin
	ASCE+ Scalp Care HRLV (ExoSCRT™)		10 billion	Scalp
	Exomage TF skin barrier ampoule		15% exosomes	Replenish skin barrier
Benev, USA	Exosome Regenerative Complex powered by ExoSCRT™	MSC-derived, lyophilised	2.5 billion	Skin
	Exosome regenerative complex + powered by ExoSCRT™		5 billion	Skin and scalp
Kimera Exosomes, USA	XoGlo®	Perinatal MSC-derived	1 billion/mL	Cosmetic
Exocel Bio, USA	Exovex Revive	Placental MSC-derived	5 billion in 1 mL	Skin
	Exovex Renew		12 billion in 2.5 mL	Skin
	Exovex Reveal		25 billion in 5 mL	Skin, neck, décolleté
Regen Suppliers, USA	ReBellaXO™	Wharton's jelly-derived	15 billion/mL	Soft tissue, hair, facial
Direct Biologics, USA	XoFlow™	Bone marrow MSC-derived (allogeneic)	>10 billion/mL	Wound healing, hair, aesthetics
Infusio, Germany	n.r	Placental MSCs	≥15 billion per treatment	Anti-ageing

and dermatological applications. All products utilise adult or neonatal stem cell sources for EV production to deliver a product containing a single dosage of between 1 and 25 billion EVs. However, it is often unclear how EVs are defined in these products. Commercially, the Evovex product by Exocel Bio (USA) utilises increasing concentrations of placental MSC-derived EVs for its Revive, Renew and Reveal products for dermal applications that are purported to exert their therapeutic effects through the modulation of macrophages that are implicated in inflammatory initiation and resolution depending on their state of polarisation. ExoCoBio manufactures lyophilised human ADSC-EVs under serum-free conditions, with EV isolation performed using tangential flow filtration (TFF). The company has a strategic alliance with the California-based company Benev, through which it markets EV products in North America. The ExoCoBio product has documented positive effects on the epidermal barrier by reducing the local levels of inflammatory cytokines and inducing the de novo production of ceramides following injection in an atopic dermatitis model [74]. These formulations have also been shown to reduce dupilumab facial redness and demonstrated toxicological safety when tested on rats [55]. However, the effects of lyophilisation on the long-term integrity and potency of EV preparations is only recently starting to be determined, with storage buffer and pH found to have an considerable impact [23,75]. Infusio (Germany) market anti-ageing therapies based on the principle that EVs derived from a younger organism will lead to the rejuvenation of older cells – an observation that is supported by studies in animal models [76]. Infusio offer EVs derived from GMP certified placental MSCs that it suggests can be administered intravenously at concentrations up to 15 billion particles in a single treatment. However, we would like to emphasise that although the availability of EV treatments in private aesthetics clinics is becoming increasingly common, currently no EV therapy has been approved by the Food and Drug Administration (FDA) or European Medicines Agency (EMA) for public use, and any instances of subcutaneous or intravenous administration are being applied off label at the clinician's discretion. Evidently, this introduces considerable risks for the patients receiving the treatment and the clinician performing the procedure. Additionally, negative outcomes also have the potential to jeopardise the broader advancements of EV therapeutics outside of the cosmetics field. EVs manufactured by Kimera Labs are isolated from a perinatal MSC source and marketed as XoGlo®. EVs purchased from Kimera Labs have been applied in a forty-patient randomised double-blinded placebo-controlled study, where 5 billion particles were topically applied immediately following microneedling, with repeated administration at timepoints 30-, 60-, 90- and 120-days post-treatment. Aside from reporting no obvious adverse reactions, visual differences were suggested based on patient satisfaction score and photographic outputs coupled with 3D analysis utilising a Quantificare Imaging system [77]. However, no cellular or molecular evidence of soft tissue regeneration was provided in the study. Additionally, it should be noted that Kimera Labs previously made claims that their EV products could also prevent or treat Covid-19 infection. This statement was since withdrawn from the website [78]. The North American market also features EV products manufactured by Regen Suppliers (Whartons jelly MSC-derived) and Direct Biologics (BMSC-derived). However, there is a lack of openly available data on these products.

All available products are designed to be administered topically in line with current FDA regulations or sometimes in combination with adjunct procedures such as micro-needling or laser-based treatments, in an attempt to further enhance EV penetration and possibly improve clinical outcomes. However, current regulation surrounding the combined application of EV products with approaches such as micro-needling or other in-house cosmetic procedures requires further comment and clarification, as it is likely that the depth of skin penetration induced during these procedures is an important factor influencing regulatory compliance. Currently no injectable EV therapy has been approved by the FDA, with the application of EVs in general dermatological practice confined solely to topical administration. Although, to the best of our knowledge, no specific regulatory guidelines have been published concerning EV therapies, it is likely that EVs will be regulated as drugs and biological products under section 351 of the Public Health Service (PHS) Act and the Federal Food Drug & Cosmetics (FD&C) Act. This means that studies are required to demonstrate safety, efficacy, purity and potency of a product for a given application [79]. While in the developmental phase, these products can only be distributed for clinical use if the sponsor has an Investigational New Drug (IND) application in effect and require the approval of the regulatory agency before initiating a clinical trial [80]. Therefore, we emphasise the need for private practitioners to exercise caution when looking to combine EV therapies with adjunct treatments in their clinics. We draw the reader's attention to the fact that there are currently no approved EV or exosome-based therapies worldwide and that efforts are ongoing to evaluate the safety of EV therapeutics. As such commercial EV therapies are often classified as unproven therapies that have often not gone through the stringent trials and regulatory procedures required for conventional drugs. We refer the reader to the valuable efforts of the International Society for Extracellular Vesicles Regulatory Affairs Task Force (https://www.isev.org/regulatory-affairs-task-force).

6. Limitations and future considerations

Current lack of FDA approval of injectable EV products is likely

related to the fact that EV preparations are highly heterogeneous, and their precise MoA remain largely undefined. Defining an optimal quality control procedure will be dependent on knowledge of a given products MoA. This will also be essential for establishing functionally relevant and rigorous potency assays that are required for the regulatory approval of both stem cell and cell-derived EV products [81]. While it has been pointed out by Gimona et al. (2021) that we may be restricted to definitions of potency based on only the most relevant intended biological outcome, due to the complexity of many pathologies and the biological effects not restricted to a single molecule [82]. We should nonetheless be aware of these considerations early in the developmental process to maximise the chances of clinical adoption. Secondly, although EVs themselves do not have the capacity to differentiate or form tumours, non-specific uptake of EVs by off target cells could nonetheless have undefined and potentially negative consequences. As such, the biodistribution, dosage and off-target effects of EVs need to be thoroughly considered, particularly if we are to move towards the development of safe injectable EV treatments in the future. Safety studies are also required to demonstrate that internalisation by off-site targets does not result in negative indirect outcomes via the potential transfer of undesired and potentially harmful molecules such as chromosomal DNA fragments [83]. Emerging outcomes from ongoing clinical trials will be able to help shed light on some of these pertinent biosafety questions.

In addition to outstanding regulatory issues concerning safety and potency, we also need highlight the need for transparency and standardisation in the reporting of purity, dosage and bioavailability for EV preparations (Fig. 2). EV preparations are inherently heterogeneous and until markers of potency are defined for a given therapeutic application it will be difficult to optimise EV products and accurately predict their effects. At present EV studies largely quantify EV material based on total protein concentration, particle concentration or, more rarely, the presence of a marker of EV biogenesis (e.g. the tetraspanin proteins CD9, CD63 or CD81). Concentrations applied in vivo to murine models typically ranged between 10 and 40 μg [48,52]. While commercial EV products differ in their dosage, which typically range from 5 to 25 billion EVs/particles per application. However, in many instances, it is unclear how EVs are being defined or whether EV numbers are simply a reflection of total particle content based on measurements provided by commonly applied but non-specific particle analysis tools such as nanoparticle tracking analysis (NTA), tunable-resistive pulse sensing (TRPS) or similar. None of these assessments are EV specific, with total protein or particle measurements reflecting not only EV content but also co-isolated particulates with overlapping sizes and densities such as lipoproteins (predominantly LDLs, VLDLs and chylomicron remnants) [84]. These measurements will be highly dependent on the method applied for EV isolation and often not directly translatable or reproducible between manufacturers [85]. Consequently, there exists no transparent means of assessing the relative purity of these commercial formulations. This is not an overt criticism, as it is currently very difficult to accurately identify EVs from other materials of overlapping size and density (e.g. lipoproteins, RNA-binding proteins) that are routinely isolated in standard EV preparations. Kimera Labs applies a measure of purity that considers the ratio of particles to total protein, in addition to RNA concentration. This is a modification of a method proposed by

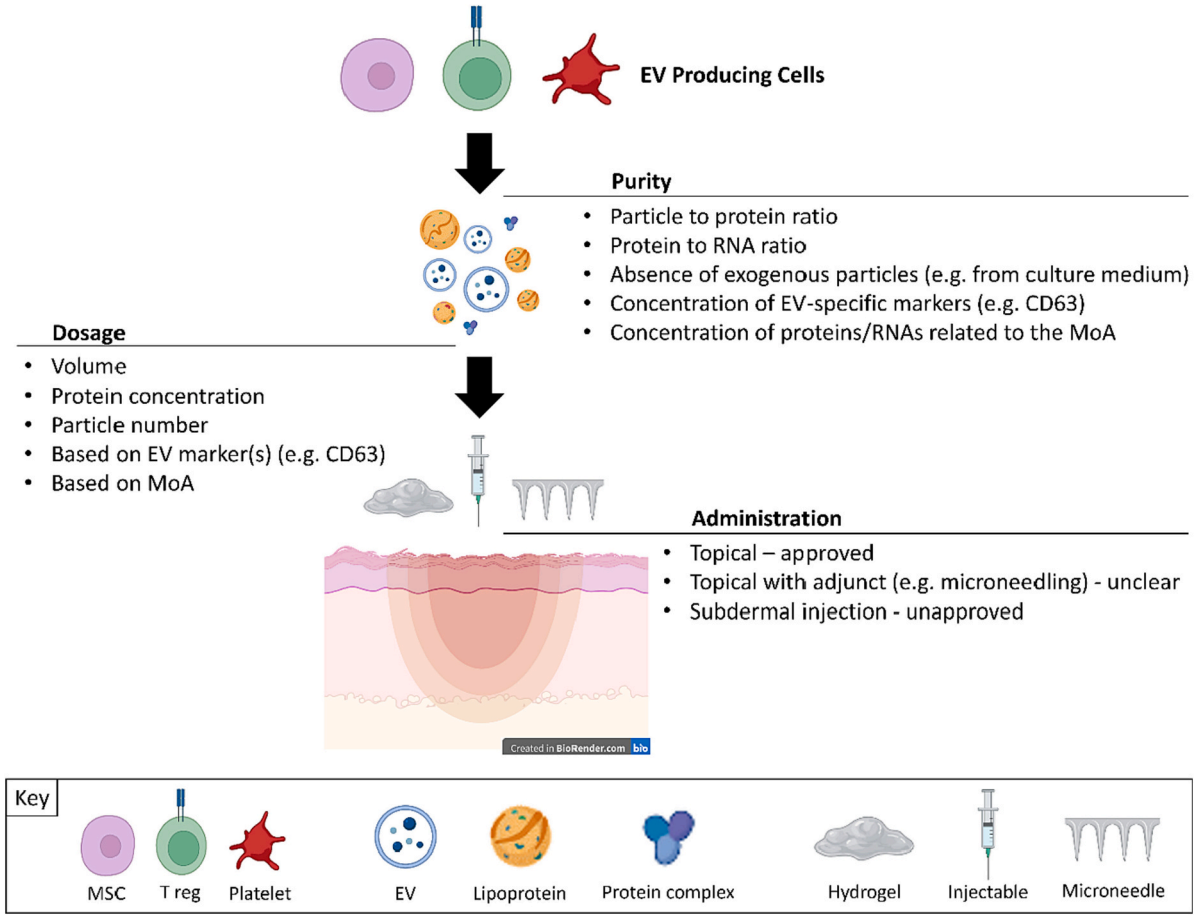

Fig. 2. Overview of post-production parameters that need to be considered when formulating EV preparations for translational regenerative dermatological applications. Parameters include the purity of each preparation, the dosage applied and route of administration. Options for determining/defining each parameter are provided, with the strength of each approach increasing as with each bullet point. Routes of administration are classified as approved or unapproved in line with current FDA regulation.

Webber and Clayton in 2013, while accounting for RNA recovery [86]. However, the calculation appears to treat all RNAs with equal importance and it is unclear whether those present within the EV are distinguished from those potentially co-isolated with the EVs on their surface or in association with RNA-binding proteins such as AGO2 [87]. This is an important point, since it is possible that heterogeneous RNAs could exert differential and potentially antagonistic biological effects if stringent quality controls are not in place. It is worth noting that more specific flow cytometry methods are becoming available that allow for the quantification of common EV surface proteins (e.g. the tetraspanins CD9, CD63 and CD81) or other contents of interest at the single EV level [88]. However, while the quantification of a given tetraspanin protein is certainly more specific, it is limited by the fact that not all EVs individually express all tetraspanin proteins [89]. Furthermore, these proteins may also not be directly indicative of potency. For example, if unidentified protein X is the primary marker of therapeutic potency and it correlates with only 10% of a given EV marker (e.g. $CD9^{+}$), there is a risk of developing suboptimal product manufacturing and selection criteria. As our understanding of the therapeutic MoA of EVs improves, it is important that we begin to apply stringent approaches to quantify therapeutically relevant proteins and RNAs to develop potency assays that will serve to improve safety, reproducibility, enhance efficacy and satisfy regulators, as we shall discuss in the following sections [90].

Evidently, there is clear advantage to developing off-the-shelf EV products in which EVs are lyophilised to make them amenable to storage at room temperature or at 4 °C. This would reduce logistical considerations and cost implications of EV therapies and permit their application in a variety of contexts, from regenerative dermatology to military medicine. It would also eliminate many of the current uncertainties surrounding the effects of storage conditions on EV integrity and the development of optimal cryopreservation strategies [91,92]. Commercial examples of lyophilised EV products are available (e.g. ExoCoBio, Table 4) and there are examples in the scientific literature where lyoprotectants including trehalose have been shown to be effective in retaining some of the therapeutic effects [93]. However, to the best of our knowledge no study has conclusively proven that conformational changes do not occur in the EV membrane that could negatively impact their ability to target and become internalised by recipient cells. Furthermore, rupture of EVs during lyophilisation could result in the release of biological cargo, which could lead to the rapid degradation of RNAs and proteins. This would ultimately reduce the therapeutic efficacy of EV treatments.

Since EVs are to be restricted to topical administration in the short-term, it will be important to further investigate their penetrance using full thickness human skin models (Fig. 2). To date, few studies have assessed the penetrance of EVs across the skin barrier. This is critical parameter that will ultimately define the commercial and clinical success of topical EV therapies, with injectable treatments such as collagen or hyaluronic acid dermal fillers often providing improved and prolonged outcomes when compared with topical alternatives [94]. Studies analysing EV penetration within the skin suggest that the majority of EV material localises within the stratum corneum, with no observable migration within the underlying stratum granulosum after a period of 24 h, with migration only being enhanced when skin permeability was increased through the application of adjunct procedures including the use of marine sponge *Haliclona* sp. spicules [95,96]. Although the aforementioned example demonstrated that penetration within the stratum corneum alone was sufficient to induce potent immunoregulatory effects, this outcome does emphasise an opportunity to enhance base formulations or utilise material delivery systems such as biodegradable porous hydrogels to enhance EV penetration or modulate their release kinetics [97,98]. Delivery of EVs in combination with existing treatments that puncture the dermis (e.g. microneedling) also provides an opportunity for the enhanced delivery of EVs (Fig. 2). A study by Cao et al. (2021) demonstrated that the addition of EVs could enhance collagen density and organisation when compared to microneedling alone. However, the study lacked EV only controls, so the added benefit of microneedling could not be determined [17]. An alternative approach could be via the local injection of EVs. For example, Hu et al. (2019) utilised a needle-free injector for the autologous transdermal delivery of EVs derived from human dermal fibroblasts (HDFs), resulting in dispersion between the dermis and hypodermis with no obvious administration injury [99]. Trans-dermis delivery of autologous HDFs is approved by the FDA and could provide a viable alternative to MSC-EVs providing the cells can be isolated and expanded in sufficient numbers. However, given the autologous nature of this approach, it is likely that outcomes will be dependent on the age and status of prospective donors. Lastly, it should be noted that all demonstrations of the in vivo efficacy of topical and injected EVs have been restricted to murine models. Human skin is comparatively thicker, which may negatively impact EV penetration and therapeutic efficacy in clinical trials and further increase the need to combine topical EV delivery with adjunct treatments that enhance dermal absorption. As the field continues to advance, it is vitally important that further studies are conducted to monitor the penetration and biodistribution of topical and injected EVs in relevant skin models.

To date, the field of regenerative aesthetics has been almost entirely focused on small EVs released by stem cells. However, it may also be pertinent to further investigate the effects of EV messengers released by dying cells, as these apoptotic bodies and apoptotic exosome-like vesicles have been found to exert important functions in regulating inflammation and indirectly promoting wound healing through the polarisation of macrophages [100,101]. Additionally, it will be valuable to assess how cells can be primed or 'educated' to enhance EV yield or boost their therapeutic content. Several studies have 'primed' EV producing cells with inflammatory mediators or factors that positively influence cell growth or motility. In the remit of wound treatment, studies have applied melatonin or the fibrinolytic drug mesoglycan, with increased mobilisation of fibroblasts and endothelial cells observed [102,103]. Alternative approaches have utilised include short periods of hypoxic cellular conditioning ($\leq$ 5% O_2), with general trends observed in the upregulation of pro-angiogenic factors such as miR-612 and increased vascular tube formation [104,105].

In conclusion, it is evident that EVs have huge potential as regenerative therapeutics in dermatological medicine through their observed roles in promoting cell proliferation, migration, and modulating ECM turnover and the local inflammatory environment. There is growing commercial interest in the production of EV therapeutics, with several companies manufacturing topical stem cell EV products for regenerative and cosmetic applications. There are also additional products in the research and development pipeline that are harvesting EVs from alternative cell sources including T regulatory cells and human platelets. However, if the considerable promise of EV therapies is to be fully realised, it is essential that we focus on identifying markers of therapeutic potency and establishing robust assays that can facilitate the regulation and manufacture of safe and effective products. It is also important that individuals working in aesthetic medicine are aware of current regulation pertaining to the application of EVs in clinical practice, acknowledging that EVs represent a highly complex and heterogeneous biological product with relatively little clinical and long-term safety data available.

Funding

The authors acknowledge funding from Engineering and Physical Sciences Research Council (EPSRC) and The Medical Research Council (MRC) Centre of Doctoral Training: Regenerative Medicine for S.W.'s doctoral studentship. O.G.D acknowledges support from the Academy of Medical Sciences (AMS), the Wellcome Trust, the Government Department of Business, Energy and Industrial Strategy (BEIS), the British Heart Foundation and Diabetes UK [SBF004\1090].

CRediT authorship contribution statement

O.G. Davies: Conceptualization, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing. **S. Williams:** Investigation, Writing – review & editing. **K. Goldie:** Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

No data was used for the research described in the article.

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