Bioactive Materials 16 (2022) 187–203

Contents lists available at ScienceDirect

Bioactive Materials

journal homepage: www.keaipublishing.com/en/journals/bioactive-materials

Review article

Advances in spray products for skin regeneration

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ARTICLE INFO

Keywords:
Skin
Spray
Tissue engineering
Fibrin
Hydrogel

ABSTRACT

To date, skin wounds are still an issue for healthcare professionals. Although numerous approaches have been developed over the years for skin regeneration, recent advances in regenerative medicine offer very promising strategies for the fabrication of artificial skin substitutes, including 3D bioprinting, electrospinning or spraying, among others. In particular, skin sprays are an innovative technique still under clinical evaluation that show great potential for the delivery of cells and hydrogels to treat acute and chronic wounds. Skin sprays present significant advantages compared to conventional treatments for wound healing, such as the facility of application, the possibility to treat large wound areas, or the homogeneous distribution of the sprayed material. In this article, we review the latest advances in this technology, giving a detailed description of investigational and currently commercially available acellular and cellular skin spray products, used for a variety of diseases and applying different experimental materials. Moreover, as skin sprays products are subjected to different classifications, we also explain the regulatory pathways for their commercialization and include the main clinical trials for different skin diseases and their treatment conditions. Finally, we argue and suggest possible future trends for the biotechnology of skin sprays for a better use in clinical dermatology.

1. Introduction

Skin is the largest organ in the human body and one of the most important defense mechanisms against pathogens and thermal, mechanical and chemical hazards, given that it is the outermost protecting sheath and is in direct contact with the external environment [1–6]. The skin is comprised of three main layers, a superficial epidermis, and a deeper dermis, followed by the subcutaneous

Abbreviations: ATMP, advanced therapy medicinal product; BLA, Biologic License Application; CAT, Committee for Advances Therapies; CEA, cultured epithelial autograft; CFR, Code of Federal Regulations; CHMP, Committee for Medicinal Product for Human Use; CTD, Common Technical Document; DMEM, Dulbecco's Modified Eagle's medium; ECM, extracellular matrix; EMA, European Medicines Agency; EU, European Union; FDA, Food and Drug Administration; GAGs, glycosaminoglycans; GLP, Good Laboratory Practice; GMP, Good Manufacturing Practice; HA, hyaluronic acid; HCT/Ps, human cells, tissues, and cellular and tissue-based products; ISO, International Organization for Standardization; MA, marketing authorization; NP, Notified body; OTAT, Office of Tissues and Advanced Therapies; PCL, polycaprolactone; PEG, polyethylene glycol; PHSA, Public Health Service Act; PMA, Premarket Approval; PRF, platelet rich fibrin; PRP, platelet rich plasma; PU, polyurethane; QMS, Quality Management System; TE, tissue engineering; USA, United States of America.

Peer review under responsibility of KeAi Communications Co., Ltd.

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https://doi.org/10.1016/j.bioactmat.2022.02.023
Received 29 October 2021; Received in revised form 22 January 2022; Accepted 18 February 2022
Available online 8 March 2022

hypodermis. The epidermis consists of several layers of keratinized epithelium, which is dynamic and in continuous self-renewal. Keratinocytes are the major cell type in the epidermis, and they play a crucial role in skin function thanks to their ability to differentiate and synthesize a variety of structural proteins and lipids that are important for epidermal regeneration [5,7]. Other cells within the epidermis are melanocytes, which provide pigmentation, and Langerhans' cells, which contribute to immune surveillance [8]. Corneocytes (enucleated dead cells) are found embedded in a lipid matrix in the uppermost layer of the epidermis, the stratum corneum, that plays a role against absorption of components and water loss [5]. The dermis is a thicker layer of connective tissue, consisting mainly of cells (like fibroblasts, endothelial cells, smooth muscle cells, and mast cells), extracellular matrix (ECM) and structural components like collagen and elastin fibers, which provide mechanical strength and elasticity, as well as a vascular plexus for skin nutrition [4,8]. Between the epidermis and the dermis is the basal membrane, a highly specialized ECM structure, composed of different glycoproteins and proteoglycans, that provides a stabilizing but dynamic interface and a diffusion barrier between these two layers, and where skin appendages such as hair follicles, sweat glands, and nerves are all embedded [4,5,9]. These skin appendages maintain the body's hydration and constitute a protective layer that helps to defend the body from hostile environment, among other functions [3]. Connecting the dermis to the underlying structures is the hypodermis, the connective tissue which includes fibroblasts and adipose tissue for fat storage and protection, serving as the skin's shock-absorber and the body's heat insulator [4,5,9]. The thickness of the human skin is approximately 1.5–2.5 mm, but it varies depending on its anatomical localization and its function in supporting the weight of the body [2].

Skin wounds or injuries may result from burns and other physical and chemical traumas, genetic irregularities, skin diseases (such as diabetic foot ulcers, perianal fistulae, or epidermolysis bullosa), or removal of skin during surgery [5]. Depending on the depth of the injury, skin wounds can be categorized as epidermal, superficial partial-thickness, deep partial-thickness, and full-thickness wounds [10]. For optimal treatment, wounds must be diagnosed early, although some cases may be difficult to classify accurately with an early evaluation [11,12]. Thanks to the self-healing functions of the skin, epidermal and superficial partial-thickness wounds can usually be regenerated without requiring therapeutic intervention [5].

However, in deep partial and full-thickness wounds, the skin's regenerative components are destroyed, which makes it impossible for the skin to regenerate on its own. Therefore, these wounds need immediate treatment to avoid a late re-epithelialization, which could facilitate infections, produce worse functional and esthetic healing outcomes (like contracture and scarring) and extend the patient's stay at the hospital [5,11–13]. A large surface area wound, coupled with extensive fluid loss, could even result in death [13]. Fast therapeutic interventions can allow mobility for the patient and can help regain the skin's structure and function, although full recovery of its function may not be possible unless all skin cell types are restored [1,6,14,15].

Conventional skin grafts have been used for decades for the regeneration of deep wounds, although they present some disadvantages and limitations [1,2]. However, the development of tissue engineering (TE) and regenerative medicine has offered multiple therapeutic strategies for the regeneration of skin lesions, using diverse types of skin substitutes, such as cell suspensions, hydrogels, or 3D scaffolds [16]. Moreover, another promising solution that is still not commonly used in clinical practice, but represents a potential therapeutic strategy for wound healing applications, is the use of skin sprays, given their numerous advantages such as their versatility to deliver different cell types and materials.

In this review, we discuss and summarize the main approaches and techniques for skin regeneration, and particularly focus on the latest advances in the technology of skin sprays, including both investigational and currently available commercialized acellular and cellular products, and an explanation of their regulatory pathways to be marketed. Finally, we include an exhaustive search for clinical trials using sprays to treat a variety of skin conditions and propose future trends for the biotechnology of skin sprays with a more feasible clinical application.

2. Main therapeutic strategies for skin regeneration

2.1. Conventional treatments for wound healing: skin grafts

Traditionally, conventional treatments for deep wounds have included split and full-thickness skin autografts, which consist of cutting a swath of skin from an uninjured part of the patient's body and grafting it over the injured area. This treatment is effective and represents the most common way to treat second to third degree burns, but it causes more pain for the patient and doubles the area that needs to heal [17,18]. Moreover, the use of autografts is limited by the size of available uninjured donor skin, which leads to the use of less desirable options such as temporary covering with other types of skin graft, like allografts and xenografts, which carry the risks of immune reactions and diseases transmission [1,18,19]. A reduced donor skin site can be expanded to a much larger size by meshing techniques to obtain meshed split-thickness autografts. However, they are usually accompanied by scarring of the donor site and mesh interstices, and are limited due to the difficulty of handling when their expansion rate is greater than 1:4 [4,6,20–22].

A recurrent solution used in burn care is the culture of autologous cells to amplify their number, obtaining cultured epithelial autografts (CEAs), firstly described by Rheinwald and Green in 1975, but it can take weeks for tissue cultures to mature and become available for grafting, while patients with severe burn injuries have increased chances for survival when wounds are excised and grafted early [1,18]. Even though there have been many reports of their successful clinical use and better cosmetic results when compared to wide mesh autografts, the process of growing cultured keratinocyte sheets is expensive, time consuming and labor intensive, and the sheets have shown fragility, low efficiency, variable rate of graft take and poor attachment [2,20,23–27]. Furthermore, CEAs are mostly grown from one cell type (keratinocyte progenitors), consequently lacking the full complement of cellular elements (e.g., melanocytes, hair follicles, and sweat glands) required for complete skin architecture [6,27]. Fig. 1 shows the different grafting techniques used for burns and wound healing applications.

2.2. Current therapeutic strategies

Alternatives to traditional wound healing strategies have emerged thanks to the development of biosynthetic skin substitutes that aim to contribute to wound healing by covering the wound and acting as a barrier to prevent fluid loss and infections. Several materials have been used to develop skin substitutes for clinical use in different biomedical and pharmaceutical applications that include hydrogels, films, cell suspensions, cell sheets, or 3D skin scaffolds, fabricated by different TE methods such as 3D bioprinting, electrospinning, and also cell-spray devices (more extensively discussed in section 3) [2,16,28,29].

These products can be differentiated into acellular and cellular skin substitutes, and both require the use of biocompatible and biodegradable materials that are also able to maintain a moist environment, reduce the pro-inflammatory response, have satisfactory mechanical properties and be porous enough to facilitate cell proliferation and the transport of biomolecules, nutrients, and metabolic wastes [3,20,29].

2.2.1. Biomaterials used for skin substitutes

A variety of biomaterials, both natural and synthetic, have been extendedly utilized as skin substitutes. They form three-dimensional polymeric networks that aim to mimic the structure and function of natural ECM, also serving as a platform for cellular localization, adhesion and differentiation, and thereby, stimulating skin regeneration [3,28–30].

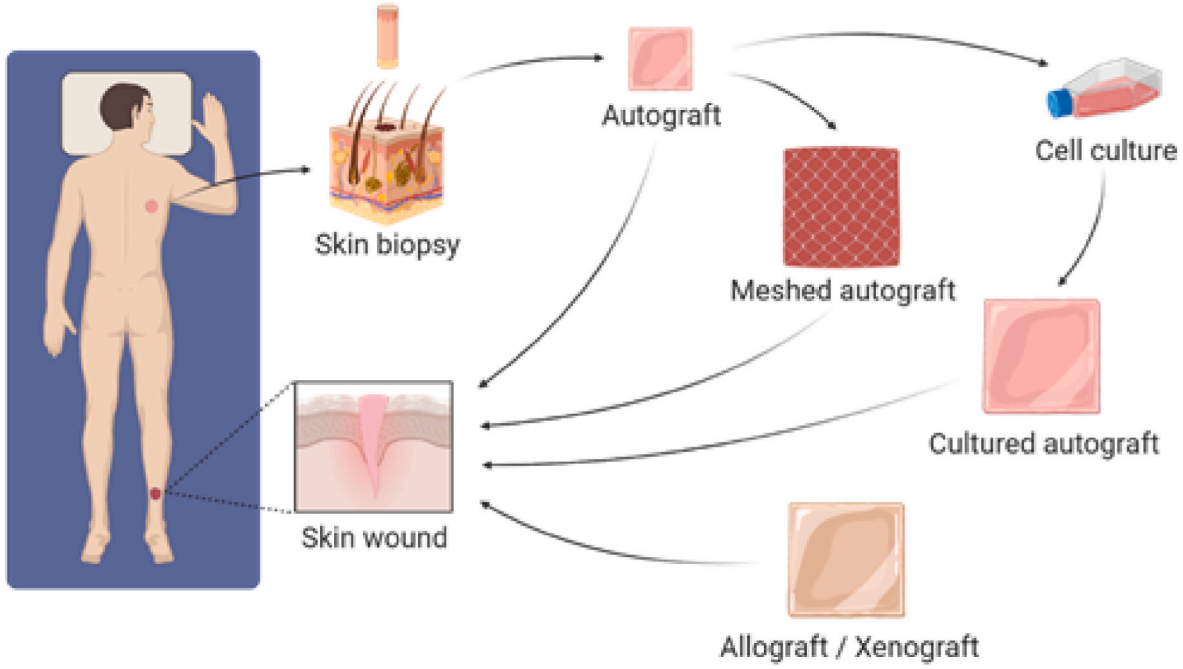

Fig. 1. Conventional grafting approaches for wound healing. Autologous skin grafts can be obtained from a biopsy of the patient's own undamaged skin, and this autograft can be either applied directly over the wound, or expanded by meshing techniques or cell culturing prior to application. On the other hand, skin grafts obtained from another individual as a donor (allograft) or from an animal (xenograft), respectively, can be applied temporarily to cover the patient's wound. Figure created with BioRender.com.

To fabricate skin substitutes that recreate the physiological conditions of the skin, several **natural materials**, such as collagen, fibrinogen, gelatin, silk, hyaluronic acid (HA) or alginate, have been employed.

Among them, **collagen** has excellent biocompatibility and is highly biomimetic because it is the main structural protein of the ECM of the dermis. Furthermore, it has good biodegradability, low immunogenicity, and a porous structure with good permeability [29,31]. There is a number of commercially available collagen-based dressings in sheet, gel, lattice, or sponge form; but these dressings often have poor biostability and low mechanical strength, and furthermore, pure collagen is expensive, and wound contraction and scarring easily occur [20,29]. The mechanical properties can be enhanced by physical or chemical intermolecular cross-linking of collagen, or by blending collagen with other materials [31,32], like glycosaminoglycans (GAGs) [33,34], agarose [35], or chitosan [36,37].

An important biopolymer in wound healing is **fibrinogen**, which is converted into a fibrin clot by the action of thrombin during the coagulation cascade, and it has been extensively used to prepare fibrin glues or sealants, which cover the wound and provide an immediate provisional matrix that will be invaded by repair cells on the wound bed [38,39]. Fibrinogen is biocompatible and biodegradable, and can be isolated from the patient's own blood, therefore limiting immunogenicity. Moreover, fibrin hydrogels have been reported to promote wound healing, epidermis regeneration and vascular growth; and also reduce scar formation [40,41]. Fibrin possesses a high elastic deformation capacity and large stretchability, but it is also one of the softest polymeric fibers, with low mechanical properties [40,41]. Nevertheless, the mechanical properties of the fibrin gels can be optimized by adjusting several factors such as pH or the concentrations of fibrinogen and thrombin; or by combining fibrin with different materials [40,41], such as HA [42], gelatin [43], or polyethylene glycol (PEG) [44].

Another biomaterial, **chitosan**, is derived from de-acetylated chitin and has many advantages for wound healing and TE, including biocompatibility, biodegradability, hemostatic activity, antibacterial properties, and stimulation of re-epithelization. In addition, it can easily be fabricated into films, gels or sponges [45,46]. However, its mechanical properties need to be improved because once it dries, it suffers severe shrinkage and deformation, which limits the application of pure chitosan to create skin substitutes [46,47]. Therefore, in order to overcome these limitations, chitosan is usually cross-linked [48]; derivatized by structural modification [46,48]; or combined with other natural and synthetic polymers [46], such as gelatin [49–53], agarose [54], or collagen [55]. For example, Soriano-Ruiz et al. (2019) developed chitosan hydrogels based on quaternary blends of three GAGs (HA, chondroitin sulfate, and dermatan sulfate) and collagen, for their use in wound healing [56].

Gelatin, a collagen-derived protein, has been studied in sponge or film forms. Advantages include its capacity to promote epithelialization and granulation tissue formation, its high biodegradability and biocompatibility, and its lower cost in comparison to collagen, but its low mechanical strength makes its use only effective when incorporated with other polymers [57–59]. For example, gelatin has been widely used in combination with chitosan to create skin hydrogels [49–53], but this blended hydrogel still has poor mechanical strength [60], which can be improved by the addition of other materials such as HA [61,62] or polycaprolactone (PCL) [60,63].

Silk fibroin, obtained from silkworms, has excellent properties for wound healing. It has good biocompatibility and biodegradability, hemostatic properties, low immunogenicity and non-inflammatory characteristics, and it is non-toxic and non-carcinogenic [64–67]. Moreover, it is known to accelerate wound healing [68], promote cell proliferation, collagen synthesis and re-epithelialization [69], and provide support for cell attachment [64,65]. Its good mechanical properties and easy fabrication make silk fibroin an interesting biomaterial for skin TE [64–67]. However, silk fibroin does not have antimicrobial properties, which often results in wound infection, but this problem can be solved by the addition of other antimicrobial materials [65]. For skin wound healing applications, silk fibroin is usually used in the form of films, hydrogels, sponges or nanofibrous scaffolds [68], either alone [64,66,70,71] or in combination with other materials such as alginate [67] or chitosan [65], among others. Roh et al. (2006) compared the wound

healing effect of a silk fibroin sponge, an alginate sponge and a silk fibroin/alginate blended sponge in a rat full thickness wound model and found that the three sponges improved the wound healing rate in comparison with the control, but with a higher increase for the blended sponge [69].

Obtained from seaweed, **alginate** is a natural polysaccharide polymer that has received much attention in the last decade, because of its outstanding features in terms of biocompatibility, biodegradability, affordability, non-toxicity, and non-immunogenic properties and chelating ability [20,72]. Various wound dressings in the form of hydrogels, electrospun mats and sponges are based on alginate, given its numerous advantages for wound healing, including its hemostatic and mild antiseptic properties, conformability, promotion of granulation tissue formation and fast epithelialization. Alginate is highly hydrophilic, and together with its ability to retain water, it allows the maintenance of a moist wound environment that can avoid drying out of the wound bed [72–74]. Moreover, its structure lacks signal sequence for cell adhesion, which makes it a significant material for wound management, as it avoids secondary injury when alginate-based dressings are peeled off [72,75]. However, alginate has poor structural integrity and mechanical properties [76,77], but these can be improved by the combination with other materials (such as chitosan [75,78], fibrinogen [79], or gelatin [80]); by immobilization of specific ligands; or by cross-linking [72]. Ionic cross-linking, using divalent cations such as Ca^{2+}, is the most common gelling method for alginate, but these divalent cations can be released and exchanged with other monovalent cations in the surrounding media, resulting in the dissolving of the alginate gel [74]. Nonetheless, alginate hydrogels can also be gelled by covalent cross-linking, thermal gelation, cell cross-linking, free radical polymerization and "click" reaction [72,74].

GAGs (including HA, chondroitin sulfate, heparin sulfate, dermatan sulfate, and keratan sulfate) play crucial roles in all stages of wound healing, which makes them essential ingredients in formulations for epidermal regeneration [81,82]. **HA** offers excellent biocompatibility and biodegradability, a high capacity to retain water, and anti-inflammatory, mucoadhesive and viscoelastic properties [82–85]. Moreover, it can inhibit tissue adhesion and scar tissue formation [86,87]. Currently, there are some HA-derived products in the market, but the clinical application of soluble HA is limited due to its poor mechanical properties and rapid degradation by hyaluronidases [88].

On the other hand, **synthetic polymers** are less expensive and more reliable sources of material as they possess lot-to-lot uniformity and can be customized to provide a wide range of physical properties [29]. Among them, polyurethane (PU) is desirable for wound healing because it is biocompatible and has good mechanical properties, and it keeps a moist environment due to its impermeability to bacteria and water but permeability to gas. PU products are cost-effective, but they have shown limited adherence to the wound bed [29,89]. Nylon, poly(glycolic acid), and poly(lactic acid) have been used in mesh form for some commercial skin substitutes. However, other polymers, including PCL or poly (L-lactide), have shown limited clinical success, because of their low rates of cell attachment and proliferation, due to the limited biological signals [29,90].

Overall, natural polymers have excellent biocompatibility and promote cell adhesion and proliferation, but some of their limitations include low mechanical strength, shrinkage or contraction, difficulty in handling, and, in some cases, high costs; while synthetic polymers offer good mechanical properties but they lack the biological signals that are present in natural polymers [29,91,92]. Therefore, in order to obtain an improved mechanical strength and maintain good biocompatibility, natural and synthetic polymers are usually combined in different ways [29,91,92], for example by blending or cross-linking the polymer solutions [29,91,92]; coating a synthetic mesh with a natural polymer [93]; or constructing hybrid meshes by forming microsponges of a natural polymer in the openings of a synthetic knitted mesh [94].

2.2.2. Skin TE fabrication methods

As mentioned before, skin substitutes are available for clinical use in different forms, such as hydrogels, films, cell suspensions, cell sheets or 3D skin scaffolds. These products are manufactured using the different biomaterials previously described, and in the case of cellular substitutes, also in combination with either allogeneic or autologous cells. These cells can be employed directly after their acquisition, or they can previously be cultured to amplify their number before being used to create skin substitutes. 3D scaffolds are the mainstay for the construction of artificial skin substitutes, as they serve as a platform for cellular adhesion and proliferation. Different scaffold designs for skin TE (e.g. nanofibrous matrices, microporous scaffolds, polymeric networks, lattices or meshes, etc.) can be prepared by traditional methods, such as freeze-drying or particle leaching, or by new TE technologies, such as 3D bioprinting, electrospinning or spraying [5,95] (Fig. 2).

3D bioprinting represents a very promising TE technique, with high flexibility and repeatability. Using computer-aided designs, biomaterials and living cells can be deposited layer by layer, fabricating custom-designed porous constructs as artificial skin tissues [1,96]. Bioprinting not only allows the generation of the different layers of the skin, but it also has the potential to include skin structures, such as sweat glands, vascular networks, and hair follicles, although this is still difficult to achieve. This could reduce problems with limited vascularization, poor healing, graft failure, immune rejection, and pathogen infection. An ideal bioprinted skin should be biocompatible and biodegradable, have the desired mechanical properties, have an appropriate surface chemistry, and be highly porous with a network of interconnected pores that will allow transportation of nutrients and removal of wound exudates, while at the same time protecting the wound from microbial invasion [1,97]. Considering that, the biomaterials generally used to formulate hydrogels for skin bioprinting can be natural (e.g. alginate [98–100], gelatin [43,98–101], fibrin [43,100,102], chitosan [101], HA [103], collagen [102,104], etc.) and/or synthetic (e.g. gelatin methacrylamide [105,106], PCL [107], PEG [108], etc.). Among the cells used for bioprinting, the most common are cultured autologous cells derived from the patient, but allogeneic cell lines, primary cells, or stem cells can also be used, including for example keratinocytes, fibroblasts, mesenchymal stem cells, or induced pluripotent stem cells [1,97].

Another promising approach is the use of **electrospinning** technology to create ECM analogs. Electrospinning applies a strong electric field to fibers of polymer solutions, either natural or synthetic, making them tight-knit together in layers and thereby forming a nanofibrous net with the same nanoscale structure and functions as native ECM [29,109]. The resulting scaffolds have a high surface area to volume ratio, which promotes cell-matrix interaction and facilitates oxygen permeability and fluid accumulation, while the pores are small enough to prevent bacterial infection. Materials like fibrinogen, collagen, gelatin, chitosan, PU, poly(lactic acid), and PCL, or some combinations of them, have been used in electrospinning. Moreover, drugs and other biologically active molecules can be added into nanofibers to obtain a controlled release system for pain management or antibacterial activity [29,109]. Although there have been reports of cells maintaining their viability after being electrospun [110,111], cell-laden hydrogels are still not commonly used for electrospinning [112]. Normally, cell-free polymer solutions are electrospun and then cells are seeded into the resulting scaffolds [113–116]. Nanomedic developed SpinCare™, a portable, lightweight, gun-shaped device that uses electrospinning technology to print a layer of nanofibers directly on the wound, creating a transparent, protective film that forms a physical barrier covering the injured skin. This method avoids any need to come into direct contact with the wound, thereby eliminating the risk of infection and pain; and once new skin is generated, the layer peels off painlessly. The product is intended for any kind of wounds that need medical treatment, including surgical and chronic lesions [117].

Recently, **skin sprays** have gained increased attention as a delivery method for hydrogels and cell suspensions, and are thoroughly

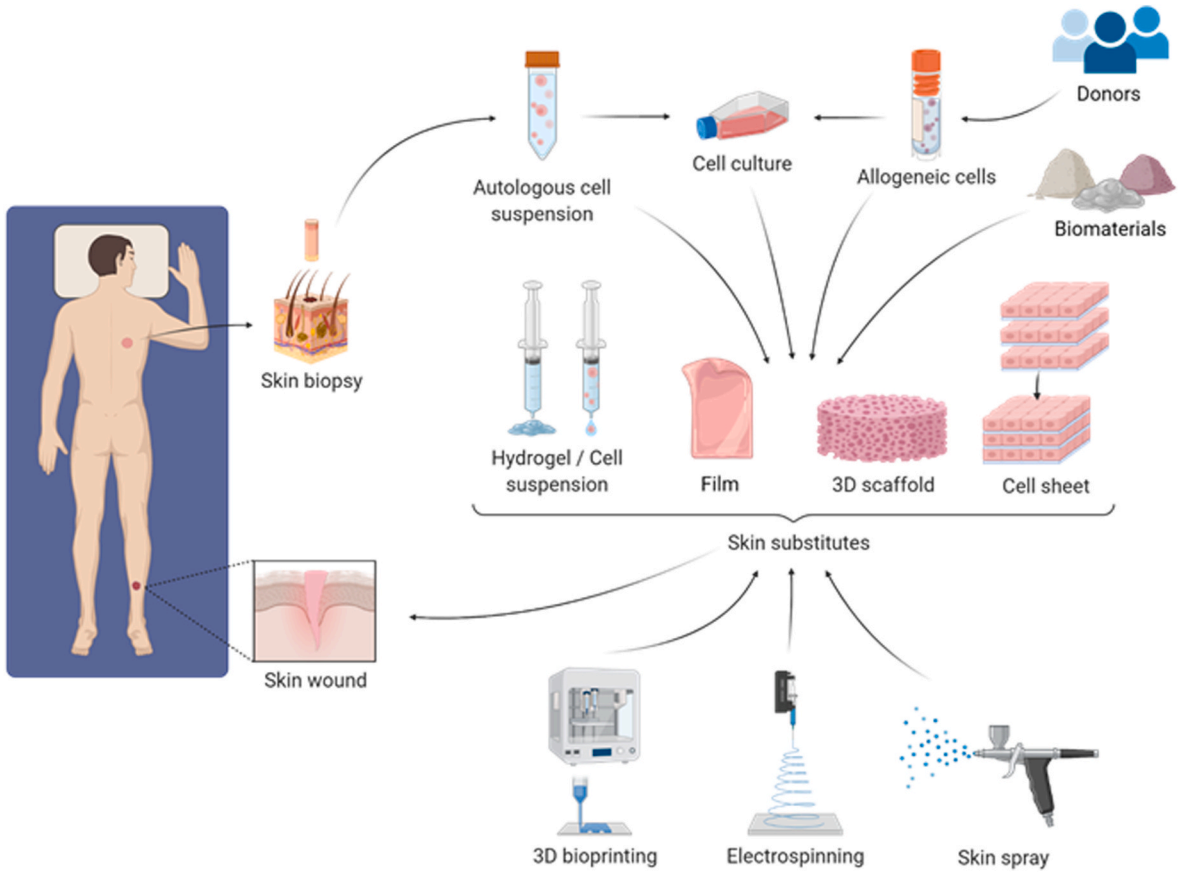

Fig. 2. Current therapeutic approaches for wound healing. Skin wounds can be covered and treated with different skin substitutes such as cell suspensions, hydrogels, cell sheets, films, or 3D scaffolds, which can be fabricated by techniques like 3D bioprinting, electrospinning or skin sprays. Skin sprays allow the delivery of hydrogels and/or cells to the wound. Cells used for cellular skin substitutes can either be autologous (obtained from a biopsy of the patient's skin) or allogeneic (obtained from skin samples of donors); and they can be used directly, or they can be cultured to amplify their number prior their application. Figure created with BioRender.com.

described in the upcoming sections.

3. Skin sprays

The use of topical sprays as an application method for hydrogels and/or cell suspensions to treat acute and chronic wounds has been of interest in clinical practice for the past few decades, thanks to their advantages such as the possibility to treat large wounds or spray over areas with unfavorable topography, the reduced time for application, and the homogeneous distribution of the sprayed suspensions [21,27,118,119].

When using spray devices to deliver biomaterials and living cells for medical applications, it is important to pay attention to the spraying parameters, such as the spraying pressure, the distance from the tip of the spray device to the receiving surface, the angle, or the sprayed volume, because these parameters can vary results significantly [13,27]. For example, in clinical practice, it has been reported that at least a 10 cm distance from the spray device to the body is necessary for a safe application without risking an air embolus [13].

For cellular sprays, it is also important to determine the amount of cells required to treat a certain wound area [27]. In the case of autologous cell spray, Esteban-Vibes et al. (2016) proposed equations to calculate the minimum skin area needed from a donor site, to obtain enough cells to treat a certain burn wound area, obtaining ratios of 1:80 to 1:100 (donor site area:wound area) [11].

Moreover, sprayed cells are expected to be damaged when they impact the target surface, with cell survival depending on numerous variables such as the viscosity of the transporting fluid, nozzle diameter, distance, velocity of the sprayed droplet, or the characteristics of the receiving surface [20,120,121]. It has been found that post-aerosolization cell viability significantly decreases with higher pressure, smaller nozzle diameter, high transporting fluid viscosity, high spraying velocity, and stiffness of the receiving tissue surface. On the contrary, cell viability is positively affected by a larger cell-containing droplet diameter [20,120,121]. Numerous studies have demonstrated the viability of epidermal cells after aerosolization [27,121–123]. For example, Harkin et al. (2006) demonstrated that cell viability was unaffected with air pressures up to 10 psi, but metabolic activity was significantly reduced at higher pressures. They also determined the approximate cell density required for re-epithelialization, concluding that, under the conditions tested, 0.2 mL bursts of $1.5{\cdot}10^6$ cells/mL sprayed from a height of 10 cm should theoretically epithelialize an area of approximately 10–15 cm^2 within 7 days [27]. Denman et al. (2007) developed a mathematical model to simulate the growth patterns of cell colonies after being applied using an aerosolized technique. This model could be used as a decision support tool for clinicians, allowing them to predict the evolution of wound coverage over time, depending on different parameters such as the number of sprayed cells and their spatial distribution, the size of initial colonies, or the wound size [124].

However, it is not sufficient to only consider post-aerosolization viability. Proliferative capacity must be assessed too, because cells experience three types of stress while being sprayed (hydrostatic, shear, and elongation stresses), which may damage the cells without disrupting their membranes, with the duration of applied stress also being a key factor on the caused impairment [125].

3.1. Acellular skin sprays

Acellular skin sprays generally consist of hydrogels that form thin layers when they are sprayed over the wound, acting as dressings and protecting the wound against infection and fluid loss. In addition, some hydrogels may help to enhance the wound healing process, and some have antibacterial properties or can act as a drug delivery system. Fibrin is the most widely used hydrogel for skin sprays and is often sprayed directly over less severe wounds to act as a hemostatic dressing or over grafts to promote adhesion and take rates [126].

3.1.1. Non-commercial acellular skin sprays

To date, there have been several studies that apply different experimental hydrogels over the wounds, shielding them from infections and avoiding water loss. They are summarized in Table 1. These specific studies were only performed *in vitro* or in animal models, generally using a simple spray pump as the spraying device, and most of the spraying parameters were not specified.

In 1981, Neumann et al. formulated a gelatin foam that is able to cool burn wound surfaces and wash and disinfect them when it is sprayed, and once it dries it adheres to the wound, creating a foam layer that reduces the rate of evaporative water loss and prevents infection [127].

Quinn et al. (2000) used an octylcyanoacrylate-adhesive spray bandage to treat traumatic abrasions in pigs. Octylcyanoacrylate polymerizes with the moisture on the wound surface and dries within 30 s, producing a thin, flexible dressing that is non-toxic, hemostatic and waterproof, it has antimicrobial activity and sloughs off with the wound eschar as epithelialization occurs [128].

Jáuregui et al. (2009) immobilized papain enzyme in a peptin gel, which forms a thin, even and smooth film when aerosolized. This gel promotes wound healing better than untreated wounds, and it stays in place on the wound bed without dripping off [129].

More recently, Catanzano et al. (2015) used a spray-by-spray deposition technique to deliver a tea tree oil microemulsion in an *in situ*-forming cross-linked alginate hydrogel. Results showed that this microemulsion is homogeneously distributed in the resulting transparent hydrogels and has potential as an advanced dressing for infected wounds, given that it has antimicrobial and anti-inflammatory properties, as well as good thermodynamic and kinetic stability [130].

3.1.2. Commercial acellular skin spray products

Among the commercial acellular sprays, plenty of them use fibrin for their products. One of the most popular is TISSEEL® by Baxter (licensed under the trademark of TISSUCOL® in some countries), a fibrin glue that has been widely used since the early 90s as an adjunct to hemostasis and sealing in open surgery and laparoscopy, derived from human pooled plasma. It offers both drip and spray applicators, that are attached to a double syringe (one for the fibrinogen solution and the other for the thrombin solution), allowing surgeons to select the right tools to address individual patient needs [126,131]. As a spray, numerous studies have used TISSEEL® fibrin sealant *in vitro* and *in vivo*, alone or alongside cells. Sometimes TISSEEL® is delivered using TISSOMAT®, a pressurized spray module (air compressor) by Baxter, whose Spray Kit has a dual syringe that supports the co-application of other materials like cell suspensions [27,123,132]. Studies using TISSEEL® (summarized in Table 2) reported that when fibrin was added to a cellular treatment (e.g. autologous keratinocytes and fibroblasts [123], or autologous mesenchymal stem cells [132]), wound healing was accelerated and wounds contracted less in comparison with wounds treated with cells alone. TISSEEL® fibrin sealant is an excellent liquid cell delivery vehicle that rapidly polymerizes upon addition of thrombin, forming an adherent network of cells embedded in a fibrin meshwork that still preserve their ability to grow and differentiate [25,27,123,132–134].

ARTISS® is another fibrin sealant by Baxter, used to adhere autologous skin grafts to the wound beds in burns and to adhere tissue flaps during facial rhytidectomy surgery, but, in contrast to TISSEEL®, ARTISS® is not employed as an adjunct to hemostasis [135]. Earnshaw et al. (2020) used ARTISS® in 50 patients undergoing lateral selective neck dissections, showing a significant reduction in the length of the hospital stay of the patients, in the drain retention time, and in the total volume drained. Moreover, they reported an easier use of ARTISS® in comparison to TISSEEL® [136] (Table 2).

EVICEL® by Johnson & Johnson is a fibrin sealant very similar to TISSEEL®, also derived from human pooled plasma, used as an adjunct to hemostasis for use in patients undergoing surgery, with the difference that its airless spray accessory does not need an external gas source, reducing setup time [137]. Reddy et al. (2017) conducted an observational prospective study to evaluate the efficiency of EVICEL® for the adherence of skin grafts. They reported better hemostasis and graft adhesion, and a reduction in surgical time [138] (Table 2).

Vivostat® has different products, with a system that prepares autologous fibrin sealant or platelet rich fibrin (PRF) from the patient's own blood without requiring additional thrombin and without using cryoprecipitation. The fibrin is shown to act as a hemostatic, as well as a glue for graft fixations in burn surgery or to adhere cells to the burn wound. Vivostat® PRF has also been successfully used to treat diabetic foot ulcers, venous ulcers, and pressure ulcers. The Spraypen is a sterile, disposable, hand-held device that delivers the fibrin sealant or PRF solution to the tissue; but besides the Spraypen, the Vivostat® system offers different types of applicators (e.g. the Endoscopic Applicator). Vivostat® also has a Co-Delivery system that makes it possible to co-deliver a desired substance, such as a cell suspension, along with Vivostat® Fibrin Sealant or Vivostat® PRF [2,24,139]. Studies using Vivostat® fibrin sealant (see Table 2) reported that its rate of polymerization is significantly faster than that of other sealants that require thrombin, allowing immediate adhesion of cells onto the wound; and since this system produces completely autologous fibrin, any risk of disease transmission is eliminated [2,24].

On the other hand, there is a wide variety of antiseptic sprays for wound cleansing in superficial wounds, for example, Prontosan® Wound Spray by B. Braun and Hansaplast® Wound Spray, composed of polyaminopropyl biguanide (polihexanide), or The Wonder Spray®, whose key ingredient is hypochlorous acid. It is worth mentioning that there are other commercial acellular spray products for healing that

Table 1
Studies using non-commercial acellular sprays.

Model	Application	Spray		Composition	Reference
		Device	Parameters		
In vitro	Infected wounds	Pharmaceutical spray pump	V: $CaCl_2$ + 140 μL Alg (3 layers) A: 36 mm Ø Petri dish	Tea tree oil microemulsion in alginate	[130]
In vitro/Rats	Wound healing/Burns	Freon 12 propellant	-	Gelatin foam (2% w/v)	[127]
In vitro/Rabbits	Wound healing	Spray pump	P: 50 psi N_2	Papain enzyme (0.1% w/v) immobilized in peptin (6% w/v) gel	[129]
Pigs	Traumatic abrasions	Spray pump	A: 3 cm Ø wounds	Octylcyanoacrylate adhesive	[128]

V: sprayed volume; D: distance from spray tip to receiving surface; P: spraying pressure; A: sprayed area.

Table 2
Studies using commercial acellular spray products.

Model	Application	Spray				Reference
		Device	Parameters	Composition		
				Cell combinations tested	Hydrogel	
In vitro	Wound healing	TISSEEL and Easyspray system (Baxter)	P: 20 psi A: 35 mm Ø Petri dishes	Stromal vascular fraction of human adipose tissue	Fibrin (TISSEEL) (Fibrinogen 80 mg/mL, Thrombin 3.3 IU/mL)	[133]
In vitro	Wound healing/Burns	TISSOMAT	V: 0.2 mL D: 10 cm P: 10, 20, 30 psi A: 10–15 cm^2	Cultured human keratinocytes (0.5–1.5 · 10^6/mL)	Fibrin (TISSEEL) (Fibrinogen 1–3 mg/mL, Thrombin 2–5 IU/mL)	[27]
Pigs	Wound healing	TISSOMAT + a 3 compartment aerosolization device	V: 4–5 mL A: 224 cm^2	Autologous non-cultured pig keratinocytes, a few fibroblasts, and dendritic cells (2 · 10^6/mL)	Fibrin (TISSEEL)	[123]
Pigs	Wound healing/Burns	Syringe with a spray nozzle	V: 2 mL/wound D: 10 cm A: 12.6 cm^2 x 18 wounds	Autologous cultured pig keratinocytes (10^6/mL)	Fibrin (TISSEEL) (Thrombin 500 IU/mL)	[25]
Pigs	Wound healing/Burns	Vivostat spray applicator	V: 1.2 - 1–6 mL/wound P: 1.4 mL/min liquid flow A: 12.6 cm^2 x 12 wounds	Autologous cultured pig keratinocytes (1.23–3.16 · 10^6/cm^2)	Fibrin (autologous, Vivostat)	[24]
In vitro/ Mice/ Patients	Acute wounds from skin cancer and chronic lower extremity wounds	TISSOMAT application device and spray set	V: 2 mL D: 1–3 cm P: 2.5–5 psi	Autologous cultured human mesenchymal stem cells (2 · 10^6/cm^2, 3 applications)	Fibrin (TISSEEL) (Fibrinogen 5 mg/mL, Thrombin 25 U/mL)	[132]
Patients	Burns	Syringe placed in a prototype cell spray device	V: 1–2 mL P: 0.16 psi (3.7 L/min airflow, 4.2 mL/min liquid flow) A: 2% body surface area	Autologous cultured human epidermal and mucosal cells (Average of 3.9 ± 4.8 millions/wound)	Fibrin (TISSEEL) (Fibrinogen 5 mg/mL, Thrombin 25 U/mL)	[134]
Patients	Burns	Vivostat co-delivery system	-	Autologous non-cultured human keratinocytes (ReCell)	Fibrin (autologous, Vivostat)	[2]
Patients	Lateral selective neck dissections	ARTISS	-	–	Fibrin (ARTISS)	[136]
Patients	Skin grafts	EVICEL	V: 0.1–0.2 mL bursts	–	Fibrin (EVICEL) (Fibrinogen 55–85 mg/mL, Thrombin 800–1200 U/mL)	[138]

V: sprayed volume; D: distance from spray tip to receiving surface; P: spraying pressure; A: sprayed area.

form waterproof, thin, transparent films or dressings, which cover the wound and protect it against infections, such as URGO® Spray Dressing, Nobecutan® by Inibsa Hospital or Hansaplast® Spray Dressing.

3.2. Cellular sprays

A variety of cell types have been utilized in cellular sprays for *in vitro* wound healing studies, but autologous epidermal cells are mainly used for *in vivo* studies and clinical trials. Cell spray autografting is an innovative technique still under clinical evaluation, consisting of taking autologous skin cells from a small biopsy of undamaged skin of the patient and either spraying the cells directly over the wound or culturing them to amplify their number before spraying. In comparison to traditional sheet autografts, cell spray autografting is a simple and cost-effective method, it avoids blister formation and needs a much smaller donor site, therefore reducing healing time and minimizing complications [12,140].

Epithelial cells can be sprayed uniformly, remain viable and proliferate on the wound bed, achieving a fast re-epithelialization, which is important for a good functionality and esthetic outcome, particularly in large wounds and in joint areas [12,21,23,140].

Moreover, compared to cultured autografts, non-cultured cell spray autografting has the advantage that grafting can be carried out *in situ*, immediately after cell isolation, avoiding a prolonged culture waiting time and the need to cover the wound with another type of graft or skin substitute during the culture time. Not culturing the cells also avoids progenitor and stem cells being lost because of their differentiation, an usual consequence of *in vitro* culture expansion [12]. Moreover, for deeper wounds, cell spray can be applied in combination with mesh grafting [20,21,141,142].

Cell suspensions, normally using lactate or normal saline solutions as carriers, can run off when applied over convex structures of the body, leading to an uneven coverage and distribution of living cells, reducing the number of viable cells that stay in contact with the wound [2,13,143]. Hence, they can be sprayed in combination with a hydrogel, such as fibrin, to ensure that cells remain attached to the wound surface. The first use of a combination of sprayed autologous cultured keratinocytes and sprayed autologous fibrin sealant for full-thickness wounds in pigs was reported by Grant et al. in 2002 [24]. Since then, many studies have combined epidermal cells with fibrin to be sprayed for different applications, and several spray products have been developed.

3.2.1. Non-commercial cellular skin sprays

The delivery of cells *via* spray or aerosol has been evaluated in many

studies. Table 3 collects the main studies that utilized experimental cell-spray suspensions for burns and wound healing applications, normally making use of syringes with spray nozzles or airbrushes as the delivery devices.

The *in vitro* studies showed in Table 3 demonstrated the feasibility of sprays as a delivery system for fibroblasts and keratinocytes. In these cases, cells maintained high viability and good proliferative capacity after being sprayed, although viability decreased as spraying pressure increased [13,121,125].

Navarro et al. carried out two studies using skin sprays in pigs. In one study, a greater wound re-epithelialization (in terms of a more complete confluence, epithelial coverage, and basal cell thickness) and a more organized dermal-epidermal junction were observed in wounds treated with non-cultured autologous pig keratinocytes when compared to wounds treated without cells. In the second study, they demonstrated the feasibility of delivering melanocytes (which are necessary for the repigmentation of full-thickness wounds) to the wound *via* spray, but they were not able to demonstrate macroscopic pigmentation, and further work was required [141,144].

The studies in patients used autologous keratinocytes to treat burns. Complete wound closure and re-epithelialization were achieved, with excellent functional and cosmetic outcomes, and no adverse events were observed in any patient. However, these studies did not include control groups to compare the results with other treatments [118,145,146].

Among all of these, only one *in vitro* study sprayed the cells in combination with a hydrogel, gellan gum. In this study, gellan gum produced a fluid gel with flexible viscoelastic properties that liquefies during spraying and self-structures post-spraying, showing no run-off when applied to a tilted surface. For clinical applications, this has the advantage of keeping the cells attached to the wound bed. Cells demonstrated high compatibility to the gellan gel, and good viability a week after the spraying process, but they showed a limited proliferation capacity in this gel, as cellular metabolic activity remained stable during the 7 days tested, in comparison to cells in culture medium, whose metabolic activity increased over time [13]. In contrast, for the rest of these studies [118,121,125,141,144–146], sprayed cells were suspended in culture medium, Hank's Balanced Salt Solution or Ringer's lactate solution, which could run off the wound [2,143]. In order to keep the cells attached to the wound bed, wounds were covered with dressings, dry gauze and bandages after the spraying process.

3.2.2. Commercial cellular skin spray products

In 1993, Australian surgeon Dr. Fiona Wood developed an aerosol system to spray a layer of the patient's own skin stem cells onto the wounded area, called "spray-on skin", consisting in taking a small biopsy from the patient's undamaged skin, suspending the skin cells in solution, and then spraying said solution over the burn wound. This permitted the coverage of an area 80 times the size of the biopsy [147,148]. Based on this, Avita Medical marketed a single-use, step-by-step kit, dubbed ReCell, which contains an enzyme solution to loosen and isolate the skin cells from the biopsy. Lastly, cells are suspended in a sodium lactate solution in a 10 mL syringe, and can either be dripped onto the wound (if the volume in the syringe is less than 2 mL) or sprayed through a spray nozzle attached to the syringe (if the volume in the syringe is greater than or equal to 2 mL). The cell suspension can be sprayed directly to partial-thickness wounds or over meshed autografts for full-thickness wounds. The ReCell non-cultured regenerative epidermal suspension includes keratinocytes, fibroblasts and melanocytes, a combination that may promote a better and faster healing outcome than one cell type alone. The FDA approved ReCell as the first spray-on skin treatment for burns in 2018 [122,147,149–151]. ReCell has been widely used for burns, but also for the treatment of other skin diseases and disorders such as vitiligo [152], piebaldism, leukoderma, treatment of scars, etc. [147]. Some studies using ReCell are summarized in Table 4. They report minimal cell breakdown during the spraying process, faster wound healing due to the reduced time of immediate post-harvesting application of the non-cultured cells, and a small harvested area for donor cells, with the downside of additional costs for the ReCell kits and a longer preparation time [2,122,153].

Professor Joerg C. Gerlach and colleagues employed a different enzymatic isolation technique from Wood et al. and ReCell to obtain the patient's own regenerative skin cells from a small biopsy, and in 2008

Table 3
Studies using non-commercial cellular sprays.

Model	Application	Spray				Reference
		Device	Parameters	Composition		
				Cells	Hydrogel	
In vitro	Wound healing	Badger 100G airbrush	V: 1 mL P: 6, 8, 10, 14, 18 psi	Cultured bovine fibroblasts ($2 \cdot 10^5$/mL)	–	[121]
In vitro	Wound healing	Syringe with a spray nozzle	V: 36 mL A: 35 mm Ø Petri dish	Cultured human keratinocytes ($6 \cdot 10^4$/mL)	–	[125]
In vitro	Burns	Badger 360 Universal airbrush	V: 25, 50 μL D: 10–15 cm P: 15 psi	Cultured human dermal fibroblasts ($1–2 \cdot 10^6$/mL)	Gellan gum (0,9% gellan + 20 mM NaCl)	[13]
Pigs	Wound healing	Syringe with a spray nozzle	V: 0.5 mL/wound D: 2, 5, 10 cm A: 9 cm^2/wound	Autologous non-cultured pig keratinocytes ($2.8 \cdot 10^4/cm^2$)	–	[141]
Pigs	Hypopig-mentation	Syringe with a spray nozzle	V: 0.5 mL/wound A: 9 cm^2/wound	Autologous non-cultured pig epidermal basal cells (melanocytes) ($3.2 \cdot 10^5$/mL)	–	[144]
Patients	Burns	Syringe placed in a prototype cell spray device	V: 1, 2 mL P: 0.16 psi A: ~ 2% body surface area	Autologous cultured human keratinocytes ($3.9 \pm 4.8 \cdot 10^6$/wound)	–	[145]
Patients	Burns	Syringe placed in a pneumatic cell spray device	V: 2 mL x 1–5 syringes	Autologous non-cultured human keratinocytes (10^6/mL)	–	[118]
Patients	Burns	Syringe with a 30G needle	V: 2 mL x 3 syringes	Autologous non-cultured human keratinocytes (10^6/mL)	–	[146]

V: sprayed volume; D: distance from spray tip to receiving surface; P: spraying pressure; A: sprayed area.

Table 4
Studies using commercial cellular products.

Model	Application	Spray				Reference
		Device	Parameters	Composition		
				Cells	Hydrogel	
In vitro	Burns	Syringe with a spray nozzle	V: 5 mL	Non-cultured human keratinocytes, fibroblasts and melanocytes (ReCell) ($1.7 \cdot 10^6/cm^2$)	–	[122]
Patients	Burns	Syringe with a spray nozzle	V: 5 mL A: 80–320 cm^2	Autologous non-cultured human epidermal cells (ReCell)	–	[153]
Patients	Burns	Vivostat co-delivery system	-	Autologous non-cultured human keratinocytes (ReCell)	Fibrin (autologous, Vivostat)	[2]
Patients	Burns	TISSOMAT spray device	V: 6 mL A: 2985.6 cm^2	Autologous cultured human keratinocytes (Keraheal®) ($3 \cdot 10^7$/mL)	–	[22]
Patients	Burns	–	A: 4668 ± 2596 cm^2	Autologous cultured human keratinocytes (Keraheal®) ($3 \cdot 10^7$/mL)	Fibrin (Thrombin 250 IU/ml)	[119]
Patients	Burns	SkinGun	V: 10 mL D: 20 cm P: 2.2 mL/min liquid flow, 3185 mL/min air flow A: 2646, 1479.25, 5719, 2961, 1955, 1730 cm^2, respectively	Autologous non-cultured human keratinocytes (7559, 6353, 4162, 15198, 8695, 15607 cells/cm^2, respectively)	–	[12]

V: sprayed volume; D: distance from spray tip to receiving surface; P: spraying pressure; A: sprayed area.

they developed a skin gun to spray the cells onto the wound [12,146]. The process takes about an hour and a half, and the electronically controlled pneumatic device has a 10 mL syringe where the cell solution is contained, which is pushed out of a 30G needle (with a 2.2 mL/min liquid flow) and into an air stream (3185 mL/min airflow), gently forming liquid droplets without injuring the cells [12]. The SkinGun was acquired by RenovaCare in 2013, and the patient's own skin stem cells solution received the name of 'CellMist Solution', which is placed in the SkinGun to be sprayed onto the patient's wound [154]. According to Esteban-Vives and Gerlach (2016), only a maximum wound area of 320 cm^2 can be covered with the cells isolated using the ReCell kit, while their SkinGun's cell isolation method has no maximum wound coverage, and the patients treated with the SkinGun generally achieved complete re-epithelialization with good esthetic outcomes (Table 4) [12]. This device system requires further clinical evaluation and, at this time, it is an investigational system and is not available for general use or sale in the United States yet [154]. Although they are still currently seeking the FDA's approval, in August 2020 they did receive a conditional Investigational Device Exemption approval to start a clinical trial [155].

Both the ReCell syringe and the SkinGun only deliver the cells suspended in a lactate solution, which doesn't adhere to the wound by itself. Consequently, in order to avoid run-off, some studies have applied the cells harvested with the ReCell kit using a different spray device that allows them to co-deliver those cells with fibrin, like Johnstone et al. (2017), who used the Vivostat's Co-delivery System to co-deliver ReCell's cell suspension with Vivostat's fibrin to assist the anchoring of the viable keratinocytes onto the wound surface (Tables 2 and 4) [2]. Meanwhile, other cellular products, like the ones described next, include the use of fibrin to adhere the cells onto the wound.

KeraHeal® by Biosolution Co., Ltd. is a spray-type cultured epithelial autograft for burn wounds consisting of a suspension of autologous keratinocytes. A small biopsy is taken from the patient's undamaged skin and keratinocytes are isolated and cultured for their expansion for about two weeks. In order to prevent differentiation and to maintain a high take rate, once they reach 70–80% confluence, the pre-confluent keratinocytes are transferred into a vial at a cell density of $3{\cdot}10^7$ cells per 1 mL of Dulbecco's Modified Eagle's medium (DMEM) and are then sprayed over a wide meshed autograft that has just been applied to the wound bed. Right after the KeraHeal® suspension of cells has been sprayed, fibrin glue is often used to facilitate the attachment of epithelial cells to the wound [22,119,142,156]. KeraHeal® received product approval in 2006 by the Republic of Korea's Ministry of Food and Drug Safety [157]. Table 4 shows two studies reporting positive results when using KeraHeal® to treat burn patients.

The downside of these autologous cell-spray methods is that the need for taking a biopsy, even if it is small, creates another wound and can produce more pain for the patient when they are already in such a delicate state. If the wounded area covers almost the entire body, it may even be difficult to find a suitable area for taking that small biopsy. These drawbacks can be avoided with the use of allogeneic cells, as long as they do not entail a risk of immune reaction and rejection.

Biosolution Co., Ltd. also offers KeraHeal®-Allo, based on allogeneic keratinocytes contained in syringes at a density of $2{\cdot}10^7$ cells per 1.5 mL of Poloxamer 407 and DMEM [158].

Although not commercialized, it is worth mentioning HP802-247 by Healthpoint Biotherapeutics, which was an investigational allogeneic spray-applied cell therapy using fibrin, used mostly to treat venous leg ulcers, and based on cryopreserved, growth-arrested fibroblasts and keratinocytes derived from neonatal foreskin. The fibrinogen solution was sprayed on the wound bed first, followed by the thrombin solution where the previously thawed cells were suspended. Allogeneic cells do not engraft, but according to *in vitro* studies, it was believed that HP802-247 released several cytokines and growth factors into the wound's micro-environment, which were expected to interact with the patient's own cells to encourage wound healing [26,159,160]. However, this product failed to demonstrate efficacy during phase 3 clinical trials. The main reason behind its failure is thought to be the batch to batch variability caused by changes in the phenotype of the cells, particularly for the keratinocytes, as a consequence of the age of the cell banks [161].

Therefore, there is a need for the development of new allogeneic products and therapies, which should carry out a thorough characterization and quality control of the cells before their application to patients.

4. Regulatory approaches for the clinical translation of spray products

The design, development and subsequent commercialization of a skin spray must be carried out in accordance with the legal and technical aspects defined by specific regulatory bodies in each jurisdiction. Based on whether the skin spray is made up of cells or not, its regulatory pathway to be marketed will be different. The acellular skin sprays are

classified as medical devices [162,163]; and the cellular skin sprays, as advanced therapy medicinal products (ATMPs) [164]. Thus, the appropriate categorization of skin sprays is critical since it will define the specific requirements to be met and, therefore, the applicable rules governing their commercialization.

Acellular and cellular skin sprays must be authorized by competent authorities before appearing on the market. Their approval is based on the scientific evidence evaluation of the skin spray and on the quality data of manufacturing [165,166]. Both types of skin sprays must be manufactured under Good Manufacturing Practice (GMP) guidelines [166,167]. The data that should be provided in the authorization request for each type of skin spray will depend on its classification and its jurisdiction [164]. Thus, the regulatory framework of two of the main jurisdictions is described below: European Union (EU) and USA.

4.1. Regulatory pathway of acellular skin sprays

Regarding the legal requirements, an acellular skin spray for skin regeneration is considered as a medical device, because its activity should not be caused by chemical, pharmacological, immunological, or metabolic processes, or it would otherwise be considered as medicine [168].

Medical devices are classified according to their risk to the patient, based on their intended use, indications for use, the level of surgical invasion or body contact, structural characteristics, duration and activity [162]. Based on this classification, acellular cell sprays are considered as medical devices with the moderate risk being defined as class IIa (non-invasive) and IIb (invasive) devices under the European Medicines Agency (EMA) regulation in the EU [169] or class II (subject to general and special controls) under the FDA regulation in USA [170,171]. Nevertheless, if the medical device acts as a wound dressing, it must be classified as a class III due to the high risk of the device. In both jurisdictions it is necessary for the safety and efficacy data of an acellular skin spray to be submitted and evaluated by the health agency or a specific organization authorized for this purpose. The submission should include pre-clinical data based on *in vitro* and *in vivo* studies which determine the biocompatibility, local and systemic toxicology, activity, shelf life, microbiology, etc. of the medical devices [163,165]. These studies should be performed under Good Laboratory Practice (GLP). When the use of an acellular skin spray involves a high risk of illness or injury, clinical data should also be provided [172].

In the EU, for acellular skin sprays to be put into market as medical devices, they must fulfil the safety and effectiveness requirements, which are evaluated in a conformity assessment procedure by a notified body (NB). The NB evaluates the submitted technical documentation (scientific data and benefit-risk analysis) and audits the manufacturer's Quality Management System (QMS) [172]. This inspection is not required if the manufacturer has an International Organization for Standardization (ISO) 13485 certification [173]. Once the acellular skin spray is approved by the NB, it issues a declaration of conformity for manufacturers, allowing them to affix a CE marking to the medical devices, according to Regulation (EU) 2017/745 [174]. Acellular skin sprays that bear a CE mark can be then marketed in the EU and European Economic Area member states.

Access to the acellular skin sprays market in USA is regulated by the FDA, which requires that acellular skin sprays classified as Class II medical devices acquire clearance through the Premarket Notification 510(k) [175,176]. The application (administrative and scientific data) is reviewed by Accredited Persons and then, after the FDA receives this recommendation, the FDA must issue a final verdict. This pathway often does not require the provision of clinical data [163]. For acellular skin sprays considered as class III medical devices, a Premarket Approval (PMA) application is required, which is evaluated by FDA [177]. A PMA application must provide administrative, non-clinical laboratory studies and clinical data, in addition to a report that the manufacturer's quality control system has been audited. If the scientific evidence about safe and effective data are suitable enough, a marketing approval will be issued by the Center for Devices and Radiological Health of the FDA [171,178,179].

4.2. Regulatory pathway of cellular skin sprays

Regarding cellular skin sprays, they are considered medicinal products and represent an innovative strategy to repair, replace, restore, or regenerate wounds and skin injuries through their pharmacological, immunological, or metabolic activity [180]. The main characteristics of these types of products are that they must be made up of living cells (autologous, allogeneic or xenogeneic), and in addition, they might incorporate other components such as biomaterials, signaling molecules, non-viable cell derivatives, etc. [164,181].

Cellular skin sprays are subjected to different classifications, which are associated with the specific legislation where the medicinal product will be authorized. Thus, in the EU, cellular skin sprays are classified as tissue engineering products or combined advanced therapy medicinal products (incorporating a medical device), and therefore, they are considered ATMPs [182]. In USA, they are defined as human cells, tissues, and cellular and tissue-based products (HCT/Ps) [183]. The access to the market for the cellular skin sprays in both jurisdictions requires a prior marketing authorization (MA) by EMA and FDA, respectively [164,184]. The MA is based on the evaluation of the efficacy, safety, and quality data of the cellular skin spray. The request must include pre-clinical and clinical data similar to that described in the case of acellular skin sprays. In addition, data related to cell biological characteristics (viability, proliferation, migration, differentiation, identity, purity, potency, proliferative capacity, genomic stability, tumorigenicity, efficacy, and dosage) should be provided [166,185]. All this information must be compiled in a Common Technical Document (CTD).

In the EU, the procedure to request a MA for cellular skin sprays must be conducted under Regulation (EC) No. 1394/2007. This pathway involves the submission of a CTD, which is reviewed by the Committee for Advanced Therapies (CAT). Then, the CAT issues a draft opinion with its recommendation about the MA approval to the Committee for Medicinal Product for Human Use (CHMP). The CHMP is responsible for granting the final MA approval [186]. In some cases, the CHMP can issue a conditional MA and request additional post-authorization studies [164]. According to the USA regulatory framework, there are different regulatory pathways for the commercialization of cellular skin sprays, based on the risk of the product. If the risk is low or middle, they are classified as 361 HCT/Ps and marketed under Section 361 of the Public Health Service Act (PHSA) and under 21 Code of Federal Regulations (CFR) 1271 [183,187]. This pathway does not require any premarket approval but the administrative information, preclinical and clinical data should be provided [188]. However, if the risk is high, the cellular skin spray is classified as 351 HCT/Ps and regulated under Section 351 of the PHSA, and 21 CFR 1271, hence requiring a premarket review named Biologics License Application (BLA) [183,187–189]. In this regulatory pathway, data on the efficacy, safety, and quality of the cellular skin spray together with the administrative data of the sponsor must be submitted through a CTD to the Office of Tissues and Advanced Therapies (OTAT). When the evaluation is positive, the OTAT issues an approval for the marketing of the cellular skin spray [164].

5. Clinical trials for skin sprays

To provide insight into the clinical advances in this field, www.clinicaltrials.gov was searched up to May 2021 for the use of skin sprays to treat skin-related conditions. Search terms used were 'skin AND spray', 'skin aerosol', 'skin aerograph' and 'skin airbrush', as well as the name of some products known to be applied on the skin as a spray, such as 'ReCell', 'KeraHeal', 'skin Tisseel' (which also searched for 'fibrin tissue adhesive', 'fibrin sealant', and 'fibrin glue'), 'Vivostat', 'SkinGun', and 'HP802-247'. In all searches, recruitment status was

filtered to exclude 'Unknown status', 'Terminated' or 'Withdrawn' (with the exception of one trial that was included in which the recruitment had been terminated based on the outcome of another similar trial, but it has a completed follow-up study), but no filter was applied for the date of the trials.

Some of these products (e.g. TISSEEL Fibrin Sealant) can be applied topically in different ways that are not sprays, such as liquid form. Therefore, trials that used products that are not marketed specifically as a spray or that did not specify that the product is applied *via* spray, were excluded.

All of the trials taken into account for different skin diseases and conditions (including burns, chronic wounds and ulcers, psoriasis, dermatitis, etc.) are compiled in Supplementary Table 1 (Clinical trials using acellular skin sprays) and Supplementary Table 2 (Clinical trials using cellular skin sprays). The results also displayed 23 trials (not included in Supplementary Tables 1 and 2) related to anesthetic or cooling sprays (many of them being ethyl chloride anesthetic sprays), 6 related to cosmetics or sunscreen sprays, and 29 related to a variety of other conditions not focused on wound healing and regeneration, and therefore, not relevant for this review.

We found a total of 104 clinical trials for skin sprays, 9 of which were observational studies and the rest were interventional studies. The majority of the interventional trials were in Phase 2 (23 trials) or Phase 3 (22 trials), followed by 20 trials in Phase 4. Meanwhile, only 7 trials were in Phase 1, and a few others were in between Phases 1 and 2, or in between Phases 2 and 3. Moreover, 16 trials appeared as "Not Applicable" (N/A), and therefore, without FDA-defined phases (Fig. 3).

As can be seen in Fig. 4, psoriasis is the skin condition with the most clinical trials related to skin sprays, with 45 trials found. It is followed by 16 trials for chronic wounds (7 of which were for venous leg ulcers, 7 for diabetic foot ulcers, 1 for pressure ulcers, and 1 for all of those: venous, diabetic foot, and pressure ulcers). Afterwards, there are 9 trials for burns, and 9 clinical trials for dermatitis and eczema, including atopic dermatitis, radiation-induced dermatitis, and one trial for dermatitis caused by diapers in babies. The 8 trials included as wound healing are for a variety of conditions or surgical procedures where the main goal of the trial is to heal the wound. There are 7 trials for alopecia, in which the spray is applied to the patients' scalp. 3 trials are directed towards acne vulgaris and another 3 towards vitiligo and piebaldism. Finally, there are 5 other trials for different conditions: rosacea, erythema, keratosis pilaris, and 2 for tinea pedis.

In regards to the different types of spray applied to treat these skin conditions, they can be differentiated into acellular (Supplementary Table 1) and cellular (Supplementary Table 2) spray treatments, with the latter being the significantly larger group (Fig. 5a). The cellular sprays for these clinical trials contain epidermal cells, generally keratinocytes and fibroblasts, with most of them being autologous cells harvested from the patients themselves; except for those trials evaluating the product HP802-247, which, as stated previously, contains allogeneic human fibroblasts and keratinocytes in combination with fibrin (Fig. 5b). In contrast, the majority of the acellular skin sprays in clinical studies are antiseptic solutions or anti-inflammatory drugs, like corticosteroids, most times not clearly specified. However, some of the acellular sprays in these trials consist of polymeric biomaterials like fibrin, chitosan, or dextran, and a few others apply autologous platelet rich plasma (PRP) (Fig. 6). Only two of the observational studies did not apply any treatment to the patients, because they were follow-up studies to other interventional trials.

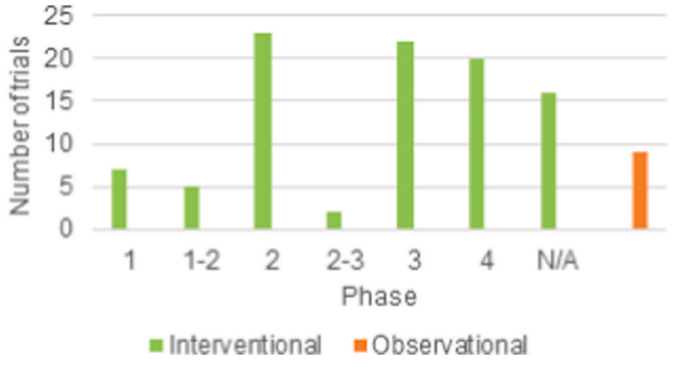

Fig. 3. Number of clinical trials per phase. There was a total of 95 interventional trials in different phases (7 trials in Phase 1, 5 trials in Phase 1–2, 23 trials in Phase 2, 2 trials in Phase 2–3, 22 trials in Phase 3, 20 trials in Phase 4, and 16 trials with Not Applicable phase), and 9 observational trials.

As can be seen in Supplementary Tables 1 and 2, ReCell is the most used spray product for burn treatment, with 7 out of the 9 trials occurring for burns. Additionally, ReCell was also used in one trial for wound healing of acute skin defects and another one for venous leg ulcers, and in 3 trials for vitiligo and piebaldism. Avita Medical, the company that marketed the ReCell kit, either sponsored or acted as a collaborator in all of these trials except for one. One of the trials for ReCell included in Supplementary Table 2 is registered on the Australian New Zealand Clinical Trials Registry (ANZCTR) instead of ClinicalTrials.gov.

Baxter Healthcare Corporation sponsored 3 clinical trials for their fibrin sealant ARTISS to assess its efficacy and evaluate skin graft adherence and wound healing when used in spray form. One of the trials dealt with burns and the other two with wound healing in patients undergoing surgical procedures like facelifts or abdominoplasty.

Regarding chronic wounds, 7 trials were found for venous leg ulcers, all of which used HP802-247 except one that used ReCell, and 9 for diabetic foot ulcers and pressure ulcers, which used a variety of spray treatments. Those 6 trials for venous leg ulcers with HP802-247 were sponsored by Healthpoint Biotherapeutics, and two of them (NCT01853384 and NCT01656889) had the same objective, so the recruitment for NCT01853384 was terminated based on the outcome of trial NCT01656889. There were also two observational safety follow-up studies: NCT01853384 was followed by NCT01970657, and NCT00852995 was followed by NCT00900029.

The results show that the most prescribed topical treatment for psoriasis are corticosteroids. Out of the 45 trials for psoriasis, 11 of them used Clobex® Spray by Galderma Laboratories, which is a potent topical corticosteroid (clobetasol propionate 0.05%) formulation prescribed for the treatment of moderate to severe plaque psoriasis; Galderma sponsored or collaborated with 9 of the trials with Clobex®. Promius Pharma sponsored 3 trials for a corticosteroid called DFD01 Spray (betamethasone dipropionate 0.05%). 15 trials, all sponsored by LEO Pharma except one, used Enstilar (LEO 90100) Aerosol Foam, which is a calcipotriol/betamethasone dipropionate cutaneous spray-on foam, also used for the topical treatment of plaque psoriasis [190]. Taro Pharmaceuticals sponsored 7 of the 8 trials using Desoximetasone (Topicort) 0.25% topical spray, another corticosteroid.

AOBiome sponsored 5 trials for their patented product B244, 2 of them for atopic dermatitis or eczema, 2 for acne, and 1 for rosacea. B244 contains *Nitrosomonas eutropha D23*, a strain of beneficial ammonia-oxidizing bacteria, intended to repopulate the skin microbiome with these bacteria typically found on the body that are washed away by soap. Once it is applied topically, B244 converts ammonia to nitrite, which has antibacterial properties, and to nitric oxide, which mediates inflammation and vasodilation [191].

ST266 by Noveome Biotherapeutics is an investigational multi-targeted, non-cellular biologic platform, produced by collecting the secretome from a novel population of cells generated by a proprietary method of culturing amnion-derived epithelial cells from donated full-term placentas, normally discarded after birth. Those cells produce hundreds of biologically active proteins and other factors, found in amniotic fluid, that are crucial to neuroprotection, the modulation of inflammation, cell recovery, and healing. These various proteins and factors are delivered in physiologic concentrations that have been

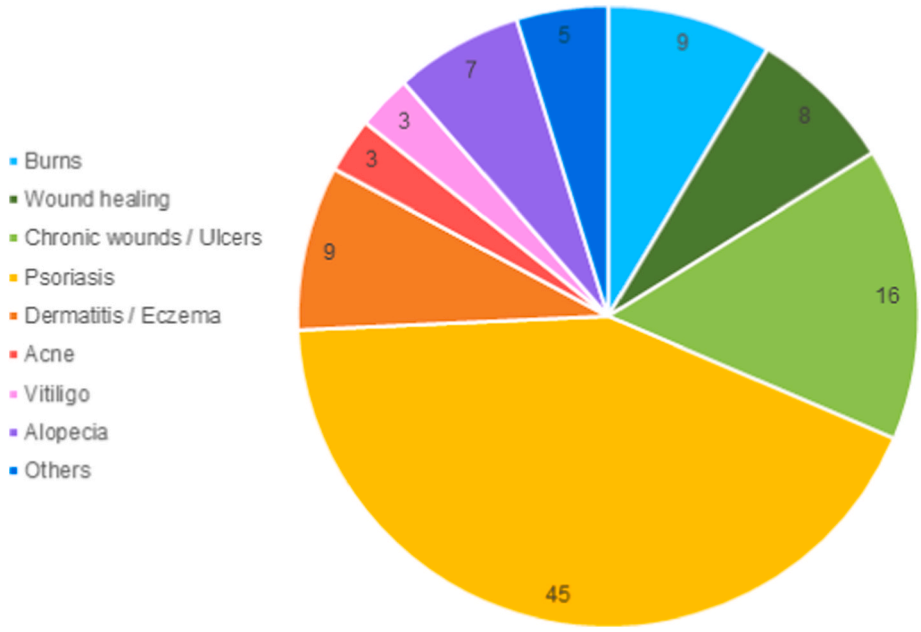

Fig. 4. Number of clinical trials per condition. The graph shows the number of trials found for each skin condition or disease, with psoriasis being the most numerous, with 45 trials. It is followed by ulcers and chronic wounds (16 trials), burns (9 trials), dermatitis and eczema (9 trials), wound healing applications (8 trials), alopecia (7 trials), acne (3 trials), vitiligo (3 trials) and other conditions (5 trials).

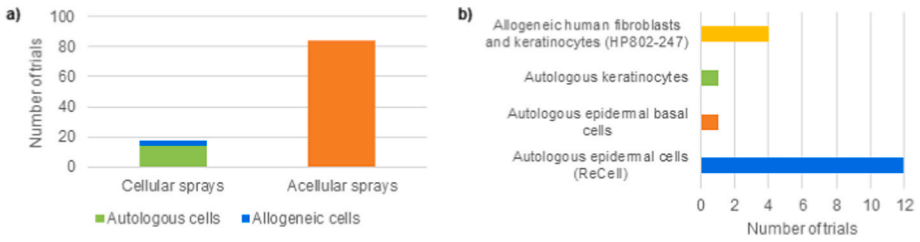

Fig. 5. Types of skin sprays used in clinical trials: (**a**) Number of clinical trials depending on the types of treatment: 84 clinical trials with acellular sprays and 18 trials with cellular sprays. Among the cellular group, 14 used autologous cells and 4 used allogeneic cells; (**b**) Number of clinical trials that use each cell type: 12 trials using autologous epidermal cells from ReCell, 1 trial using autologous epidermal basal cells, 1 trial using autologous keratinocytes, and 4 trials using allogeneic human fibroblasts and keratinocytes from the product HP802-247.

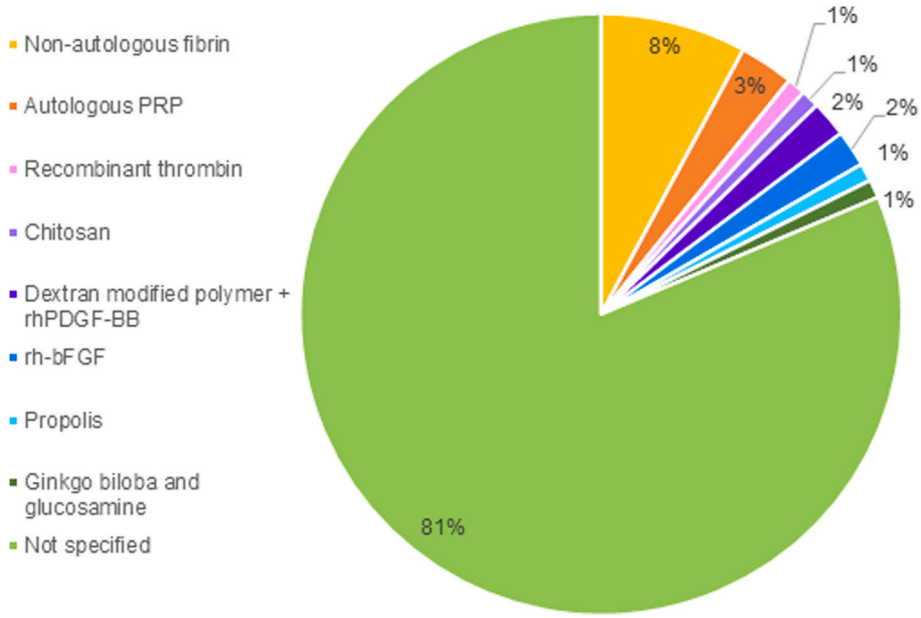

Fig. 6. Percentage of clinical trials depending on the material used for skin sprays. 4 out of the 8 trials using fibrin were in combination with cells (from the product HP802-247), and 1 out of the 3 trials using PRP was in combination with keratinocytes. (PRP: platelet rich plasma; rhPDGF-BB: B isoform dimer of recombinant human Platelet-Derived Growth Factor; rh-bFGF: recombinant human basic Fibroblast Growth Factor).

shown, in preclinical and clinical studies to date, to be well-tolerated with demonstrated biologic activity [192]. Noveome sponsored 2 trials where ST266 was sprayed topically: one for UV light burn wounds, and another for radiation-induced dermatitis in patients undergoing treatments for breast cancer.

For alopecia, Legacy Healthcare sponsored or collaborated with 3 trials for their product CG428, which is a novel patented botanical lotion made of a citrus, cocoa, guarana, and onion blend, aimed to improve hair recovery after loss due to chemotherapy [193].

6. Conclusions and future trends in spray products

Skin regeneration, especially in deep wounds, remains a challenge for many healthcare professionals. There are numerous and diverse strategies, but none of them provide a perfect solution. The use of sprays as a delivery system for hydrogels and cells allows the treatment of large wounded areas with much more ease and reduced time than other methods like traditional skin sheet grafts or bioengineered skin substitutes. There are some spray products for skin on the market, but cell spray grafting is mostly still under clinical evaluation.

Among the investigational and commercially available skin spray products, fibrin is the most widely used material, sometimes as a hydrogel alone, and other times as a carrier for cells. In general, cellular sprays for skin regeneration apply epidermal cells like keratinocytes and fibroblasts, but the incorporation of stem cells or growth factors could provide better stimulation for the healing of the skin. In the same way, the enrichment of fibrin hydrogels with other molecules, or the combined use of other materials present in the natural ECM (including components such as HA or collagen; or decellularized tissues, such as decellularized dermis) would most likely enhance the proliferation of cells at the wound bed matrix. Moreover, when formulating hydrogels to be used in skin sprays, it is necessary to carry out rheological studies to optimize the mechanical properties of the hydrogels. Viscosity is an important parameter to take into account, as hydrogels need to be able to pass through the spray nozzle without clogging, and then remain on the wound bed without running off. Furthermore, in the case of cellular sprays, hydrogels viscosity can affect cell viability. Since cells can be damaged when passing through the nozzle, an ideal hydrogel should have shear-thinning properties, having a high enough viscosity to maintain good mechanical properties, but reducing that viscosity when passing through the nozzle, and therefore reducing cellular damage. The viscoelastic properties are also important, as the materials sprayed on the skin need to be elastic and flexible to endure the body's movements. These parameters like viscosity and viscoelasticity can be adjusted by the combination of different materials or biopolymers at different concentrations, and thus the formulation of hydrogels using diverse materials that mimic the natural ECM of the skin and have satisfactory mechanical properties is an important factor for skin sprays.

When it comes to the translation of skin sprays to clinical practice, there are very few clinical trials studying cellular regenerative spray products, but many are acellular spray products using drugs like corticosteroids to relieve the irritation caused by some conditions like psoriasis. Therefore, it is important to develop more regenerative spray products for skin containing hydrogels and cells, and then translate them to clinical practice, which could benefit thousands of patients around the world suffering from burns, chronic wounds, and other skin conditions.

Finally, improved spraying devices should be developed. To date, only a small number of devices have been approved for commercialization and clinical use. Many preclinical studies utilize simple syringes with spray pumps, and they do not give much focus to the spraying parameters such as the pressure, distance, angle or volume, and therefore, these studies may hardly be reproducible, as these parameters can affect results significantly. New spraying devices should be able to regulate these parameters, especially the pressure (for example by adding a manometer or pressure gauge to the device) and volume. It is essential that new studies optimize these parameters for the device and the material used. Currently, most devices are designed to spray only one material at a time, with the exception of Baxter's double syringes for TISSEEL and ARTISS or the Vivostat's Co-Delivery system, but these are intended for the use of their own fibrin products. In case of wanting to deliver other types of hydrogels, it would be interesting to look at new devices that permit the spray delivery of two or more different materials or gels, simultaneously or subsequently. For example, in the case of hydrogels needing a gelling agent, it would be desirable if both materials could be sprayed simultaneously, so as to gel right as the material is applied on the wound (i.e. this is the case of the TISSEEL and ARTISS double syringes, spraying fibrinogen and thrombin to form fibrin, but it could also be used to spray other materials such as alginate and calcium). The use of diverse materials, such as different composite hydrogels sprayed in subsequent layers, could also be useful to simulate the different layers of the skin, and could consequently, greatly improve the healing of deep wounds.

Funding

This research was funded by Fundación Mutua Madrileña (FMM-AP17196-2019), the Consejería de Economía, Conocimiento, Empresas y Universidad de la Junta de Andalucía (ERDF funds, projects B-CTS-230-UGR18, PY18-2470, A-CTS-180-UGR20, SOMM17-6109, PYC20 RE 015 UGR and P18-FR-2465), and by the Ministry of Economy and Competitiveness, Instituto de Salud Carlos III, ERDF funds (DTS21/00098 and DTS19/00145).

Authors' contributions

Paula Pleguezuelos-Beltrán: Conceptualization, Investigation, Writing – Original Draft and Editing, Visualization; Patricia Gálvez-Martín: Writing – Original Draft, Review and Editing; Daniel Nieto-García: Writing – Review and Editing; Juan Antonio Marchal: Writing – Review and Editing, Supervision; Elena López-Ruiz: Conceptualization, Writing – Review and Editing, Supervision. All authors have read and approved the final manuscript.

Declaration of competing interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2022.02.023.

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