ISSN 1993-6842 (on-line); ISSN 0233-7657 (print)
Biopolymers and Cell. 2018. Vol. 34. N 3. P 171–195
doi: http://dx.doi.org/10.7124/bc.000979

UDC 612.014.3/616-001.4-002:57.089.67/611.018.013

Regenerative medicine advanced therapy for the non-healing cutaneous wound

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Regenerative medicine therapy is inspired by current research advances in cellular biology, genetic engineering, synthetic biology, material sciences and so far contributes to the traditional therapy, making resistant diseases curable. Nowadays, chronic wound healing is possible due to cell-based regenerative technologies and recent non-cell therapeutic approaches. Here we review clinical applications of human stem cells, as well as cellular and tissue products as alternatives to the traditional therapy of non-healing wounds. The cell-based technologies for tissue regeneration and bioengineering utilize stem cells that are either injected into bloodstream or positioned directly into the target area. Cell-free regeneration technologies require either stem cell products, *i.e.*, secretomes or their separate components, extracellular membrane vesicles, or tissue products. The stem cell therapies are designed to replace critically absent components of wounded or degenerative tissue. The stem cell secretome can promote the repair of damaged tissues independently of parent cells. Extracellular membrane vesicles mimic and recapitulate the mechanisms of stem cells in tissue regeneration and therefore might be promising for chronic wound and severe burns healing. The tissue products traditionally remain efficient wound healing remedies along with emerging advanced technologies.

Keywords: wound healing, cell and cell-free technologies, stem cells, secretomes, extracellular membrane vesicles, tissue products

Introduction

The skin is a protective barrier of the body systems against harmful environmental factors, and its damage can result in either temporary or full disability, sometimes followed by lethal outcome. The process of cutaneous wound healing represents a cascade of overlapping molecular and cellular events that are well-regulated by a complex highly interconnected and multifaceted system. The impairment or loss of regeneration system components leads

to delay in tissue regeneration. Chronic wounds have lower levels of growth factors and chemokine production, a slower vessel formation, fibroblast proliferation, the interrupted inflammation in a tissue reparation process [1]. If traditional conservative and surgical therapies do not aid at curing chronic wounds, the latter accepted as non-healing. The non-healing wounds are often either in the state of cellular aging or lack of resident Stem Cells (SC), or other cells needed for a healing progress [2, 3]. The tissue regeneration efficiency is complicated by various factors, such as aging, stress, diabetes, obesity, alcohol consumption *etc.* The impaired skin healing represents a burden for both patients and the healthcare system and creates a social-economic problem. In the developed countries, 1.0–2.5 % of population suffer from incurable wounds [4]. Chronic wounds, especially venous and arterial ulcers, are called a 'silent epidemy'. In recent years, tremendous progress has been made in delineating the factors and mechanisms involved in a tissue repair, and this academic knowledge has provided a basement for advanced wound healing. The contemporary cell and cell-free tissue regeneration technologies are in use to overcome the limitations of traditional medicine (Fig. 1).

The cell-based regeneration therapy involves two fundamental components: stem cells and bioengineered scaffolds. Stem cells are the significant players in the formation of the *de novo* tissue, whereas scaffolds serve to mimic extracellular matrix and support SCs [5]. Another approach is based on the cell-free regeneration technologies, involving the use of products generated by SCs — full secretomes — [6] or their separate components, extracellular membrane vesicles (EMVs) [7], as well as EMVs of other cell types [8]. Gene therapy also belongs to the regenerative advanced therapy, however, it is not in the focus of this review. The tissue products, such as extracts remain efficient wound healing remedies along with advanced technologies.

Cell therapy for the treatment of chronic wounds

In this review, we are paying attention to a vital role of SCs in the maintaining of adult tissues and injury repair, not discussing other roles. Their functions are not restricted to the tissue regeneration, and SCs are known to take part in the homeostasis of damaged tissues, *e.g.,* by inhibiting the formation of reactive oxygen forms or modulating the immune response [9, 10]. The cell regeneration technologies are based, first of all, on the capacity of undifferentiated SCs to differentiate into diverse lin-

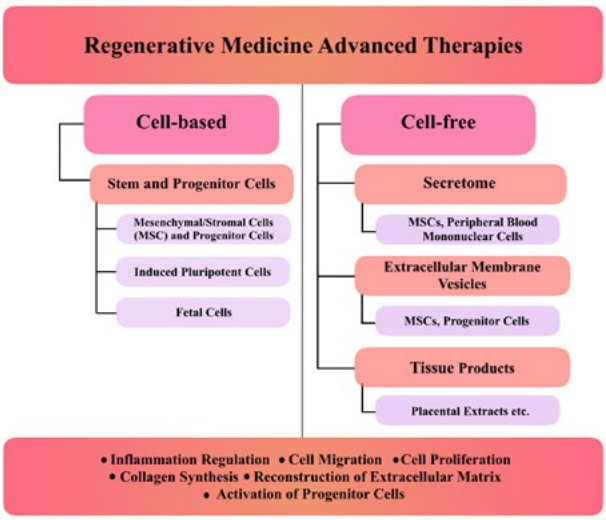

Fig. 1. Cell and noncellular regeneration technologies in the treatment of chronic wounds.

eages of specialized cells. Different SCs represent valuable reserves of an organism that can be employed, when needed, to develop into specialized cells, to restore healthy cells or to replace damaged or aging ones.

Stem cells have been classified by their capability to differentiate into various types of cells (by potency) and by their origin [11]. *Totipotent* cells (first cells from a zygote division) differentiate into all possible types of cells and organs. *Pluripotent* cells also have unlimited capacity to differentiate and give rise to almost all cell lineages derived from mesoderm, endoderm and ectoderm (germ layers). *Multipotent* cells are capable of differentiation into several types of closely related cells such as hematopoietic SCs that can become red and white blood cells or platelets. Another example is Mesenchymal/stromal Stem Cells (MSCs) from the bone marrow stroma and other tissues, which can differentiate into various cell lineages, including classical mesodermal ones — bone, cartilage and fat. *Olygopotent* cells differentiate into a limited variety of cell lineages, *e.g.*, into lymphoid or myeloid stem cells. *Unipotent* cells are able to regenerate only the cells of their own type.

There are four main types of stem cells classified by origin: embryonic stem cells (ESCs), adult organism SCs, fetal SCs (from the fetal part of a placental complex — chorion, amnion, placental villi — and the umbilical cord) and induced pluripotent stem cells (iPSCs). ESCs originate from the blastocyst, an early stage of embryonic development, and have the highest potential for differentiation. However, there are ethical issues associated with the production of ESCs that restrict their use. The adult SCs (also referred to as somatic or tissue specific stem cells) are more specialized than embryonic stem cells and serve in the body for the regeneration of aging or injured tissue. The progress in SCs research led to the emerge of a new SCs type, called iPSCs, when the adult stem or progenitor cells were reprogrammed to an embryonic stem cell-like state by introducing genes responsible for growth factors important for maintaining the essential properties of ESCs [12]. These reprogrammed adult cells create a new powerful way to "de-differentiate" cells and use iPSCs in regenerative medicine, as well as a new tool for drug development and disease modelling. Fetal stem cells have the advantage over adult SCs due to a younger age and therefore fewer acquired mutations. Phenotypically, placental MSCs are similar to MSCs of the bone marrow and the fat tissue, however, this cell lineage possesses a higher capacity to differentiate into other cell lineages — myogenic, angiogenic, pancreatic, cardiogenic, neurogenic *etc.* as compared to adult SCs [13].

Progenitors of the specialized cells are early descendants of SCs that can differentiate, generally giving rise to a single cell lineage, but they cannot divide and proliferate unlimitedly, *i.e.,* they are more limited than the SCs in terms of division and differentiation. Progenitors act as the element of a tissue reparation system, and these cells are used by an adult organism to replenish specialized cells in blood, skin and intestine tissues. Most progenitor cells remain quiescent and can be activated, if the tissue is either damaged or aging [14].

The undifferentiated cells were found in the nineteenth century, however, their existence was proved not long ago, in the 1960s. During

the last two decades, exogenous SCs were administered in regenerative medicine to treat degenerative tissues and traumas with limited regenerative capacities. Therapeutic potential of SCs has three main mechanisms of action: (a) self-guidance, or the ability to migrate into the damaged area, following chemical gradients; (b) differentiation into various lineages of cells, which leads to a renewed functionality of damaged tissues; (c) a secretion of bioactive factors that can potentially impact both local and systemic physiological processes [11] (Fig. 2).

MSCs are some of the usable cells in regenerative medicine and tissue bioengineering due to their advantages, such as immunocompatibility, proliferation and multipotency. Initially, in regenerative medicine, the adapted hematopoietic MSCs were used for the replacing of lost cells occurred during specific diseases or traumatic events. At the end of the last century, adult SCs were considered to be more promising because they have much wider developmental potential *in situ* and can be relatively easy and abundantly obtained from different tissues of humans or animals, such as the bone marrow or the fat tissue. Presently, the most common source of MSCs is the bone marrow, however, harvesting and processing of the bone marrow MSCs have major drawbacks and limitations [15]. The fat tissue-derived mesenchymal stem cells (adipose MSCs) are multipotent stem cells that can differentiate into various cell types and, most importantly, can be harvested from own fat tissue abundantly. Adipose MSCs play a key role in reconstructive or tissue engineering medicine [16]. Currently, the iPSCs offer a promising strategy to generate patient-specific SCs lines [17]. Substantially, the iPS cell technology is a hallmark in regenerative medicine and revolutionizes the medical field. However, the technical challenges must be overcome before this strategy can be deployed in translational regenerative medicine; there are also open questions remaining, *e.g.*, regarding the potential tumorigenicity of the iPSC-derived cells or the impact of epigenetic background.

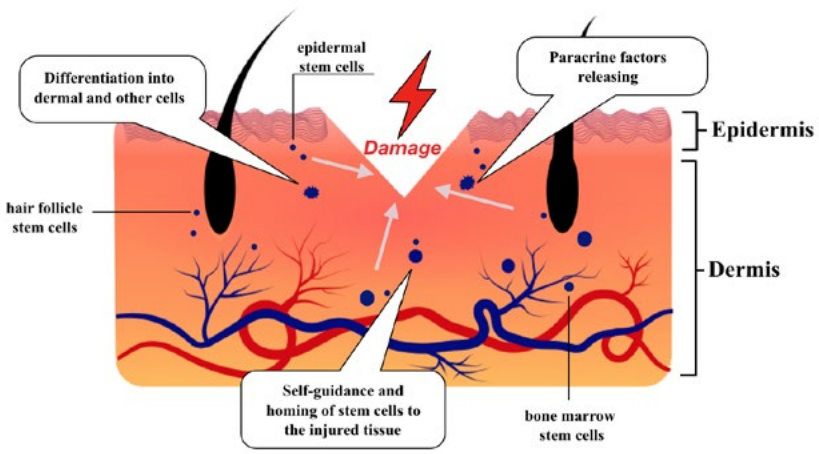

Fig. 2. Bioeffects of stem and progenitor cells in wound healing. Epidermal, dermal cells and cells of hair follicles, as well as circulating in the bloodstream bone marrow mesenchymal stem cells, migrate to the damaged skin and take part in its regeneration [21]. Stem cell therapeutic efficacy in cutaneous wound healing is associated with the ability to migrate into the damaged area, following chemical gradients (a); differentiation into various lineages of cells, which leads to a renewed functionality of damaged tissues (b); a secretion of paracrine factors (c) [11].

The emerging regenerative therapies, based on fetal SCs, show their high potential for clinical applications [18].

For the cell and tissue regeneration, undifferentiated SCs are collected from the patient's body or from donor material. Cell therapy can be autologous, when own cells are used, or donor SCs are used for the allogeneic transplantations. Although MSCs are present in multiple tissues, their overall quantity in the body is scarce. Therefore, cell expansion *in vitro* is needed before administration. According to the criteria of the International Cell Therapy Society, SCs are considered usable for regenerative medicine, if (a) produce enough cells (lg6-lg9 CFU/mL) *in vitro*; (b) differentiate into several lineages of cells; (c) can be taken out by minimally invasive procedures; (d) must express Clusters of Differentiation (CD) 73, 90 and 105, and (e) attach to plastic surfaces during the culturing process (see more in [19]). Importantly, MSCs can be obtained from the patient through a rather cheap procedure (from the fat tissue) and even a non-invasive one (from urine). The stem cells treatment can be based on both their intravenous injection similarly to drugs and the local use in the target tissue. The cell therapy is successful in the treatment of many diseases, as well as in healing chronic wound of various etiologies.

Stem cells in the treatment of cutaneous wounds

Various methods and drugs for the treatment of wounds are strictly oriented on the stage of the wound process, which does not depend on the mechanism of the wound formation and occurs according to a universal scheme, unifying 4 overlapping phases: hemostasis, inflammation, proliferation and remodelling (well reviewed in [20]). For a better understanding of the SCs role (and their secreted components, as well) in wound healing, here we represent a short description of successive events in tissue regeneration under this process (Fig. 3). The *hemostasis phase* proceeds due to fibrin and platelets, which trigger a cascade of blood coagulation in normal lesion healing. The formation of the platelet plug and fibrin matrix leads to the release of cytokines and growth factors, which elicit an inflammatory reaction that is necessary to progress the tissue repair. Fibrin derived from thrombocytic fibrinogen acts as a matrix for macrophages and fibroblasts that appear in the lesion site. The *inflammation phase* begins once the neutrophils are attached to the endothelium within a few minutes of the injury. Neutrophils use elastase and collagenase to facilitate migration in the extracellular space, where they capture bacteria, degrade the matrix proteins and attract additional neutrophils and macrophages. Macrophages are perhaps the most important of inflammatory cells in acute healing as they destroy pathogenic microorganisms, degrade damaged tissue in the wound and stimulate the formation of granulation tissue and blood vessels via growth factors such as platelet derived growth factor, transforming growth factor beta (TGF-β), fibroblast growth factor and proinflammatory cytokines: interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-a). Therefore, a deficiency of neutrophils and macrophage cells is responsible for the inhibition of the inflammatory response phase and the healing process stagnation. Also, the excessive release of IL-6 and

IL-1β from neutrophils and macrophages leads to the formation of chronic wounds. Lymphocytes are the last to fall into the wound and play an important role in the production of interleukin-2, which in turn plays a role in attracting fibroblasts. The participation of fibroblasts begins with the production of matrix metalloproteinases that facilitate the movement of fibroblasts in the matrix. However, in chronic wounds, elevated metalloproteinases have been shown to degrade the extracellular matrix (ECM). Chronic wounds often remain in a prolonged inflammatory phase and lack the capability to transition into the subsequent phases to complete the wound healing process, resulting in an open wound. The *proliferation phase* encompasses fibroplasia, granulation, epithelization, angiogenesis and proceeds within 24 hours after the formation of the lesion. The newly formed fibrin matrix allows keratinocytes to migrate from the edge of the wound and from the follicles of the hair to wound bed. By decreasing the proteolytic activity of metaloproteinases, fibroblasts begin

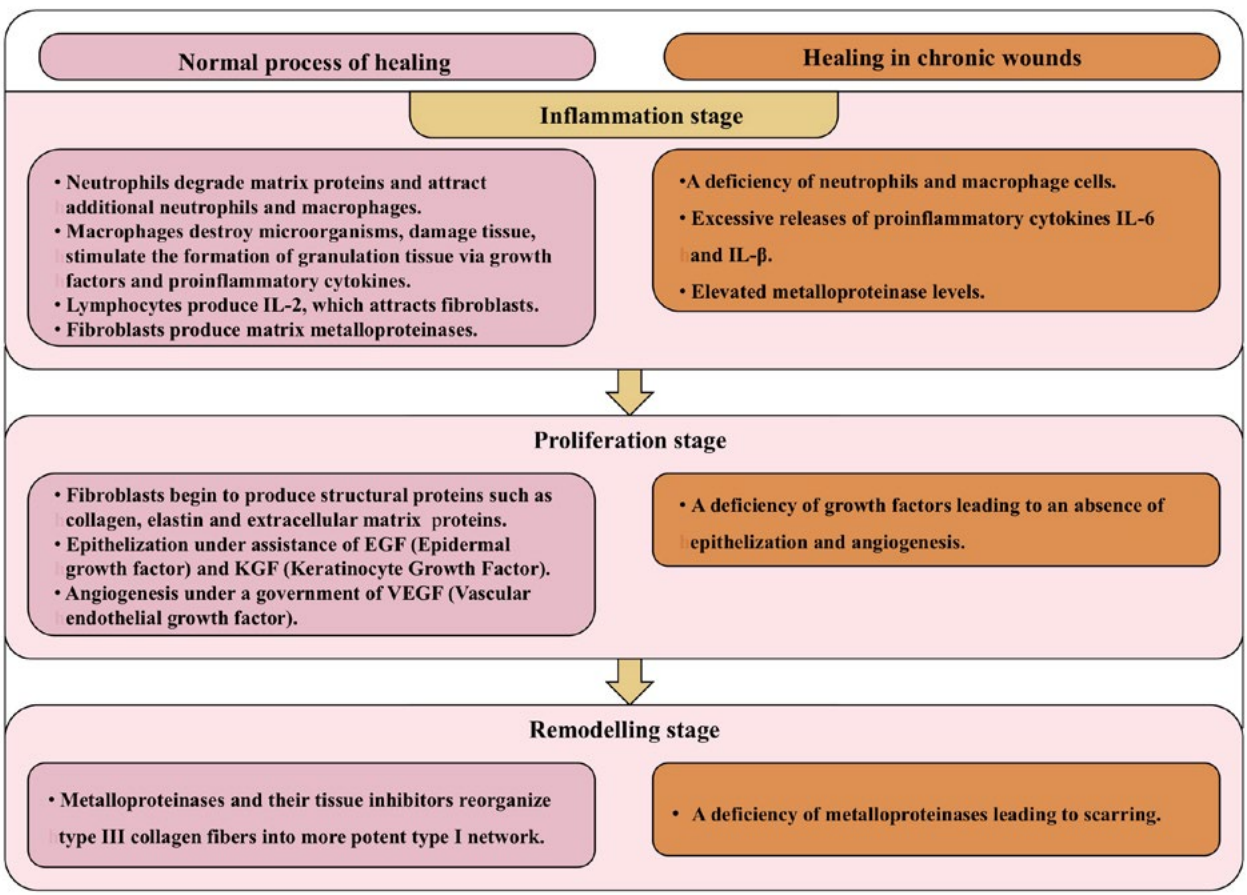

Fig. 3. A schematic course of healing in normal and chronic wounds [20].

to produce structural proteins such as collagen, elastin and extracellular matrix proteins, *i.e*., fibroblasts are important for the proliferation of the dermal matrix. This stage is regulated by two growth factors: TGF-β, secreted by platelets and macrophages, as well as connective tissue growth factor, which is secreted by the fibroblasts. As a result, epithelization occurs by the propagation and migration of epithelial cells under assistance of both epithelial and keratinocytes growth factors, as well as other factors. At the same time, the vascular endothelial growth factor promotes angiogenesis. *Remodelling* phase is a part of the wound healing completion. The granulation tissue is gradually remodelled, forming scar tissue. The contraction of the wound begins due to phenotypic changes in fibroblasts and their transformation into myofibroblasts. Metalloproteinases and their tissue inhibitors reorganize type III collagen fibers into a more potent collagen type I network. The process of remodelling takes weeks to several years.

The lesion healing progression depends on a well-coordinated interplay of cell-signalling events at the wound site and surrounding tissues, in which endogenous SCs are vital players. In case of skin tissues damage, SCs from several locations are mobilized via the peripheral bloodstream to the injured site for differentiation into specific progenitor cells, which in turn differentiate into specialized cell types necessary for tissue regeneration. Epidermal, dermal cells and cells of hair follicles, as well as circulating in the bloodstream bone marrow MSCs, can migrate to the damaged skin and take part in its regeneration [21].

For chronic wound healing, populations of SCs from epidermis, dermis and hair follicles are not easy to isolate and support, so MSCs of the fat tissue, the bone marrow and placenta are in use to replace skin SCs. MSCs source selection is based on the kind of wound, a patient's age and condition. One of the first successful treatments of chronic non-healing wound was performed with the bone marrow-derived MSCs [22]. The wound, which previously failed to heal with both bioengineered skin and autologous skin transplants for more than 1 year, has been closed after the application of MSCs. There were previously unseen signs of curing in the wound such as an increase in reticulin fibres and in overall vascularity. During the last ten years, it has been proven that the adipose MSCs had a favorable effect on wound healing through inflammation inhibition, an induction of angiogenesis, a proliferation of fibroblasts and keratinocytes, a reconstruction of extracellular matrix [23]. In the treating of complex wounds, such as diabetic ulcers, it is important to promote the process of blood vessel regeneration. That is overcome by the iPSCs potential, which can efficiently influence the revascularization of wound bed [24]. Proangiogenic endothelial progenitor cells have also been shown to improve healing in cutaneous wounds by enhancing the neovascularization of granulation tissue *in vivo* [25]. Placental MSCs have a potential for the treatment of chronic wounds, since they have a number of advantages over other stem cells: (a) possess more pronounced immunomodulating effect and (b) higher proliferation rates; (c) demonstrate lower immunogenicity; (d) can differentiate into various cell lineages [19, 26].

Taken together, all these studies show that human SCs play a vital role in damaged tissue regeneration and are used in curing different

types of wounds, increasing the rate of healing and reducing scarring. The evidence suggested that the healing effects were largely due to the inflammation regulation by factors released, stimulation of new vessel and granulation tissue formation, guidance of the epithelial keratinocytes and fibroblasts, improved dermal matrix deposition.

Currently, there are a few finished clinical trials of SCs in the treatment of wounds, ulcers and burns. For example, the successful treatment of diabetic foot ulcer using the placental MSCs, incorporated in a sodium alginate hydrogel, has been achieved recently [26]. The progress in the clinical trials of novel MSC-based technologies can be followed at the National Institute of Health web-site www.clinicaltrials.gov.

A delivery of stem cells

Systemic administration of SCs is limited by the difficulties of tissue targeting. In some cases, delivery via the circulatory system could be harmful and lead to toxic effects. Inefficient delivery of SCs to the targets followed by low cell levels or their weak differentiation and engrafting causes problems in treatments. Therefore, for many cutaneous wounds curing, the regeneration therapies are delivered to the specific site of tissue repair.

The challenge of successful *ex vivo* expansion of SCs is that the cells need to proliferate and preserve their stem cell properties. It promotes researchers to elaborate new approaches to deliver SCs or progenitor cells into wound bed and bioengineering neo-tissues. For wound healing, SCs are delivered preferentially inside a three-dimentional (3D) architectural constructs resemble to a skin. *In vivo,* the SC microenvironments (niches) are multi-dimensional, multi-factorial systems that regulate the quiescence, proliferation, activation of SCs and play an essential role in the differentiation process [27]. This known fact is applied in bioengineering to modulate more favorable conditions for the SCs within a 3D than 2D matrix. Bioengineered scaffolds that can also be enriched with bioactive molecules such as cytokines, growth factors *etc.* should support cells by mimicking the function of a native ECM. The effectiveness and efficiency of biomimicking materials are dependent on several factors, such as the choice of material, the cell type, the physio-spatial properties within the construct and so on. Different 3D systems such as cell-encapsulating hydrogels and other porous biomimetic scaffolds are discussed in [28]. In this background, current research is focused on the developing of an artificial skin that could fully imitate a natural skin architecture and its functionality needed for the SCs transplantation. The cell-free biomaterials, *e.g.*, a pig skin, amniotic membrane *etc.*, were used to create a 3D-template optimal for SCs growth and differentiation [29]. Another approach is encapsulating SCs in hydrogels, which provides a true 3D system that supports several SC properties. Hydrogels can be made from natural or synthetic polymers [30]. The natural materials used for hydrogel synthesis include collagen, hyaluronic acid (HA), alginate, chitosan, bacterial cellulose. The latter is a biodegradable porous scaffold sponge-like 3D material that serves for cell attachment and supports their growth. For the *in vitro* expansion of the SCs, several experiments have been performed using cellulose, *e.g.,* within composites of cellulose and HA or gelatin, the

dental pulp MSCs were grafted and well distributed [31]. A combination of SCs with the highly biocompatible and clinically relevant bacterial cellulose renders new possibilities and potential in clinical applications of 3D scaffold systems. For better mimicking, biopolymer scaffolds are functionalized by needed biomolecules. For example, to recapitulate the adhesive properties of the ECM, the hydrogel was equipped with short RGD (the tri-amino acid sequence, arginine-glycine-aspartate)-peptides, to which cells can adhere via integrin receptors [32]. Currently, the regenerative cell technologies also employ synthetic polymers, including polyvinyl alcohol, polycaprolactone *etc.*, as a niche for SCs to improve their survival and paracrine activity required for a better wound healing [33].

Scaffold-based SC delivery is becoming increasingly popular. It does not only provide wound dressing, but also protects SCs and significantly accelerates wound healing, compared to injection techniques, both in animal models of wound healing and clinical settings [33, 34]. Meanwhile, a 3D- printing is a potential solution to fabricate skin constructs using biomaterial scaffolds with stem cells. The 3D-printing is a method based on the digital data of a 3D-structure to be converted into the real objects. In contrast to applications of solvents or molds, the 3D-fabrication enables to create the data on digital platform. Bioprinting is applied to develop skin equivalents for wound healing therapy [35].

Along with the elaboration of efficient MSC delivery methods, researchers develop the strategies for producing stem cells of the next generation with improved regenerative capacities [36]. Using the genetically modified MSCs, capable to secrete novel bioactive molecules, *e.g.,* the growth factors, contributes to both higher local MSCs concentrations and potentiating novel bioeffects in the target area [37]. The emerging technologies of genome editing, especially, transcription activator-like effector nucleases or clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein-9 nucleases (Cas9), have provided robust tools for biomedical research [38–41]. The generation of iPSC from patients with inherited disorders and subsequent genomic correction of the disease-causing mutations could provide an ideal therapeutic approach. The next challenge is to utilize these studies for the generating of full complex organs as a complete cell therapy and bioengineering. However, there is a growing concern about the ethical aspect of the SCs gene editing. At the same time, the technology of genome editing may safely serve in the area of wound healing, *e.g.,* researchers have recently reported engineering MSCs that overexpress platelet-derived growth factor B with improved wound healing capacity [42]. CRISPR-Cas9 technology was also used to eradicate the functions of porcine endogenous retroviruses, targeting the porcine genome, in the cells used for making the donor graft [43].

Genetically modified MSCs are promising agents for current and future regenerative medicine, however, even a simple preliminary treatment of MSCs with chemical, physical or biological factors improves the cell survival and activity [44].

Limitation of stem cell therapy

MSC-based therapy is capable of what the traditional therapy cannot accomplish: it pro-

vides missing cells for regeneration of tissues and organs. This novel approach in regenerative medicine is especially indispensable in relation to aging and age-related diseases. At the same time, MSC therapy makes some concerning challenges, including the genetic instability of MSCs, immune-related rejection and poor engraftment, limited survival rate, *etc.* All signalling pathways remain unknown yet. Besides, the tumor formation is a major concern of MSC therapy in clinical applications. There are some limitations in the MSC isolation, large-scale production, storage and distribution. However, these major drawbacks and limitations depend on the depth of current research and technical challenges.

Cell-free technologies: secretomes of stem and other cell types

In the 1990s, the evidence appeared that SCs carried out their functions via paracrine effects [45]. The non-cellular therapy concept was born, and in this light, MSC secretomes (MSC-sec) can be considered as a valuable source of therapeutic agents, such as growth factors, cytokines *etc.* that can be therefore used for regenerative medicine purposes. Accordingly, the interest to the full MSC-secs as a healing remedy is growing. Secretome denotes all secreted organic molecules, as well as organic and inorganic substances, by biological cells, tissues, organs and organisms. Secreted factors (soluble proteins, *e.g.,* cytokines, chemokines, growth factors, as well as lipids, nucleic acids and EMVs) [46] can change the order of cell division, differentiation, apoptosis, immune status and affect other processes. Various SCs differ in secretion of bioactive cell components; the variations in MSC-sec also depend on the extrinsic cues [47]. Two main groups of secretome components — the set of bioactive molecules and EMVs — can trigger the regeneration and repair to some extent by themselves, independently, and may allow an increased cell proliferation, differentiation, angiogenesis and neurogenesis, as well as a reduction in inflammatory responses. Overall, this can lead to the improvements in tissue regeneration and the rates of healing, including wound healing, successfully proven in preclinical studies [47–51]. Recent preclinical *in vitro* study has demonstrated that the MSC-sec activated the Phospho-Inositide 3-Kinase (PI3K)/Akt [52] or Focal Adhesion Kinase (FAK)/extracellular signal-regulated kinase (ERK)1/2 [53] signalling cascades. The first pathway is known to play central regulatory roles in MSC survival, proliferation, migration, angiogenesis, cytokine production and differentiation, and another mentioned pathway is represented by mitogen-activated protein kinases, like ERK, which governs the cell movement by phosphorylating FAK and plays so far crucial roles in focal adhesion and cell migration. Thus, secretome enhances the proliferative and migratory abilities of various types of skin cells, such as fibroblasts, keratinocytes and vascular epithelial cells in the same way as MSCs do. Major components of the stem cell secretome were recognized as epidermal growth factor, basic fibroblast growth factor and hepatocyte growth factor, which could be responsible for promoting migration and proliferation of skin cells, such as dermal fibroblasts, keratinocytes and vascular endothelial cells and so far for upregulation of appropriate signalling pathways, leading to the acceleration of wound contraction [54]. The presence

of another epithelial growth factor, TGF-β, vascular endothelial derived growth factor, keratinocyte growth factor and others in secretome of MSCs play an important role in wound healing [55].

In the clinical research, the conditioned medium "ASC" (Adipose Stem Cells) was used as an agent for healing the patients' skin wounds after a laser irradiation, and it was shown that the medium improved wound healing and reduced side effects of laser therapy [56]. The MSC-sec is profitably employed in situations, where bandages are not easily applied, *e.g.,* to heal ulcers of ocular epithelium [57].

Secretomes of platelets [58] or peripheral blood mononuclear cells (PBMC) [59] also contain the components essential for wound healing. Platelets contribute to physiological hemostasis and pathological thrombo-inflammation and are the first cells to respond to an injury. Platelets secretome is enriched in growth factors, chemokines, cytokines and other signalling molecules. These components have diverse effects on the biochemical, molecular and cellular environment at the wound site in the context of inflammation; recruitment of neutrophils, macrophages and fibroblasts; anti-microbial response; angiogenesis; extracellular matrix synthesis *etc.* [60].

The secretome of apoptotic PBMCs obtained by irradiation — APOSEC (APOptotic PBMC SECretome, a secretome from apoptoticPBMCs) — had a number of cytokines, lipids, proteins, nanovesicles. In preclinical studies, this rich bioactive material had a high potential for regenerative therapies and treatment of various diseases and chronic wounds [61, 62]. In Phase I research, it was shown that APOSEC did not cause complications, however, it did not affect the wound closing [63]. On the basis of a long-term research of the APOSEC preparation, the authors considered that elucidating the mechanism/s of skin regeneration was impossible at that stage. These results show that, firstly, there is still much to learn about the secretome content, regulation, interaction and complementarity of the specific components in wound healing. Secondly, the environmental factors, such as general state of patient's health, age, sex *etc.*, affect the healing, and this ought to be taken into account. Thirdly, it is necessary to apply standard approaches to production and application of the preparations of clearly defined content with the expected mechanism of action.

In spite of still unfruitful clinical trials results, emerging MSC- and other secretome-based non-cellular therapies have the future in regenerative medicine because of several advantages over cell-based technologies: (a) an overcome of the safety problem, potentially linked to living cell transplantation and included immune compatibility, the possibility of tumorigenicity, embolism and a transfer of infections; (b) a prolonged store of the secretome preparations without potentially toxic crioconservative agents with no loss of functionality; (c) secretome is more practical for a clinical application, since it does not require the invasive protocols of cell acquisition; (d) a large-scale production of secretomes through specific cell lines in controlled lab conditions; (e) time and cost of keeping cultured SCs can be reduced; (f) the biological product, obtained for a therapeutic use, can be modified for getting the desired cell effects.

As mentioned above, the whole stem cell secretome contains a mixture of valuable bio-

molecules. For clinical purposes, the identification and production of the whole stem cell secretome in accordance with good manufacturing practice of single MSC-sec-components will be challenging in the nearest time. MSC-secs-derived valuable therapeutics such as cytokines, growth factors, hormones, peptides, miRNA *etc.* are produced biotechnologically, using modern approaches such as culturing MSCs in 3D scaffolds, growing under influence of such stress factors as magnetic field, oxidation, hypoxia [55].

Extracellular membrane vesicles as potential novel therapeutics in regenerative medicine

Human cells secret several types of membrane vesicles with different physiological properties that include exosomes, microvesicles (known also as microparticles, shedding microvesicles) and apoptotic bodies [64]. The smallest of them are exosomes (30–100 nm), which originate from the late endosome multivesicular bodies (MVB) formed inside the eukaryotic cell to sort various transport vesicles. Exosomes are secreted when MVBs integrate with plasma cell membrane and are released into the extracellular compartment. This process is regulated by the protein machinery known as the Endosomal Sorting Complex Required for Transport (ESCRT) system [65]. ESCRT-independent exosome formation relies on ceramide generation by neutral sphingomyelinase 2, a key cell signaling enzyme. The microvesicles (MVs) are formed by "budding" of the cell membrane with a size from 60 to 1000 nm, and they also require for the ESCRT system together with scramblase and flippase activities [66]. Lastly, large complex vesicles, apoptotic bodies (500 nm to 2 μm), are secreted through exocytosis by dying cells; this type of EMVs is out of this review. All types of EMVs are formed imperatively by phospholipid membranes and secreted by cells outwards. Nanovesicle biogenesis is different, and molecular cargo is different, respectively. For example, transmembrane proteins, such as tetraspanins (CD9, CD63, CD81) from the endosomal sorting complex, are mostly associated with exosomes, whereas proteins from the endoplasmic reticulum, Golgi, mitochondria or the nucleus are preferentially found in microvesicles and rarely — in exosomes [67].

Theoretically, the heterogeneous populations of EMVs are produced by all known types of cells, from archaea to mammals. They are present in the body fluids, including blood, saliva, tears, urine, cerebrospinal and amniotic fluids, milk and sperm. EMVs, derived from different cells, have different activities associated with either cellular functions or pathological states, depending on their content. Having no metabolism and the capability to self-replenish, EMVs carry valuable cargo molecules produced by the host cells, such as proteins (cytokines, growth factors, transcription factors, enzymes, receptors *etc.*) and nucleic acids (messenger RNA, miRNA, long noncoding RNA, mitochondrial and chromosomal DNA) [64, 66] (Fig. 4). Recent studies have shown that EMVs carry membrane-derived bioactive lipids (eicosanoids) that perform an important function in the immune system. EMVs protect bioactive lipids (as well as other biomolecules) from degradation and play a role in the transcellular synthesis of prostaglandins and leukotrienes [68].

EMVs are recognized as mediators of paracrine signals and play a role in cell-to-cell communications. EMVs can interact with target cells using different mechanisms, *e.g.*, clathrin-mediated endocytosis, micropinocytosis and lipid raft-dependent endocytosis [69]. In short- and long-distance communications, small noncoding (nc) RNAs along with bioactive protein factors packed and carried by vesicles are the key modulators of biological effects in the target cells. Long noncoding RNAs induce epigenetic modifications in recipient cells by recruiting epigenetic regulators [70]. In 2013, J. Rothman, R. Schekman and T. Sudhof won the Nobel Prize in Physiology/Medicine for discovering molecular principles of the governed cargo molecules delivery to target cell by membrane vesicles. This event considerably promoted the research in biomedical area. Due to unique properties, such as nanoscale, a transversion of the blood-brain barrier and a penetration of dense structural tissue, EMVs became a promising tool for the treatment of a wide spectrum of diseases, either as unmodified agents or as functionalized carriers for targeted drug delivery. Noteworthy, the healing effects were observed in studies with both exosome and microvesicle populations, and often it was mentioned with no regard to the distinction between them. In the background of regenerative medicine research, MSC-derived exosomes are the most studied and appear to be responsible for many of the traits commonly associated with MSCs, including tissue healing. Many other types of cells also secrete exosomes, which are promising for healing.

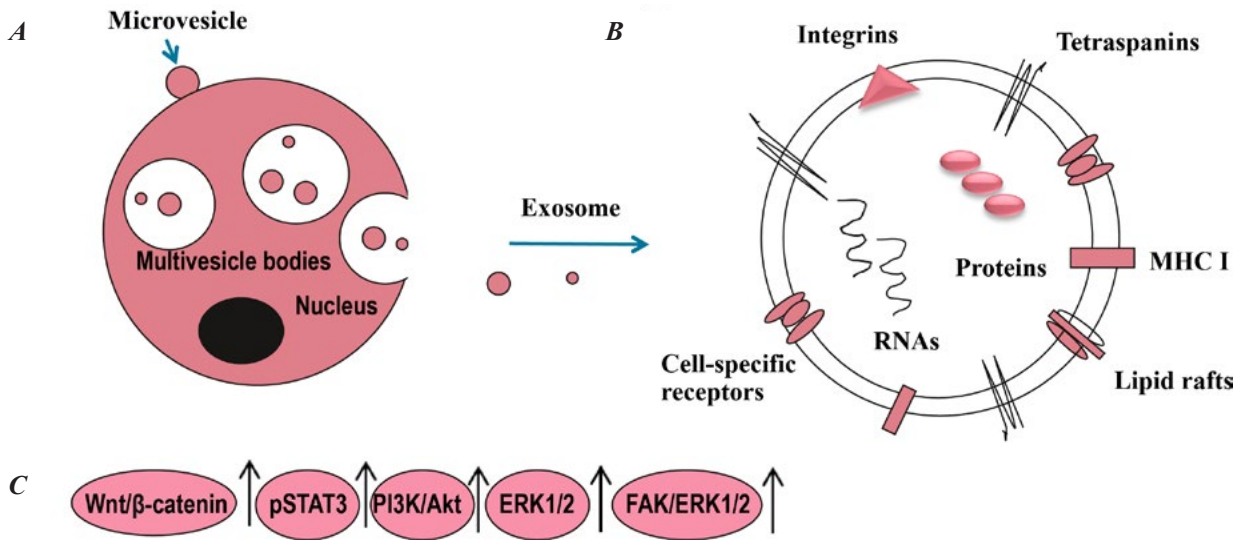

Fig. 4. Biogenesis, molecular cargo and signalling of extracellular membrane vesicles (EMV). *A* — The EMVs can be secreted into the extracellular environment through either fusion of multivesicular bodies with the cell surface (exosomes) or budding pathway (microvesicles). *B* — Molecular composition of exosomes. Exosomes are membrane-bound phospholipid nanovesicles secreted by all cell types. They bear cellular components, including mRNAs, miRNAs, DNAs, various proteins, lipids. *C* — EMVs trigger signalling pathways that suppress inflammation, modulate proliferation, migration, angiogenesis, collagen production and reconstruction of extracellular matrix [52, 53, 79–83].

Extracellular membrane vesicles of stem cells and progenitor cells in wound healing

EMVs, secreted by MSCs, mimic and recapitulate some of the parent cell functions, such as self-guidance into the target/injured site, immunomodulation and reprogramming of cells required for the tissue regeneration [71]. Remarkably, exosomes were shown to possess procoagulatory activity, essential for healing the bleeding wounds [72]. In the wound healing process, EMVs take part at all stages of wound healing [69, 73]. EMVs derived from different sources demonstrated similar characteristics in wound healing. The topical or systemic use of exosomes and microvesicles showed their efficiency in non-healing wound treating at the same rate, as SCs did [74–78]. At the cellular level, EMVs administration has been found to enhance endothelial cell proliferation and migration, tube formation, promoting angiogenesis and blood vessel maturation, skin remodelling, resulting in suppressed myofibroblast accumulation and reduced scar formation *in vivo*. In other words, MSC-EMVs act directly upon epithelial cells, endothelial cells and fibroblasts during wound healing or via EMVs molecular cargo at a distance, modulating intracellular signalling pathways within the recipient cells. Activation of signalling cascades resolves chronic inflammation (*e.g.*, by the activating Signal Transducer and Activator of Transcription 3 (STAT3)/AKT (protein kinaseB) and promotes angiogenesis (*e.g.*, via ERK1/2 or Wnt4 pathway, which induced β-catenin activation [79, 80] (see Fig. 4C). Exosomes promote synthesis of collagen I and III at the early stage of wound healing, whereas at the late stage, exosomes prevent collagen deposition and scarring [81]. More specifically, such a regulation can be performed by regulatory molecules that mediate a wound healing such as proteins, *e.g.*, HSP90a, p-STAT3, p-AKT, p-P65 [82], and by small non-coding (nc)RNAs, regulating gene expression at the level of translation [83]. At the injury sites, ncRNAs contribute to the regulation of matrix remodelling, epithelial mesenchymal transitions and the attraction of fibroblasts. Additionally, 'anti-inflammatory' RNAs potentially orchestrate the resolution of inflammatory responses and immune alleviation to facilitate healing processes.

Exosomes originated from MSCs also have been shown as efficient therapeutics in the regeneration of joints, bones, a heart muscle after myocardial infarction, a brain traumatic injuries *etc.* [84–87].

Extracellular membrane vesicles of other cell types in wound healing

In addition to EMVs of stem and progenitor cells, the membrane vesicles of other cell types also have healing potential. Exosomes of platelets are the most widespread EMVs in human peripheral blood (known as a platelet 'dust') and comprise more than a half of all the EMVs in blood. Platelet EMVs bear pro-coagulant anionic lipid needed for the formation of protrombinase complex and other factors of wound healing [88]. Such nanostructures take part in several essential biological and pathological processes through blood clotting, angiogenesis, inflammation, immunoregulation *etc.* [89]. Torreggiani and co-authors showed that the platelet exosomes contained main growth factors of parental cells and so far could be one of the effectors of the well-known action of the platelet-rich plasma (PRP) and

platelet lysate [90]. Based on this conclusion, the attempts to use them for a therapy of chronic wounds have been undertaken. In the diabetic rat model, it was shown that platelet exosomes had properties similar to PRP: they improved cell proliferation, migration and angiogenesis in healing wounds [91]. Up to date (20.06.2018), the clinical trial (Phase 1) has been listed on the clinical trials website (https://clinicaltrials.gov/), the purpose of which is to evaluate the effect of autologous EMVs preparation from rich plasma on cutaneous wound healing.

More research is required to find new sources for the harvesting of EMVs with their further translation into clinical applications. Cheaper EMV-based therapeutics for non-healing wounds may be found among vesicleomes of safe human commensal microorganisms, especially, for curing infected and rotting wounds. Outer membrane vesicles (OMVs) of gram-negative bacteria, rich in biologically active substances, are the major factor in their interaction with the macroorganism [92]. Proteomic and biochemical analyses have shown that they contain a variety of bacterial components from the outer membrane, like lipopolysaccharide (LPS) and lipoproteins, periplasmic proteins, DNA and RNA. The EMVs secretion mediates bacterial adaptation to various niches [93]. The surface OMV components act as pathogen-associated molecular patterns and stimulate the innate immune receptors, such as Toll-like receptors and Nod-like receptors, to modulate the innate immune responses. OMVs of human commensal gut bacteria have an anti-inflammatory effect, enhancing the T(reg) cells in the intestine and protecting against colitis [94]. OMVs are the vehicle that delivers LPS in host cells to trigger the immune response [95]. Bacterial nanovesicles induce cytokine synthesis needed for the injured tissue regeneration and wound healing [96]. Importantly, bacterial OMVs, secreted by *Burkholderia* spp., inhibit the development of antibiotic resistant bacteria as well as the production of a biofilm [97]. Besides, gram-negative bacteria (*Citrobacter* sp., *Enterobacter* sp., *Lysobacter* sp. *etc.*) are known to produce a complex of bacteriolytic enzymes localized in the OMVs that are efficient against gram-positive pathogens [98, 99]. Remarkably, there is no available information on the role of bacterial vesicles in tumorigenicity unlike exosomes, which appeared to impact the process. Taking together, it is easy to predict the therapeutic potential of OMVs, loaded with bacteriolytic enzymes, against antibiotic-resistant bacteria (that infect open wounds and complicate the treatment process), as well as to imagine, how cheaper might be bacterial nanovesicle therapeutics in comparison with MSC-exosomes and MVs.

Driving forces of exosome industry

The EMVs attract attention as possible ideal therapeutic agents because of their long circulation time without the loss of the quantity and quality. Exosomes and microvesicles are non-living creatures, and so far as therapeutics are advantageous over the cell-based technologies in regenerative medicine, especially, considering the safety problem. Unlike SCs, where there is the risk of spontaneous or induced transformation and necessity of the cytogenetic control, exosomes cannot differentiate into any cells. Exosomes are much easier to manipulate and store. In contrast with syn-

thetic drugs, exosomes are physiological and perceived as own, so they can systemically, even overcoming the hematoencephalic barrier, deliver small (femtomole) doses of biomolecules or drugs [100]. Finally, using the autologous exosomes reduces the risks of pathogen transfer and immune non-compatibility.

Bioengineering is in use to construct smart-vectors based on EMVs for different types of healing factor delivery that would selectively enter certain cells and correct the healing process (see reviews [101, 102]). Healing factors can be loaded into the exosome interior, in the periplasmic space or represented as superficial constructs (displays) on the EMV surface [103]. Currently, the EMV engineering for enhanced therapeutic effect has not yet been extensively pursued in the area of wound healing. However, there are three approaches elaborated to improve efficiency and specificity of the EMVs action, when needed: a) a treatment of cells-donors of EMVs with therapeutic agents; b) a genetic modification of EMVs cells-donors; c) a direct modification of EMVs. For example, the preconditioned with lipopolysaccharide MSCs showed enhanced paracrine effects and the therapeutic efficacy for chronic inflammation and wound healing via the loading of miRNAlet-7b, which so far mitigated inflammation in the wound through the regulation of anti-inflammatory cytokines expression [104]. Depending on the etiology of non-healing wounds, exosomes can be filled with different miRNA, enhancing the signalling pathways, responsible for healing, *e.g.,* with miRNA-126–3p, which stimulates a fibroblast proliferation [105]. Placing protein ligands on the surface of EMVs allows a delivery of molecular cargo into the target site [103].

Exosomes are easy to enter the body, in particular, by systemic or local therapy. The reported studies have utilized both local and systemic (intravenous) administration in wound healing. Locally applied EMVs have been delivered by direct topical application, injection into or around the wound and integration into biomaterials. For wound healing topical application, *e.g.,* sponges or dressings, filled with EMVs are in use. Biocompatible carriers are successfully employed to deliver EMVs and release their contents in controlled quantities into the wound. Recent researches have shown that for topical application, polysaccharide-based matrices are the efficient carriers of EMVs. For example, a combination of MSC-exosomes and chitosan-derived hydrogel can efficiently help to heal the skin wounds in diabetic rats by reepithelization, synthesis and remodelling of collagen, angiogenesis and production of neurons [106]. The *in vivo* diabetic wound healing study showed that the treatments using platelet exosomes loaded into chitosan-derived hydrogel, enhanced with polysaccharide from the rhizomes *of Curcuma zedoaria,* accelerated wound contraction and decreased ulcer [107].

At present, the interest in the application of exosomes and other membrane vesicles in regenerative medicine is constantly growing. Market forces driving growth of the exosome industry have been expanding over the past two years, with increasing investment flowing into research, development of tools, new therapeutics, entering various stages of development. First exosome biotech companies from the USA and the European Union appeared on

the world market in 2015. Using academic and commercial research as a background, they started the exosome bioproducts manufacturing for regenerative medicine. The most benefited are patients with posttraumatic syndromes and degenerative diseases. Exosome-based therapeutics are in use also for chronic wounds healing. For example, the ExoWound product, based on the exosomes of umbilical cord blood MSCs produced by Exogenus Therapeutics from Portugal has recently launched on the European market (http://www.exogenus-t.com/). Another exosome-based product known as XoGlo™ for topical application in wound and burn healing is practicized by Kimera Lab (the USA) (http://www.kimeralabs.com/).

Meanwhile, the exact mechanisms of indirect reparative effect of EMVs remain insufficiently studied. It also remains to elucidate, whether morphology, integrity and interaction of EMVs with target cells and their biological activities do not alter during the obligatory purification from viruses. The rate of the regenerative capacities of EMVs depends on the type of mother cell, and it remains unclear, which donor cell type provides the most powerful effect. Despite the emerging possibilities of treatment by EMVs, the novel nature of EMV-mediated therapy causes concern on their safety. The International Society for Extracellular Vesicles established the minimal set of biochemical, biophysical and functional standards for the EMV research, which can be used in the countries where this background is not developed. Therefore, the obtaining of expected positive results from clinical trials of EMVs as therapeutics requires in-depth research for reaching their full potential.

Tissue products in wound healing

Extracellular matrices are in use in regenerative medicine for wound healing and tissue regeneration in various traumas. In addition to their use as scaffolds for tissue reconstruction, fibrous matrices are considered as valuable pools of bioactive molecules that can mediate tissue regeneration through modulating the immune response and recruiting and stimulating stem and progenitor cells [108]. The temporary organs, *e.g*., a placental complex, necessary for the development and birth of the fetus, are known as such matrices. The amniotic membrane is the most used in clinical practice within hundreds of years. The amnion is a placental membrane that envelops the fetus and is found to be useful in therapy due to its unique structure and valuable contents for the damaged tissue regeneration. The membrane is rich in collagen, contains no blood and lymph vessels or nerves and has very low antigenicity. The transplanting of the membrane promotes reepithelization, inhibition of inflammation and scarring, relieves pain and so far it is especially efficient in burns healing. Amniotic tissue is also used in the treatment of diabetic wounds, in particular, in the lower limb or other wounds resistant to traditional therapy [109, 110]. Current data show that the remarkable healing effect occurs because of the amnion products, which stimulate secretion of growth factors, cytokines, proteases and increase synthesis of stromal cell factors [111].

The placental extracts obtained by lysis of the placental tissue are rich in amino acids, proteins, minerals, steroid hormones, cytokines *etc.* and have curing effects. Placental products are used in the treatment of traumatic and chronic wounds, as well as non-healing ulcers

and burns, due to the bioactive substances with anti-inflammatory properties, which also promote reepithelization, reduce infiltration and relieve a pain syndrome through the inhibition of cyclooxygenase-2. The extracts also have immunoregulative, neuroprotective, anti-allergic and antitumor properties. Through their complex action, placental extracts are promising agents in the treatment of diabetic neuropathy and angiopathy [112]. The mechanism of placental extract action depends on the presence of bioactive corticotrophin-releasing factor that possibly increases the production of TGF-β and reduces the level of proinflammatory TNF-α [113].

In spite of the remarkable composition of bioactive substances, the placental tissue products do not overwhelm the world market of therapeutics, *e.g.,* for wound healing. However, the placental tissue extracts were used in the development of the burn wound treatment therapy at the Institute of Cell Therapy (Kyiv, Ukraine). The application of placental tissues in burn treatment allows shortening the time before autodermoplasty, stimulates granulation and epithelization and reduces inflammatory reaction and intoxication (http://www.stem-cellclinic.com).

Conclusion

Regenerative medicine advanced therapy opens new possibilities in the treatment of wounds resistant to traditional therapy. Stem cells are known as highly relevant agents for healing chronic and non-healing wounds due to valuable secretomia, self-guidance into the target place, self-regeneration and differentiation. This novel approach in regenerative medicine is especially indispensable in relation to aging and age-related diseases, because of the SCs capability to replace lost cells for regeneration of tissues and organs. Despite the successes in applying SCs and progenitor cells, some barriers prevent efficient therapeutic action, *e.g.,* limits of transplantation areas and a poor SCs engrafting into the recipient injured tissues. Current approaches, such as preconditioning and using the next generation SCs, modulation of the SC-secretomes, using extracellular membrane vesicles lead to maximization of the SC bioeffects. EMVs of different origin can be used in the treatment of non-healing wounds either by themselves or in combination with other therapeutics. On the background of the newest reconstructive technologies, the tissue products remain reliable means for the rescue of patients with chronic ulcers. Considering that the mechanisms of wound healing may differ, depending on the localization, size and depth of the wound, infection by pathogenic microorganisms, patient immune system state, there is an urgent need to develop personalized medicine for healing patients in accordance with the individual protocols based on the patient's advanced examination.

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Сучасні технології регенеративної медицини у лікуванні незагоювальних шкірних ран

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Сучасні напрямки регенеративної медицини, інспіровані досягненнями у галузі клітинної біології, генної інженерії, синтетичної біології, матеріалознавства, довершують традиційну терапію, що робить можливим лікування хронічних недуг. У наш час лікування хронічних ран можливе через використання клітинних регенеративних технологій та більш пізніх неклітинних терапевтичних підходів. В огляді ми розглядаємо альтернативні підходи до лікування незагоювальних ран за допомогою клінічного застосування стовбурових клітин, а також клітинних та тканинних продуктів. Для клітинних технологій регенерації тканин та біоінженерії використовують стовбурові клітини, які вводяться у кров›яне русло або безпосередньо у рану. Інший підхід грунтується на регенераційних неклітинних технологіях, які потребують продуктів стовбурових клітин, тобто, секретомів або їхніх окремих компонентів — позаклітинних мембранних везикул, а також тканинних продуктів. Технологія стовбурових клітин призначена для заміни критично відсутніх компонентів пошкоджених або дегенеративних тканин. Секретом стовбурових клітин, незалежно від стовбурових клітин, може сприяти відновленню пошкоджених тканин. Позаклітинні мембранні везикули здатні імітувати механізми стовбурових клітин в процесі регенерації тканин і, отже, є переспективними у лікуванні хронічних ран та ускладнених опіків. Тканинні продукти традиційно залишаються ефективними засобоми загоювання ран на тлі сучасних технологій.

Ключові слова: загоєння ран, клітинні і неклітинні технології, стовбурові клітини, секретоми, позаклітинні мембранні везикули, тканинні продукти

Современные технологии регенеративной медицины для лечения незаживающих кожных ран

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Современная терапия регенеративной медицины находится под влиянием достижений в области клеточной биологии, генной инженерии, синтетической биологии и материаловедения, которые совершенствуют традиционную терапию и делают возможным лечение хронических недугов. В наше время лечение незаживающих ран возможно благодаря использованию регенеративных клеточных технологий и более современных неклеточных терапевтических подходов. В обзоре рассмотрены альтернативные методы лечения незаживающих ран с использованием стволовых клеток, а также клеточных и тканевых продуктов. Для клеточных технологий регенерации тканей и биоин-

женерии используют стволовые клетки, которые вводятся в кровяное русло или непосредственно в рану. Другой подход основан на регенеративных неклеточных технологиях, которые используют продукты стволовых клеток, то есть, секретомы или их отдельные компоненты — внеклеточные мембранные везикулы, а также тканевые продукты. Методики с использованием стволовых клеток предназначены для замены критически отсутствующих компонентов поврежденных или дегенеративных тканей. Секретомы стволовых клеток, независимо от стволовых клеток, могут способствовать восстановлению поврежденных тканей. Внеклеточные мембранные везикулы способны имитировать механизмы регенерации тканей стволовыми клетками и, следовательно, являются перспективными для лечения хронических ран и осложненных ожогов. Тканевые продукты традиционно остаются эффективным методом заживления ран среди других современных терапевтических технологий.

Ключевые слова: заживление ран, клеточные и неклеточные технологии, стволовые клетки, секретомы, внеклеточные мембранные везикулы, тканевые продукты

Received 03.05.2018