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Strategies to increase the effectiveness of wound healing therapy with mesenchymal stem cells in diabetic patients

ABSTRACT

Administration of mesenchymal stem cells (MSC) into the wound seems a promising therapy in the management of hard-to-heal wounds in diabetic patients. We still do not have algorithms that would precisely define appropriate use of this therapy. However, the latest research results indicate multidirectional effects of MSC therapy (improvement of blood supply and tissue granulation within the wound, formation of superficial skin layers), as well as differences in the treatment efficacy between transplantation of cells obtained from a healthy person (allogeneic graft) and transplantation of cells obtained from the diabetic host (autologous graft). Various modifications of MSC therapy lead to more rapid wound healing, and this therapy may be a breakthrough in the treatment of chronic wounds in diabetic patients. In this article, we reviewed the up-to-date knowledge on the use of MSC in the treatment of hard-to-heal wounds in diabetes. (Clin Diabetol 2021; 10; 2: 226–233)

Key words: mesenchymal stem cells, chronic wounds, diabetes

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Introduction

The management of wounds in a diabetic patient is a challenge for clinicians. Wounds developing in diabetes heal slowly and often become chronic. Factors specific for the biology of chronic wounds in diabetes include leukocyte dysfunction, abnormal production of growth factors and extracellular matrix, reduced fibroblast activity, and increased (or unbalanced) protease production (excess metalloproteinase activity) [1]. An additional factor affecting abnormal would healing processes is impaired wound perfusion both at the macrovascular and the microvascular level, the latter due to multidimensional endothelial dysfunction. This is compounded by the effects of progressive neuropathy, placing the patient's foot at the risk of mechanical damage and thus contributing to recurrent ulcerations and possible complications during therapy. One the most common forms of ulcerations in diabetes is the diabetic foot syndrome. Most ulcerations within the distal leg are due to trauma. Due to poor long-term effectiveness of available therapies, diabetic foot syndrome is the most common cause for non-traumatic lower limb amputations.

A cohort study [2] in 158 diabetic patients with diabetic foot syndrome indicated that the rate of wound healing at 12 months was only 67%, and wounds were recurrent in as many as 31% of patients. In addition, the median survival without wound in the cured group was only 233 days.

In the study by Pound et al. [3], permanent lower limb wound healing was not possible in as many as 32.7% of patients with diabetic foot syndrome, and amputation was necessary in 9.9% patients. The wound often becomes superinfected with pathogenic bacterial flora. If infection ensues, the prognosis becomes worse, with the 12-month mortality rate of 15.1% [4].

The awareness of potentially adverse outcomes of wound treatment in patients with diabetes prompted a search for better therapeutic strategies. Among various approaches to wound healing, potential benefits of allogenic mesenchymal stem cell (MSC) transplantation are increasingly noted. The aim of the present article is to summarize the current knowledge on modifications of MSC therapy in the management of hard-to-heal wounds in diabetic patients with the aim of improving the effectiveness of multimodal therapy.

Mechanism of action of mesenchymal stem cells in wound healing therapy in diabetes

In the last several years, stem cell therapy has become a new therapeutic approach in many diseases, including wound healing and tissue regeneration. One of the most commonly used stem cells are MSC which may be retrieved from various compartments including bone marrow (BM-MSC), umbilical cord, adipose tissue (adipose derived stem cells, ASC), molars, and amniotic fluid. Regardless of the origin, all types of MSC act in a similar way, accelerating healing via two major mechanisms, i.e., the paracrine effect and direct differentiation into skin cells (fibroblasts and keratinocytes) [5].

MSC release many active substances including insulin-like growth factor (IGF), hepatocyte growth factor (HGF), transforming growth factor beta-1 (TGF-β1), vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF), fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), fibronectin, and collagen I9 [5]. At the molecular level, these factors are involved in various stages of wound healing including stimulation of angiogenesis and proliferation of skin cells and extracellular matrix [6].

The effect of direct MSC differentiation into specific skin cells has not been entirely elucidated but it was shown that MSC may differentiate into various types of resident cells following their application into the wound. It is believed that local secretion of various substances is the main mechanism leading to differentiation of stem cells into endothelial cells, fibroblasts, or keratinocytes. Thus, strategies to increase paracrine MSC secretion by modulating the wound environment may increase the effectiveness of healing. When using MSC, the current stage of wound healing should be taken into account. This means that the research should target modifications of the content of suspension applied into the wound, e.g., bubbles containing appropriately selected secretome.

Of note, currently there are no data from randomized studies that would confirm the efficacy and safety of MSC application in the management of wounds. Use of MSC remains a subject of active clinical research [7].

Attempts to increase treatment efficacy by modifications of MSC application into the wound

The approach of using MSC for treating chronic, hard-to-heal wounds in diabetic patients remains a subject of active research to increase its efficacy and accelerate tissue healing. One potential point for therapy modification that focused researchers' attention is the way MSC are applied into the site of its prospective action. One of such approaches involves applying stem cells on specific scaffolds. This issue was a subject of multiple research papers and clinical studies, and the materials serving as cell carriers show multidirectional biological properties. One of such materials is gelatin-sericin (GS) scaffold covered with laminin (GSL). Sericin acts via the c-Jun pathway to improve migratory properties and adhesibility of mammalian cells. In addition, it may increase cellular proliferation via a hitherto poorly characterized mechanism and shows mitogenic properties. Gelatin, both itself and in combination with sericin, binds to cells and enables them to proliferate. An increased fibroblast and keratinocyte proliferation was shown on GS scaffolds *in vitro* compared to no-scaffold models. GS scaffold improves scavenging of free oxygen radicals (which are one factor that impairs angiogenesis) and protects stem cells from oxidative stress, while laminin, an endothelial basal lamina protein, accelerates in-wound vascularization which contributes to more rapid healing and improved treatment effectiveness. The study by Tyeb et al. [8] evaluated the time to wound healing treated with MSC on GSL scaffold in rats with streptozotocin-induced diabetes. It was also compared how each of the component material of GSL scaffold affected the healing process (Fig. 1). The best results were obtained in the MSC therapy group with GSL scaffold. As shown in Figure 1, the most rapid decrease in wound dimensions was obtained when ASC were used on GSL scaffold.

Another scaffold used by Wu et al. [9] is silk fibroin (SF)/chitosan (CS) composite which served as a medium to administer ASC graft to the wound in rats with induced diabetes. This study also included two control groups, one with ASC graft only application to the wound, and the other with no graft application. Secretion of factors involved in wound healing, such as epidermal growth factor (EGF), tumour growth factor beta (TGF-B) and VEGF, was highest in the study group, while VEGF level was significantly higher in the graft only group. These findings support using such scaffold to administer ASC.

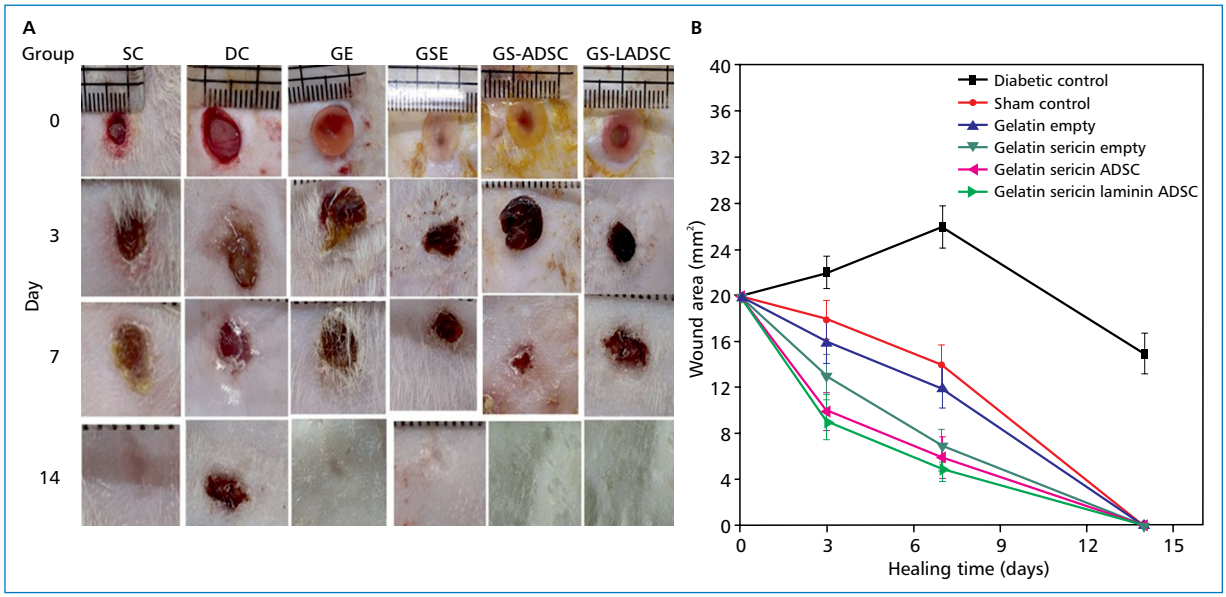

Figure 1. A — digital images of wound healing progress over a 14-day period; B — graph presenting the area of wounds on days 0, 3, 7 and 14 respectively. Analysis of the wound healing process using scaffolds: gelatin-sericin coated with laminin in combination with stem cells (GSL-ADSC), gelatin-sericin in combination with stem cells (GS-ADSC), gelatin-sericin empty (GSE), gelatin empty (GE), sham control — non diabetic rats without treatment (wound covered only with Tegaderm) (SC), diabetic control — diabetic rats without treatment (DC). Adapted with permission from [8]

Collagen-based scaffolds are another material that may replace the extracellular matrix until it regenerates, serving as a medium for MSC and increasing their proliferation and penetration to the wound. Unfortunately, most available collagen-based gel scaffolds have poor physicochemical properties and rapidly undergo degradation. The latter effect may be avoided by using the electrochemical deposition method to produce densely packed, damage-resistant collagen wound matrix (CMW) [10]. This collagen modification, with or without MSC, was tested in the diabetic mouse wound model and showed regenerative properties. Wounds treated using this approach had a brighter colour post-healing, and the tissue was more elastic, bearing more resemblance to the healthy skin compared to the non-CMW treated group. Wound histological analysis to evaluate granulation tissue area showed that both CMW and CMW+ASC grafting stimulated tissue regeneration, with significant benefits compared to the controls.

Another substance injected together with stem cells to the wound niche is Pluronic F-127 hydrogel. Pluronic F-127 is a good medium for ASC grafts, supporting stem cell proliferation by simulating three-dimensional extracellular matrix with excellent cellular affinity [11]. This hydrogel was often successfully used as the graft medium in cellular therapies [12, 13]. The specific content of the hydrogel medium differs depending on the manufacturer. An example of another substance from the same group is chitosan/silk hydrogel used by Shi et al. [14] which showed appropriate swelling and moisture retention properties. In vivo studies showed that addition of gingival mesenchymal stem cell (GMSC)-derived exosomes may effectively promote wound healing in diabetic patients. The wound size following treatment was significantly smaller compared to both the control group and the hydrogel only group. In addition, the wound size in the hydrogel only group at 1 and 2 weeks of treatment was significantly smaller compared to the control group.

Use of MSC which increase blood supply to the wound opens new possibilities as an artificial skin graft can be used along with stem cells. Until now, these skin graft were rejected due to impaired circulation within the wound, neuropathy, and the size of skin damage. In the study by Kato et al. [15], an artificial skin graft was not only accepted but also served as a three-dimensional scaffold for ASC by keeping them in a moist environment and protecting them from trauma and infection. In addition, the skin graft promoted formation of a connective tissue matrix resembling natural dermis, and wounds treated this way tended to heal much more rapidly.

Pharmacological modifications of MSC therapy

Another approach to treatment modification, which remains poorly characterized, are attempts to improve MSC therapy outcomes using pharmacologi-

cally active substances which may increase the secretory function, survival, and acceptance of the cellular graft.

Eunhui et al. reported a synergistic effect of subcutaneous administration of ASC with exendin-4 (Ex-4), a glucagon-like peptide receptor-1 agonist [16]. The combination of cellular therapy with this drug accelerated wound healing in diabetic mice compared to the control groups receiving each component separately. Proliferation was found to be increased by either Ex-4 or ASC, and the best result was achieved with the combination. It was also shown that both Ex-4 and ASC increased VEGF expression, while the combination had no synergistic effect in this regard.

Oses et al. [17] showed that preconditioning of ASC with 150 μM or 400 μM deferoxamine (DFX) for 48 hours induced a dose-related increase in hypoxia-inducible factor 1 alpha (HIF-1α) expression. The authors also noted significant increases in mRNA levels of proangiogenic (VEGF-A and angiopoietin-1 [ANG-1]), neuroprotective (glial cell line-derived neurotrophic factor [GDNF], nerve growth factor [NGF], and neurotrophin-3 [NT3]), and anti-inflammatory (interleukin [IL]4 and IL5) factors as evaluated by qualitative reverse transcription polymerase chain reaction (RT-qPCR). Using ELISA, it was then shown that the increase in mRNA levels of the above factors correlated with an increase in VEGF and IL4 levels in ASC secretomes, and only NGF did not show a significant increase. Oses et al. also showed that ASC preconditioning using DFX led to a marked increase in antioxidative properties of the secretomes that was related to DFX level. The authors also proved the cytoprotective role of ASC preconditioning using DFX, as the procedure markedly increased the survival of dorsal root ganglion neurons in the settings of high glucose levels compared to neurons subjected to untreated ASC.

Another group of agents that may pharmacologically enhance MSC effects are statins. Li et al. [18] used flow cytometry to show that MSC pretreatment with atorvastatin (ATV) increased expression of chemokine receptor 4 (CXCR4) which plays a major role in MSC migration to their target sites. The authors induced cardiac ischemia in three groups of rats which were than administered MSC (pretreated with ATV), MSC and phosphate-buffered saline (PBS). When evaluated using confocal microscopy, myocardial specimens sampled 3 days after cell therapy application showed an increased number of MSC in the ATV-pretreated group. The longest graft survival was also noted in this group, which indicates that pretreatment with ATV resulted in an increased MSC survival. However, these promising effects of ATV pretreatment on MSC require further studies on cell survival, particularly within the wound.

The effect of wound environment in diabetic patients on the efficacy of MSC treatment

Conditions of hypoxia present in the wound environment in diabetic patients, along with increased glucose levels, are a major problem limiting cell survival, differentiation and function, and impair effective healing of the damaged skin covering in the diabetic foot syndrome. Multiple studies that evaluated the effect of hypoxia on ASC survival in the wound showed that cells were able to adjust to these conditions. Lafosse et al. [19] reported that the microenvironment of hypoxia and hyperglycaemia did not affect the proliferative capabilities and survival of ASC. In addition, the authors showed that in the settings of hypoxia, VEGF secretion by ASC increased significantly compared to the normoxic settings. It was also shown that the conditions of hyperglycaemia and hypoxia had no effect on the secretion of growth factors by ASC (Fig. 2).

Multiple studies evaluated benefits of incubating BM-MSC in hypoxic conditions prior to their injection into the ischemic limb. They study by Tong et al. [20] involved placing BM-MSC on a three-dimensional biomimetic scaffold and then translocating this graft to a wound on a ischemic limb of a rat with induced diabetes. It was shown that maintaining the graft in hypoxic conditions prior to application to the wound resulted in an increased expression of proangiogenic factors in BM-MSC, including HIF-1α, VEGF, and PDGF. In animals treated with a hypoxia-incubated graft, more rapid wound healing in the ischemic limb was shown compared to the group grafted with normoxia-incubated BM-BMC. Graft biopsy showed nearly physiological layer restoration, including complete reepithelialization with hair follicles present, in the group treated with a hypoxia-incubated graft, while only epidermal thickening and poorly vascularized granulation tissue were noted in other treatment groups. The theory of benefits from MSC incubation in hypoxic conditions prior to their use in vivo has been supported by the study by Liu et al. [21] who showed that this procedure significantly increased expression of VEGF-A, HIF-1α, HGF, basic fibroblast growth factor (bFGF), matrix metallopeptidase 9 (MMP-9) and PDGF in BM-MSC incubated in hypoxic conditions (2%, 5% or 7% O_2). It was also noted that gene expression was most increased in BM-MSC incubated in 5% O_2 for 48 hours. The authors also showed that MSC incubation in hypoxic conditions for 48 hours did not affect their survival and phenotype but was associated with an increased expression of antiapoptotic Bcl-2 protein.

Another report on changes in the morphology and function of ASC subjected to hypoxia comes from

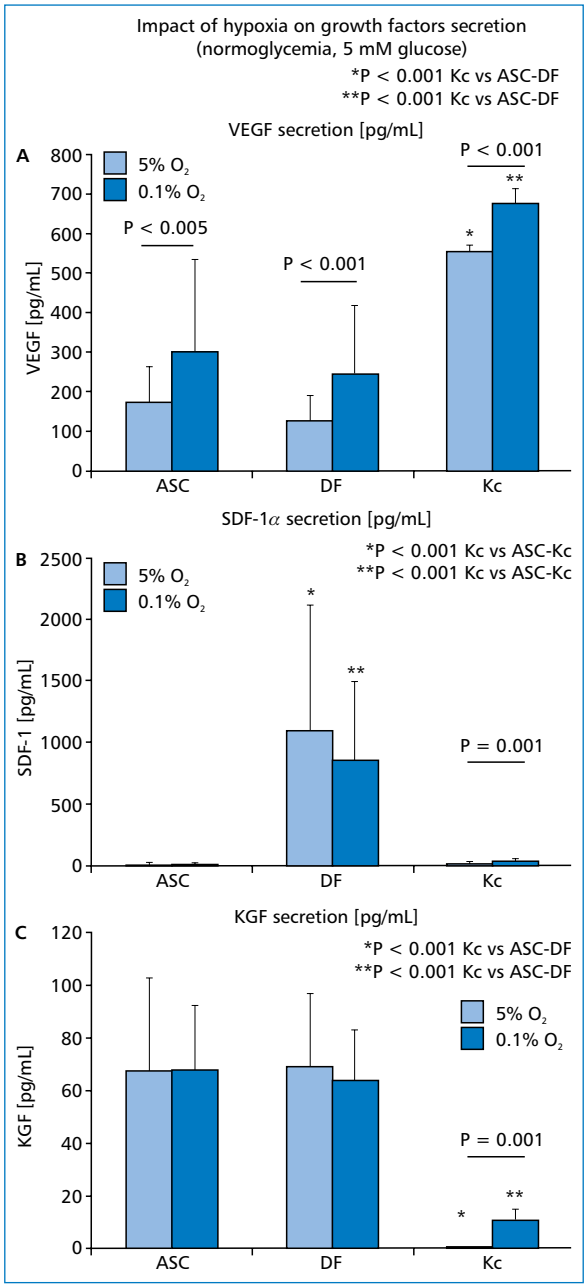

Figure 2. Influence of the hypoxic environment on secretion of VEGF, SDF-1a, KGF by: ASC, fibroblasts located in the skin (DF), keratinocytes (Kc). A — influence of the hypoxic environment on VEGF secretion by ASC, DF, Kc. VEGF secretion was significantly higher for ASC in hypoxia environment (compared to normoxia environment) for ASC ($P < 0.005$), DF ($P < 0.001$) and Kc ($P < 0.001$); B — influence of the hypoxic environment on secretion of SDF-1a by ASC, DF, Kc. SDF-1a was mostly secreted by DF ($P < 0.001$ compared to ASC and Kc). ASC did not secrete SDF-1a. Kc significantly increased secretion of SDF-1a in hypoxia ($P = 0.001$); C — influence of the hypoxic environment on secretion of KGF by ASC, DF, Kc. KGF was secreted mostly by ASC and DF, but its secretion changed only by Kc under hypoxic conditions ($P = 0.001$). Used with permission PLOS Medicine, based on [19]

a 2017 study by Schive et al. [22] in which ASC were harvested from patients and incubated for two days in the conditions of hypoxia (1% O_2, PT-ASC) or normoxia (21% O2, NT-ASC). Cellular viability, growth and differentiation, surface markers, and factors released to the incubation medium were compared. In addition, pancreatic islets were harvested from another group of patients and then subjected to the substances present in the incubation media in the PT-ASC and NT-ASC groups. In these settings, pancreatic islet cell secretory function and their propensity to apoptosis following exposure to proinflammatory cytokines were evaluated. The authors showed that incubation in hypoxic conditions was well tolerated by ASC which also showed increased production of VEGF-A, FGF2, and β-nerve growth factor (βNGF) compared to the cells incubated in the normoxic conditions. However, reduced expression of HGF, IL8, and chemokine ligand 1 (CXCL1) was shown in the PT-ASC group. In addition, the authors showed that pancreatic islets incubated in the medium from the PT-ASC group were characterized by an increased secretory function and lower rate of apoptosis following exposure to proinflammatory cytokines compared to those incubated in the medium from the NT-ASC group. The last step of the study was to evaluate the cytoprotective function of ASC incubated in the hypoxic conditions. For this purpose, they were administered, along with ASC incubated in the normoxic conditions, to immuno-incompetent mice with pancreatic islet inflammation induced by streptozotocin. The control group were animals administered PBS. In mice in the PT-ASC group, significantly lower plasma glucose levels were noted as early as at 4 days, accompanied by lower blood glucose levels in the oral glucose tolerance test (OGTT). Murine organs were tested with polymerase chain reaction (PCR) for the presence of human DNA. Most samples showing human genetic material were derived from mice in the PT-ASC group, which may suggest longer graft survival. The study by Chen et al. [23], which evaluated MSC functioning in the conditions of hypoxia, showed that by releasing exosomes, these cells may protect others from hypoxia-induced apoptosis. The authors showed that the rate of beta-cell apoptosis in the hypoxic settings was as much as 28.5%, compared to only 10.9% in the normoxic settings. To evaluate cytoprotective properties of MSC, the authors incubated beta cells in the hypoxic settings with an addition of increasing levels of exosomes produced by MSC (0, 6.25, 12.5, 25, 50, 100, 200 μg/mL). The showed that low MSC exosome doses (6.25, 12.5 μg/

/mL) had no effect on beta cell survival in the hypoxic conditions, while high exosome doses (25, 50, 100, 200 μg/mL) markedly improved beta cell survival in the hypoxic conditions in a dose-related fashion.

Stem cells used in the above studies were derived from bone marrow where hypoxia is physiologic, with the mean oxygen content ranging from 5.4% O_2 to 7% O_2 [24]. For this reason, studies are also need to evaluate the effect of hypoxia on the function and efficacy of MSC derived from places other than bone marrow.

Impaired function of stem cells derived from the adipose tissue in diabetic patients

Adipose tissue derived stem cells harvested from diabetic patients are much less efficient in accelerating wound healing compared to those harvested from healthy subjects. One reason for this reduced effectiveness may be reactive oxygen species. In a study in a murine model published in 2019 by Lian et al. [25], ASC were harvested from mice with induced diabetes and incubated with a mitochondrial enzyme scavenging reactive oxygen species (mitoTEMPO), a non-mitochondrial version of the enzyme (TEMPO), or without these enzymes. The authors showed that in the hypoxic settings, expression of proangiogenic *Hif-1a*, *Vegfa*, and *Sdf-1a* genes was higher in diabetic ASC (dASC) treated with mitoTEMPO (mitoT-dASC group) compared to the dASC group. In addition, elevated levels of VEGF-A, HGF and FGF2 were detected in the medium in the mitoT--dASC group. The authors concluded that incubation with mitoTEMPO increased the mitochondrial antioxidant capacity of dASC, as evidenced by increased levels of antioxidant enzymes. The authors postulated that the increased amount of mitochondrial antioxidants contributed to an elevated proangiogenic potential of these cells. To determine whether cellular preincubation with mitoTEMPO may increase the efficacy of dASC in vivo, the authors developed a model of critical limb ischaemia in diabetic mice. Ischaemic mouse limbs were injected intramuscularly with PBS, ASC harvested from non-diabetic mice (nASC), dASC, or mitoT-dASC. Survival of the transplanted ASC in diabetic mice was then evaluated using bioluminescence imaging. It was shown that the intensity of bioluminescence signal over the following days was lower in the dASC group compared to nASC and mitoT-dASC. Bioluminescence signal was not detected in the dASC group at 28 days after cell transplantation, while it was still present in mice treated with nASC or mitoT-dASC, which suggests that prior incubation with mitoTEMPO increased dASC survival in the critically ischemic mouse limb. Incubation with mitoTEMPO also improved the function of dASC. Limb loss or necrosis were noted at 28 days in nearly all mice in the PBS group, while the rates of limb loss or necrosis were lower, and the rates of limb salvage were higher in mice treated with nASC and mitoT-dASC compared to those treated with dASC. The amount of VEGF, a key promoter of angiogenesis, was higher in the nASC and mitoT-dASC groups compared to the dASC group, as evaluated by the Western blot. Staining for angiogenesis marker CD31 showed more microvessels in the nASC and mitoT-dASC groups compared to the dASC group.

Support for superior quality of an allogeneic graft from a non-diabetic donor compared to an autologous graft from the diabetic host was also provided by a 2017 study by Peng et al. [26] who compared the functionality of ASC harvested from mice with induced diabetes (dASC) and the same cells modified using lentivirus (E-dASC) to increase expression of glyoxalase-1, an enzyme that utilizes reactive oxygen species. Both types of cells were compared in the setting of increased glucose levels. The authors reported that the study group with an increased expression of glyoxalase-1 showed increased proliferation and reduced apoptosis, as measured using the Annexin V/PI kit and the Western blot. In addition, modified ASC showed an increased expression of antiapoptotic Bcl-2 protein and a decreased expression of proapoptotic Bax protein. The study also showed an increased migratory capability of modified E-dASC, which seems a necessary factor for these cells to reach their target sites. The authors also noted an increased expression of genes involved in angiogenesis compared to controls. To confirm increased angiogenic capabilities of these cells, the authors implanted them using their murine model of induced critical limb ischemia. Reperfusion was noted to be more frequent and more rapid when modified ASC were used. All these results using modified ASC were comparable to the results obtained using ASC harvested from non-diabetic mice.

In the study by Lafosse et al. [19], ASC harvested from patients with type 2 diabetes and healthy individuals were evaluated in vitro. Cell survival, proliferation, KGF and VEGF expression, and the effects of low oxygen and high glucose levels (conditions typical for the diabetic foot syndrome) were compared. The authors did not significant differences in terms of isolation and proliferation capabilities between ASC harvested from diabetic and non-diabetic individuals. Release of KGF was significantly lower in ASC harvested from diabetic individuals, while VEGF release was not lower in these cells.

Conclusions

Use of MSC to treat wounds in diabetic patients is one of the most promising therapeutic options. Multiple studies provided information on various modifications of this therapy to achieve optimal treatment outcomes. The most promising strategies to increase the effectiveness of MSC therapy include modifications of the medium serving as cell carrier for their application into the wound, and of incubation conditions prior to cell application. One such approach is to use specific suspensions as the medium and scaffold for MSC application. Already at this stage, opportunities arise to enrich the medium in active substances, modify the content of cellular suspension, or add specific drugs, all potentially increasing the excretion of growth factors that are deficient within the wound in diabetic patients. Treatment effectiveness is also affected by the appropriate choice of MSC donor. It was shown that transplantation of cells obtained from a healthy person (allogeneic graft) is more effective that transplantation of cells obtained from the diabetic host (autologous graft).

Studies on MSC use in human models are currently underway and they may be expected to provide better therapeutic algorithms and optimized patient treatment outcomes.

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Conflict of interest

The authors declare no conflicts of interest.

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