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CURRENT OPINION

Cell Therapy of Severe Ischemia in People with Diabetic Foot Ulcers—Do We Have Enough Evidence?

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Abstract

This current opinion article critically evaluates the efficacy of autologous cell therapy (ACT) for chronic limb-threatening ischemia (CLTI), especially in people with diabetes who are not candidates for standard revascularization. This treatment approach has been used in 'no-option' CLTI in the last two decades and more than 1700 patients have received ACT worldwide. Here we analyze the level of published evidence of ACT as well as our experience with this treatment method. Many studies have shown that ACT is safe and an effective method for patients with the most severe lower limb ischemia. However, some trials did not show any benefit of ACT, and there is some heterogeneity in the types of injected cells, route of administration and assessed endpoints. Nevertheless, we believe that ACT plays an important role in a comprehensive treatment of patients with diabetic foot and severe ischemia.

1 Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia persisting for a prolonged period. The progression of this disease can lead to several chronic complications such as microvascular (diabetic kidney disease, retinopathy and neuropathy) and macrovascular changes that can result in ischemic heart disease, stroke or diabetic foot ulcers (DFUs). Although DM represents is one of the greatest epidemics of this century, the strategies to treat DM and complications associated with this disease are still very much limited [1]. For diabetic patients with severe lower limb ischemia and DFU, autologous cell therapy was proposed to be the most promising option. However, despite trials with a wide range of application, such treatment still has several limitations. Globally there are over one million people with diabetes who lose a limb as a consequence of a foot ulcer. Therefore, DFU represents one of the major complications for people with diabetes [2]; it occurs in 15% and precedes 84% of all diabetes-related lower limb amputations [3]. In addition, increased mortality is seen after lower limb amputation (LLA) associated with DM and can be up to 50% 3 years after LLA [4]. Furthermore, 25% of patients with healed DFU experience a recurrence within 1 year after

Key Points

Autologous cell therapy is a novel treatment for patients with most severe stages of limb ischemia [mainly no-option chronic limb-threatening ischemia (CLTI)] and has been shown to be safe and effective in the improvement of ischemic parameters in the ischemic lower limb.

The current level of evidence is very heterogenous because there are different types of cell population, cell sources, mode of administration, and dose of the cell products used in these patients with no-option CLTI.

Future perspectives for this kind of treatment will be to advocate this treatment in patients with an advanced stage of ischemia or to use cell therapy as advuvant treatment to standard revascularization techniques.

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Table 1 Types of cell therapies

Cell population	Acronym	Source	Surface markers (phenotype)
Bone marrow mononuclear cells	BMMNC	Bone marrow	$CD34^+$, PROM1 $CD133^+$, KIT (C-Kit)$^+$, $CD14^-$, $CD45^-$
Peripheral blood mononuclear cells	PBMNC	Peripheral blood Cord blood	$CD34^+$, $CD14^-$, $CD45^-$
Mesenchymal stem cells	MSC	Bone marrow Adipose tissue Cord blood Muscle tissue Skin Heart	$CD73^+$, $CD90^+$, 105^+, $CD34^-$, $CD45^-$, $D11b^-$, $CD14^-$, $CD19^-$, $CD79a^-$, HLA-DR$^-$
Myeloid angiogenic cells	MAC	Mononuclear fraction of peripheral blood	CD45+, CD14+, CD31+
Endothelial colony forming cells	ECFC	Cord blood Mononuclear fraction of peripheral blood	CD31+, CD105+, CD146+, CD 34+

healing and around 75% within 5 years [5]. Therefore, it is apparent that DFU and amputations significantly decrease quality of life, with high mortality. To prevent these complications, the current therapy of DFU includes wound debridement and dressings, avoiding increased plantar pressure, antibiotic treatment, revascularization, and adjunctly also advanced therapies such as granulocyte colony-stimulating factor applications, platelet rich plasma, etc. [6]. However, all these therapies have various limitations and are not able to sufficiently prevent people developing a foot ulcer or result in healing of the ulcer. Therefore, the search for new therapies is urgently required. One of these approaches could be autologous cell-based therapy, and we discuss the trials performed using this mode of treatment, and the outcomes and clinical applications.

The aim of this article was to analyze the level of published evidence of autologous cell therapy (ACT) by means of cell populations, safety, efficacy and niche indications, as well as our experience with this treatment method.

2 Methods to Assess the Effect of Cell Therapy

2.1 Transcutaneous Oxygen Pressure ($TcPO_2$) Measurement

Assessment of the effect of tissue oxygenation is mainly done by the measurement of $TcPO_2$, which is a well-recognized and reliable standard method for non-invasive evaluation of limb ischemia, especially in diabetic patients with chronic limb-threatening ischemia (CLTI), and serves as the main parameter of the therapeutic effect in studies on stem cell therapy in CLTI [7–10]. This method measures the amount of oxygen that has diffused from the capillaries, through the epidermis, to a Clark-type electrode at the measuring site. It provides constant, continuous information about the body's ability to deliver oxygen to the tissue. Any impairment in the ability to deliver oxygen to the tissue will be observed immediately since the skin is ranked very low in the body's system of oxygenation priority, especially the acral parts. $TcPO_2$ measurements usually require at least two to three sites on the dorsum of the measured foot, preferably four or more, to indicate a good clinical response of the oxygenation status of the skin. Reference values for $TcPO_2$ are: 50–70 mm Hg for normal values, < 40 mm Hg for impaired wound healing, and < 30 mm Hg for critical limb ischemia. Panunzi et al. observed a correlation between an increase in $TcPO_2$ and the response to cell therapy using peripheral blood mononuclear cells (PBMNCs) [11]. Patients who recovered without a major amputation after 1 year had higher $TcPO_2$ levels at the end of PBMNC therapy compared to those without ulcer healing (see Table 1).

2.2 Magnetic Resonance Spectroscopy

Phosphorus (31P) magnetic resonance spectroscopy (MRS) allows non-invasive in vivo monitoring of metabolites containing phosphorus, and thus, in effect, energy metabolism [12]. Dynamic 31P MRS is a method used to study changes in muscle metabolism not only at rest but also during and after exercise, which is not possible by other methods [13]. The principle of the method is in vivo monitoring of normal or pathological biochemical processes associated with phosphorus-containing metabolites, i.e. ATP, phosphocreatine, phosphomonoesters, phosphodiesters and inorganic phosphate. In addition to these compounds, whose signals are visible in the 31P MR spectrum, other parameters characterizing the measured tissue can be calculated from dynamic MR spectra, such as pH, mitochondrial capacity, or the recovery time of phosphocreatine to its resting concentrations.

2.3 Skin Perfusion Pressure

Skin perfusion pressure (SPP) is the parameter that is required for the restoration of microcirculatory or capillary flow following controlled occlusion and subsequent flow return [14]. Overall, the advantages of SPP measurement include noninvasiveness, high reproducibility, and independence from the influence of vascular calcification compared with other indices for peripheral circulation assessment. Furthermore, SPP has been shown to be a good predictor of wound healing in patients with lower limb ischemia [15].

2.4 Contrast Ultrasound

Contrast-enhanced ultrasound (CEU) is sometimes used for the assessment of microvascular blood flow (perfusion) in various human tissue beds, and a limited number of studies have used this technique to assess leg muscle perfusion in patients with limb ischemia [16]. These studies have shown that peripheral arterial disease (PAD) is associated with a reduction in peak muscle perfusion capacity in response to ischemic provocation tests. Using real-time CEU, Meneses et al. showed that PAD has a significant effect on the muscle microvascular perfusion responses to cuff-induced ischemia and submaximal exercise [17].

3 Efficacy of Autologous Cell Therapy

Several reviews, meta-analyses, as well as randomized controlled trials (RCTs) using ACT in patients with CLTI have been published. The most recent meta-analysis published by Pu et al. [18] assessed 630 patients in 12 RCTs and reported a significant improvement in major amputation rates, ABI, $TcPO_2$, and lower limb rest pain scores. Similar results were observed in a meta-analysis published by Sun et al.; these authors also reported improved wound healing and pain-free walking distance [19]. Gao et al.'s meta-analysis included 27 RCTs, which included 1186 patients and 1280 lower limbs, although the majority of studies showed a high risk of bias. They demonstrated that ACT was more effective than conventional therapy with regard to the healing rate of ulcers, and a significant reduction was observed in amputation rates and rest pain scores [20]. These results were confirmed in the diabetes subgroup.

4 Guidelines for Use of ACT in People with Diabetes

CLTI is a new term that replaces the original "critical limb ischemia" as the most severe stage of PAD. Diagnostics and treatment of CLTI have been recently summarized in a guideline document entitled "Global Vascular Guidelines" [21]. In this guideline the recommendation is that the first treatment should be an endovascular approach by percutaneous transluminal angioplasty (PTA) to treat CLTI. A diagnosis of CLTI requires objectively documented atherosclerotic severe PAD in association with ischemic rest pain or tissue loss (ulceration or gangrene) [21, 22]. Ischemic rest pain is typically described as affecting the forefoot, and is often made worse with recumbency while being relieved by dependency. It should be present for > 2 weeks and be associated with one or more abnormal hemodynamic parameters. The decision regarding the optimal revascularization method for CLTI in patients with DFU should be assessed with respect to distribution of arterial occlusion (as described by the new Global Limb Anatomical Staging System—GLASS [21]) and by local expertise [23]. Mostly proximal or very long occlusions should be considered for a surgical approach [24]. Even though very advanced revascularization techniques are available, there are still some CLTI patients who are not eligible for any kind of standard procedures and are at high risk of imminent major amputation [25]. These "no-option" patients could benefit from ACT as one of the key parts of comprehensive therapy [20, 26, 27].

5 Cell Therapy of Chronic Limb-Threatening Ischemia —General Thoughts

Since 2002, many studies have been published on the cell treatment of CLTI, the results of which are not always clear-cut [6]. This may be due to different study designs, statistical analysis and significance, patient selection and standard of care, and several other factors that condition the composition and viability of the respective cell suspension [28]. Several papers have indicated that the angiogenic capacity of some cell populations is greatly reduced in diabetic patients. Fadini et al. demonstrated that diabetes negatively influenced bone marrow architecture and function, impairing the mobilization of immature cells into the bloodstream, thus decreasing the pool of circulating cells with regenerative potential [29]. It has recently been observed that diabetes and PAD are accompanied by bone marrow vasculopathy, neuropathy and adiposity, and the existence of a repair defect driven by the disturbed cross talk between distant organs and ischemic limbs has been described [30]. All these factors can influence the clinical outcome of ACT in diabetic patients. Moreover, it has been shown that diabetes limits the therapeutic effect of mesenchymal stem cells from adipose tissue, and impairs the angiogenic capacity of adipose tissue-derived stem cells [31, 32].

Studies have suggested that inflammation counteracts the regenerative capacity of MSC in diabetic patients, and that the bone marrow and adipose tissue in people with diabetes

are highly inflamed and therefore the MSC regenerative capacity could be reduced [33, 34].

All published studies on autologous cell therapy for CLTI should be considered primarily in terms of their endpoints, which should be based on the mechanism of action of cell therapy at the tissue level. However, the effect of cell therapy on the microcirculation is very difficult to demonstrate morphologically. Some isolated studies have attempted to assess the limb microcirculation morphologically by histological or radiological methods [35], but their reproducibility has not yet been sufficiently verified. Therefore, the question of the effect of cell therapy on CLTI has long been a subject of research.

Almost 20 years ago, some studies on CLTI provided hope for preserving a functional lower limb and avoiding major amputations in patients with otherwise intractable CLTI [36]. Nonetheless, there is still one question that researchers are asking today: Are new therapies, including bone marrow-derived cells or peripheral blood mononuclear cells, effective in treating patients with DFU and PAD for whom standard revascularization is not feasible [37]?

According to both the International DFU Consensus [37] and our own experience, the most appropriate parameter to demonstrate tissue ischemia in the lower limbs in diabetic patients is $TcPO_2$. In contrast to other parameters, it is not dependent on medial sclerosis and is based on the measurement of tissue perfusion by assessing microcirculation through oxygen diffusion into the upper layers of the skin [38, 39]. Other angiological non-invasive methods for assessing limb perfusion (e.g., ankle-brachial index (ABI), toe pressure, or laser Doppler flowmetry parameters) tend to be more variable and are significantly influenced by medial sclerosis.

Many studies use LLA or amputation-free survival (AFS) as the main target outcome. In our experience, amputation is not an optimal target parameter because the healing of diabetic foot ulcers is influenced by many factors (e.g., infection, pain, ulcer size, failure to heal), and patients with DFU require comprehensive podiatric care. The decision to amputate is also variable and is based not only on objective criteria but also on the experience of the treating team and the patient's preferences and physical capacity. It is usually based on the progression of infection and ischemia, but often also depends on psychosocial factors.

As we showed in our previous study, the total amount of vascular precursors (characterized by CD34+) and the number of monocytes and lymphocytes injected into ischemic muscles was comparable for different isolation methods [41]. We used bone marrow-derived mononuclear cells (BMMNCs) separated from iliac crest and PBMNCs isolated from stimulated peripheral blood after preceding stimulation by filgrastim with number of totally injected CD34+ cells at least 5×10^7 in 40–90 mL of cell suspension. Even according to recent publications, it is still unclear which cell subpopulation is responsible for angiogenesis or arteriogenesis after intramuscular injection of autologous precursor cells isolated from different sources [42, 43]. However, when a combination of cell subpopulations and possibly vasculogenic growth factors is applied, a potentiation of the vasculogenic effect often occurs [44].

The effect of cell therapy can also go beyond the circulation benefits. It has been proven that culture of cells in special media promotes the expansion and differentiation of endothelial precursors, increases the number of M2 macrophages (CD206, anti-inflammatory macrophages), and result in significantly less inactivated regulatory T cells (CD4+CD25+CD127+ cells). In addition, the phenotype of expanded cells also revealed a higher number of CD34+ and CD133+ cell populations (which indicates an expanded population of immature EPCs), and also increased the number of CD105+ or CD146+ cell populations. Therefore these cultured cells play an important role in controlling immune regulation and have an impact on the level of inflammatory cytokine release [45].

ACT not only promotes arteriogenesis, but also can induce M2 polarization, which is an essential step towards wound healing. The impaired transition of diabetic wound macrophages from pro-inflammatory M1 phenotypes to anti-inflammatory pro-regenerative M2 phenotypes might represent a key issue for impaired diabetic wound healing [46]. Unfortunately, in diabetic wounds, monocyte polarization towards M2 macrophages is strongly reduced, while inflammatory phenotype M1 polarization is elevated, and this results in poor angiogenesis. Moreover, vasoreparative dysfunction has been observed in diabetic CD34+ stem cells due to impaired autocrine/paracrine function and reduced sensitivity to hypoxia, while the injection of freshly isolated circulating CD14+ monocytes into the ischemic limbs of diabetic mice improves healing and vascular growth, suggesting an important angiogenesis potency of the monocyte population even in diabetic patients.

6 Significant Randomized Controlled Trials

The well-designed RCT RESTORE-CLI showed a significantly longer time to treatment failure (defined as major amputation, death, newly formed gangrene, or worsening of the defect) in the ACT group compared with placebo [44].

Another randomized trial, PROVASA, demonstrated a significant increase in $TcPO_2$ and faster ulcer healing in patients treated with intra-arterial BMMNC injection compared with placebo [47].

Another study, which was partially biased by patient selection and cell product quality, is the often cited randomized, double-blind, placebo-controlled JUVENTAS

trial, which evaluated the efficacy of BMMNCs versus placebo, and was published in 2015 [48]. This study enrolled 160 patients; BMMNCs were injected intra-arterially in three doses 1 week apart, and erythrocyte suspension was administered in the placebo group. Results showed no difference in mortality, the incidence of major amputations, or changes in ischemia parameters (ABI and $TcPO_2$) between the cell group and placebo (this result was in disagreement with the results of other studies published at the same time [47, 49, 50]). It is debatable, for example, that the patients enrolled in this study already had adequate $TcPO_2$ values at baseline (35 ± 22 mm Hg), and thus there was little margin for improvement of ischemia with cell therapy and for demonstrating a difference between the intervention and non-intervention groups. The possibility of improving tissue perfusion by influencing microcirculation with cell therapy has its limits, and the achieved $TcPO_2$ values usually do not exceed the zone of mild ischemia; on the other hand, they are usually sufficient to improve ulcer healing or to relieve pain. Furthermore, it can be argued that ABI as the main evaluation parameter could often be influenced by medial sclerosis in diabetic patients. Patients included in this study also had significant arterial stenoses above the knee in a large percentage of cases, and therefore the effect of cell therapy applied only to the calf muscle may have been questionable in these patients. In our experience, these patients are not suitable candidates for cell therapy if proximal arterial stenoses cannot be effected by other methods. Additionally, in the above study, a relatively small amount of bone marrow was collected for the preparation of the cell suspension—only 100 mL compared to the standard 250 mL in other studies.

In patients with CLTI another RCT demonstrated that individuals who received four repeated BMMNC injections versus one single treatment showed an increase in pain-free walking distance, but not in ABI or pain, 24 weeks after the injections [51]. Kang et al. confirmed that many treatments were more successful than administering a greater number of cells in a single therapy in an animal model of CLTI [52]. Furthermore, Beugels et al. demonstrated in a rat model that centrifuged human BM suspension containing low and medium concentrations of mesenchymal and hematopoietic stem cells significantly improved vascularization in limb ischemia, but that the effect was nearly lost at higher stem cell doses [53].

The cell suspension was then injected intra-arterially, which carries the risk of embolization or "washing away" of the cell suspension. Although intra-arterial injection has been used, intramuscular cell administration has been employed in the majority of cell therapy trials for CLTI. Rigato et al. published a meta-analysis and demonstrated that intramuscular ACT injection was related to better results than intra-arterial administration in no-option patients with CLTI [54]. The main advantage of intramuscular injection of a cell suspension is the local paracrine effect in the ischemic muscle; the cells are able to migrate some distance, which can enhance the therapeutic arteriogenesis in the ischemic tissue [40].

In estimating the number of patients for sufficient study power, the difference between the intervention and non-intervention groups was not appreciated, and the study power was therefore relatively weak.

However, JUVENTAS was not the only study with negative results. Another randomized trial on BMMNC used to treat no-option CLTI patient also showed negative results (MOBILE randomized double blind) where 152 patients with Rutherford stages 4 and 5 and CLTI were randomly assigned to receive BMMNCs or a placebo [55]. There was no significant difference in AFS rates between the two groups after 1 year. However, a 2-year post hoc analysis revealed a significant difference between the BMMNC group and the placebo, with a hazard ratio of 0.49 favoring cell therapy after 52 weeks. The data also found that, whereas BMMNCs provided a significant benefit to patients without diabetes at Rutherford stage 4, diabetic patients and/or those at Rutherford stage 5 received no benefit. These outcomes support previously mentioned data that diabetes has a deleterious impact on BMMNC treatment for peripheral ischemia. Moreover, a retrospective study of 367 patients revealed that Rutherford's stage 5 was the best indication for ACT in patients with PAD [56]. Fifty percent of Rutherford's stage 6 patients who initially suffered from a major tissue loss, overstepping metatarsus phalangeal level (Rutherford's stage 6), all went through a major amputation, implying that treatment was performed in some patients at a too advanced stage

7 Niche Indications of ACT

The question arises whether ACT would not also be beneficial for patients with moderate limb ischemia, either alone or in combination with standard revascularization. This is a change in the current way of recommending cell therapy—so far, this therapy has only been prescribed for patients with CLTI without the possibility of standard revascularization. However, these patients have a very high risk of cardiovascular complications and a relatively high mortality rate, so improving limb ischemia with ACT may not improve survival unless other risk factors for atherosclerosis are modified or if cardiovascular disease is already advanced [57, 58]. Thus, there is still a need for discussion with vascular surgeons and interventional radiologists regarding whether we should consider ACT in people with diabetes who are candidates for repeated PTA, because they should be treated by PTA preferentially.

The concept of ACT as an adjuvant therapy was described in a preliminary study by Persiani et al., who also suggest an adjuvant role of autologous peripheral blood mononuclear cells (PBMNC) implanted in diabetic patients with CLTI [59]. In this study, 32 patients were treated by PBMNC associated with endovascular procedure, and a significant increase in $TcPO_2$ and decrease in visual analogue scale was observed. As mentioned in a recent review [60], one of the first studies that used BMMNC as an adjuvant therapy to vascular bypass was published in 2010 by Kolvenbach et al. [61]. They treated only eight patients in this pilot study and in five of them observed an increase in ABI.

Therefore, we suppose that combination treatment of ACT and PTA should have an additive effect, because the therapeutic intervention is targeted not only to the macrocirculation, but also to the microcirculation. Cell therapy may potentially affect ischemia by tissue remodeling and positive chemotaxis—increase of cell motility [62]. Our assumption is based on cardiological studies that demonstrated improvement of left ventricular ejection fraction after combining cell therapy with standard revascularization [63, 64].

Another interesting area of research has been the indication of ACT for CLTI in patients with renal impairment who have a significantly worse prognosis for healing of DFU [65]. When treating patients with the most severe form of CLTI, our own studies often included patients with severe renal impairment [66]. In most studies, these patients are almost always excluded, and our study was the first to evaluate the effect of cell therapy in patients with severe diabetic kidney disease (sDKD). We demonstrated that cell therapy leads to a significant rise in $TcPO_2$ even in patients with severe kidney disease, and that it leads to a longer AFS in patients with sDKD compared to conservative treatment [67]. All published studies evaluating the effect of DKD on healing, amputation, and ischemia parameters agree that higher stages of DKD or dependence on hemodialysis significantly worsen all these parameters [65, 68]. On the other hand, the US Nationwide Inpatient Sample study showed at 10-year follow-up that even in patients diagnosed with DKD, endovascular and surgical revascularization treatment is appropriate, as these treatments have been shown to reduce the incidence of high amputations and improve quality of life [69].

8 Do All Patients Respond to Cell Therapy?

During our long-term experience with CLTI cell therapy, we have observed a lack of effect of this treatment in some patients (so-called non-responders). Therefore, we decided to analyze the factors responsible for the reduced response of patients to autologous cell therapy [70]. The most striking finding was that the main independent factors in non-responders were thrombophilic mutations (Leiden and MTHFR), while classical expected factors such as low number of injected CD34+ cells, low product viability, infection or duration of diabetes showed no significant difference between responders and non-responders. We believe that innate thrombophilias may contribute to micro thrombosis downstream of vasculogenesis after cell therapy application; however, other pathogenetic factors may also play a role such as mural thrombosis, smooth muscle cell proliferation and increased inflammatory response.

Also, other studies demonstrated that the number of CD34+ cells was not correlated with amputation and/or wound healing. Liotta et al. showed that while the number of CD14+CD34low cells from peripheral blood was positively correlated with calf time to peak (TTP) and muscle reperfusion, there was no significant correlation between TTP and total leukocytes or number of CD34+ cells [71]. These results are in accord with the outcomes observed by Moriya et al. where CD34+ cells were not correlated, but the levels of VEGF were correlated, with increase response to the ACT [72]. From the published data as well as from our own experience we believe that the revascularization effect of ACT is based on a different cell population than CD34+.

Gemnati et al. demonstrated that the MTHFR 677 mutation may increase the risk of not only venous but also arterial thrombosis [73]. In the study by Pan et al. evaluating response to cell therapy, the following factors were identified: older age, lower baseline $TcPO_2$, lower CD34+ cell dose, increased fibrinogen, and arterial occlusion above the knee, all of which led to worse outcomes [74]. However, in the latter study, unlike ours, only 6.8% of the subjects had diabetes and 78% had a diagnosis of thromboangitis obliterans, and therefore only 9% had PAD; also, their definition of non-responders differed—it was defined as lack of remission of critical limb ischaemia within 6 months.

In another study published by Madaric et al., a non-responder was defined as a patient with major amputation or progression of critical ischemia as defined by the Rutherford classification [75]. In this study, low baseline $TcPO_2$ and a low amount of injected CD34+ cells were found to be the most important factors; however, the difference from our study was that in the Madaric study half of the patients treated with BMMNCs were treated intra-arterially, and in our study the BMMNC suspension was injected intramuscularly.

9 Safety of ACT?

Very few adverse events have been reported in studies of cell therapy for PAD. The most frequently described major amputation rates are thought to be a consequence of pre-existing disease (diabetic foot and severe PAD) rather than

a side effect of ACT [7]. Hypothetical side effects of this therapeutic modality such as worsening of diabetic retinopathy or acceleration of tumor growth have not been observed in previously published studies [18, 76]. The study published by Murphy et al. was primarily focused on the safety of BMMNC treatment [77]. All patients underwent physical examination, ECG, and biochemical and hematological sampling before therapy and subsequently at intervals of 1 day; 4, 8, and 12 weeks; and at 6 and 12 months. Only two patients experienced adverse effects up to 12 months into therapy: one patient developed ST depression on ECG a few days after the procedure and a decrease in hemoglobin requiring blood transfusion; cardiac enzymes were not elevated. The other patient developed microembolism on follow-up angiography performed 12 weeks after therapy; this microembolism was resolved surgically without complications. Overall, the study rates the treatment of PAD with BMMNCs as safe. No acceleration of atherosclerosis was observed in patients treated with cell therapy in any of the clinical trials [18, 78]. The authors of published papers agree on the need for more studies with longer follow-up [7].

10 Which Cell Population is the Best for Therapeutic Arteriogenesis

10.1 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are one of the cell populations involved in tissue regeneration [79]. Based on the definition of the International Society for Cell Therapy, MSCs are characterized by the expression of CD105, CD90, and CD73 surface markers and the absence of expression of hematopoietic and lymphocyte lineage markers CD14, CD31, CD34, CD45, and CD11b [80]. These cells must also be able to differentiate into osteoblasts and adipocytes under standard culture conditions and adhere to the plastic surface when cultured in vitro [81]. MSCs also induce vasculogenesis by collateral formation in ischemic rats via human basal fibroblast growth factor (FGF) [82]. They also have the ability to migrate to the site of injury. MSCs have anti-inflammatory effects and exert suppressive effects on cells of the immune system. Because of their properties, they are therefore widely used in the treatment of ischemic defects in people with diabetes [83, 84]. MSCs are involved in tissue healing by producing cytokines and growth factors and differentiating into the necessary cell types [85]. The effect of MSCs on neovascularization in ischemic muscle was described in a study comparing the effect of BMMNCs versus bone marrow-derived MSCs (BMSCs), which showed significantly more healed ulcers in the BMSC-treated group compared to the BMMNC group [86]. Huerta et al. showed in their recent review that ongoing clinical trials concerning MSC from bone marrow and adipose tissue may offer hope to a patient population that continues to expand owing to the increasing prevalence of PAD, diabetes, and cardiovascular disease [87]. Even though amputation rates have not been significantly impacted compared to placebo, several important issues should be taken into consideration [88]. Given the frailty of this patient population, studies have been of low power due to decreased sample sizes, and subsequent interpretation of long-term effects remains challenging.

10.2 Myeloid Angiogenic Cells

Myeloid angiogenic cells (MACs), an early form of endothelial precursor cells (EPCs), are characterized by the expression of the surface markers CD133 and CD117 [89]. The expression of the marker CD133 can be observed in hematopoietic stem cells, fetal brain cells, and embryonic epithelial and other cells, and is considered a marker of immaturity of the cells [90]. The marker CD117, also referred to as c-kit, is expressed in, for example, melanocytes, germ cells, Cajal cells, or epithelial cells [91–93]. Furthermore, MACs are CD45 positive but negative for the markers CD34 and CD146 [94, 95]. These undifferentiated cells play a role in the healing of many tissues due to their ability to differentiate into different cell types [96]. MACs also play a role in reducing the progression of atherosclerotic changes in blood vessels [97]. Wong et al. demonstrated that MACs can be sourced under xeno-free conditions paving the way for their safe clinical application [98]. Since these cells can be easily accessible from peripheral blood, their therapeutic potential could exceed traditional MSCs in the treatment of CLTI.

10.3 Endothelial Colony-Forming Cells

In contrast, endothelial colony-forming cells (ECFCs), which represent more mature EPCs, are characterized by surface markers of CD34 and CD31, together with vascular endothelial growth factor (VEGF) receptor and FGF receptor, von Willebrand factor production, and endothelial nitric oxide synthase [99, 100]. ECFCs appear to be more favorable candidates for vascular regeneration compared to MACs because of their vasculogenic potential even in the intact vessel wall [101, 102]. ECFCs are CD45 negative but positive for the phenotypic marker CD146. These facts are reflected in subsequent responses in ischemic tissue. If the tissue is affected by ischemia or inflammation, VEGF, FGF, and other factors are released by mature EPCs. These are subsequently responsible for a cascade of events leading to capillarogenesis and the formation of new collaterals [28].

10.4 Peripheral Blood Mononuclear Cells

A recent meta-analysis demonstrated that PBMNCs, but not other cell types, significantly reduced amputation rates and AFS. BMMNCs enhanced wound healing considerably, although both BM and PBMNCs increased ABI, $TcPO_2$, and rest pain score [54]. Moreover, PBMNC showed promising results in several clinical trials. The previously mentioned study by Persiani et al. showed the benefit of PBMNC therapy either alone for no-option CLTI patients or as an adjunctive therapy together with endovascular reconstruction [59]. The authors observed a significant increase in $TcPO_2$ and a decrease in VAS. Scatena et al. published a study that included 76 no-option CLTI patients with DFUs [103]. Thirty-eight patients were treated with PBMNC and 38 patients conservatively, which formed the control group. Only four out 38 amputations (10.5%) were observed in the PBMNC group, while 15 out of 38 amputations (39.5%) were recorded in the control group ($p = 0.0037$). The Kaplan–Meier curves and the log-rank test results showed a significantly lower amputation rate in the PBMNCs group versus the control group. Panunzi et al. treated 50 patients with PBMNCs and observed a significant increase in $TcPO_2$ (17.2 ± 11.6 vs. 39.1 ± 21.8 mm Hg; $p < 0.001$) and increased ulcer healing in 1.5-year follow-up [11].

Moreover, the PBMNC treatment was included as the most promising cell therapy in the international Union of Angiology Position Statement on no-option CLTI [104].

11 Conclusion

In this review we have attempted to provide comprehensive evidence that ACT has a very important role in the treatment of diabetic patients with no-option CLTI, mainly as a part of the comprehensive treatment of those patients. Even in accordance with the Global Vascular Guidelines [21], this treatment was scored as 1B (strong grade and moderate level of evidence), and those guidelines recommend to "restrict use of therapeutic angiogenesis to CLTI patients who are enrolled in clinical trials." ACT in diabetic patients with CLTI enhanced microcirculation in most of the published studies. This therapy does not achieve limb salvage in all cases, whereas through the improvement in small vessels it can increase the chance of healing wounds after minor amputation even in most severe ischemic limbs. Most of the studies are in agreement that cell therapy is an effective treatment approach that improves ischemia parameters and therefore improves the quality of life of ischemic patients. Several randomized controlled trials are ongoing (e.g., NCT03968198, NCT04466007, and NCT04661644), and they can help to confirm or disprove the beneficial effect of ACT on limb ischemia and quality of life of people with diabetes.

Declarations

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