Autologous Bone Marrow Aspirate Stem Cell Concentrate (BMAC) for treatment of patients with Fontaine stages III-IV peripheral arterial disease: a pilot safety and feasibility study

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Abstract

Objective: Peripheral arterial disease (PAD) is a chronic arterial occlusive disease which mostly affects the arteries of the lower extremities. PAD morbidity generally ranges from 3% to 10%, although it increases with advancing age and can be greater than 15% in subjects over 60 years of age. In small trials, autologous bone marrow aspirate stem cell concentrate (BMAC) has proven to be safe and effective in promoting angiogenesis in patients with PAD. In this small pilot, open-label, non-randomized, non-controlled study, we assessed the safety and feasibility of periarterial BMAC injection and implantation for treatment of patients with PAD who are not susceptible to conventional endovascular or open revascularization.

Patients and Methods: A total of 27 patients with non-revascularizable CLI (Fontaine stages III and IV) were enrolled between 2015 and 2019. Comorbidities and risk factors for PAD such as diabetes, hypertension, dyslipidemia and cigarette smoking were also evaluated.

Results: A total of 27 patients (M/F: 25/2) with non-revascularizable critical limb ischemia (stages III and IV according to Fontaine classification) were enrolled between 2015 and 2019. Comorbidities and risk factors for PAD such as type 2 diabetes, hypertension, cigarette smoking and dyslipidemia were also evaluated. The prevalence of cigarette smoking was high (74%; 20 patients). During the follow-up, only 4 patients underwent amputation of the limb affected by critical ischemia. In such subgroup, all patients had a Fontaine stage IV PAD. The remaining 23 patients showed a remarkable improvement in clinical features and ankle-brachial index (ABI) values, without undergoing lower-limb amputation. Computed tomography angiography (CTA) performed 3 months after the periarterial BMAC injection confirmed the presence of neovascularization in 24 out of 27 patients. The procedure was well-tolerated by all patients.

Conclusions: Our study showed that periarterial BMAC injection is a safe and feasible approach for obtaining clinical improvement and short-term induction of neovascularization in patients with severe PAD.

Introduction

Peripheral arterial disease (PAD) is a chronic arterial occlusive disease which mostly affects the arteries of the lower extremities. PAD includes different non-coronary arterial syndromes characterized by structural and functional alterations of the arteries that supply brain, visceral organs and upper and lower extremities. Atherosclerosis represents the most common cause of PAD, although other possible etiologies include arteritis, peripher-

al arterial aneurysms and thromboembolic events[1]. PAD morbidity generally ranges from 3% to 10%, although it increases with advancing age and can be greater than 15% in subjects over 60 years of age[1].

Autologous bone marrow aspirate stem cell concentrate (BMAC) has proven to be safe and effective for treatment of patients with PAD[2]. Moreover, autologous implantation of bone marrow-mononuclear cells appears to be safe and effective in patients with PAD for achievement of therapeutic angiogenesis, due to the ability of these cells to supply endothelial progenitor cells (EPCs) and to secrete several angiogenic factors or cytokines[2]. Preclinical studies have shown that implantation of bone marrow-mononuclear cells (including EPCs) into ischemic limbs promotes collateral vessel formation[3]. Critical limb ischemia (CLI), which is an advanced stage of PAD, is a serious condition, as it often leads to disability and is associated with high mortality rates[1,4]. In light of the potential contraindications or low efficacy of conventional treatments, cell therapy - particularly transplantation of mesenchymal stem cells (MSCs) - represents a promising approach to treat patients with PAD and CLI[5]. PAD is a major health problem and approximately 1% to 2% of patients with PAD progress towards CLI, which is characterized by chronic ischemic rest pain, ischemic ulcers or gangrene[6].

For a substantial number of patients with CLI, no effective therapeutic options other than amputation are available; approximately a quarter of these patients will require a major amputation during the first year after diagnosis[6]. Local and regional ischemia follow a specific clinical progression, starting with claudication, referred as muscle pain distal to the site of occlusion and induced by predictable degrees of exertion. In a more advanced stage, CLI presents with rest pain typically occurring at the level of the metatarsal bones; the pain is often unrelenting and relieved by dangling the foot. As arterial blood flow decreases below a critical perfusion pressure, ulceration or frank ischemic necrosis ensues. This symptomatic progression can be classified using Fontaine and Rutherford classifications[7].

Fontaine classification describes four stages of lower extremity arterial disease[8], namely:
- Stage I: defined as asymptomatic; this stage includes patients who are asymptomatic, but in whom a careful history may reveal subtle, non-specific symptoms, including paresthesia; physical examination may reveal reduced peripheral pulse, cold extremities, and murmurs in the peripheral arteries.
- Stage II: characterized by the presence of intermittent claudication; at this stage, patients often have a constant distance at which the pain occurs. This stage is further divided into two sub-stages: i) stage IIa, characterized by intermittent claudication after more than 200 meters of walking, and ii) stage IIb, characterized by intermittent claudication after less than 200 meters of walking.
- Stage III: characterized by rest pain (occurring especially during the night).
- Stage IV: characterized by the presence of ischemic ulcers or gangrene, which can be dry or humid[8].

The diagnosis is confirmed by peripheral vascular examination including pulse-Doppler and assessment of the Ankle-Brachial Index (ABI), which is defined as the ratio of the systolic blood pressure at the ankle to the systolic blood pressure in the upper arm. Normal ABI values range from 0.91 to 1.40 (values of 0.91 to 0.99 are considered "borderline"). ABI values ≤0.9 are considered diagnostic of PAD, whereas values lower than 0.5 suggest the presence of severe PAD. ABI values >1.40 are also considered abnormal and indicate the presence of noncompressible calcified arteries[9]. Additional imaging techniques, such as conventional angiography, computed tomography angiography (CTA) and/or magnetic resonance angiography (MRA), can help to localize the sites of arterial occlusion and to determine if arterial bypass surgery is feasible[6].

Cell-based therapies have also been proposed as promising alternatives for treatment of PAD, since they provide cell progenitors capable of stimulating the neoformation of blood vessels in the ischemic areas. Bone marrow-derived mononuclear cells (MNCs) and peripheral blood MNCs containing a population of EPCs have been used in various clinical trials for vascular insufficiency with varying degrees of success[3]. Periarterial injection of non-selected or selected CD34^{+} MNCs ameliorates the symptoms associated with poor distal blood supply[7].

Stem cell injection represents a novel alternative for a point-of-care cell-based therapy for symptomatic patients with non-reconstructable CLI, poten-

tially resulting in ischemic pain relief, improved healing of chronic wounds and tissue preservation[10]. Cell-therapy based on the injection of autologous progenitor cells (bone marrow-derived progenitor cells or adipose-derived progenitor cells) administered intra-arterially or periarterially has been shown to improve claudication symptoms and ABI values in small studies[11]. We, therefore, conducted a small pilot, open-label, non-randomized, non-controlled study aimed to assess the safety and feasibility of periarterially BMAC injection for treatment of patients with severe PAD who are not susceptible to conventional endovascular treatment or surgical procedures.

PATIENTS AND METHODS

In this pilot, open-label, non-randomized, non-controlled study, a total of 27 patients with non-revascularizable CLI (Fontaine stages III and IV) were enrolled between 2015 and 2019. Comorbidities and risk factors for PAD such as diabetes, hypertension, dyslipidemia and cigarette smoking were also evaluated.

INCLUSION CRITERIA

1. Claudication, as defined by the ability to walk one-half block or less
2. Rest pain
3. Non-healing ulcer for more than 4 months
4. Inoperable PAD due to the presence of clinical contraindications
5. Patients with PAD who are not susceptible to endovascular treatment or surgical procedures

EXCLUSION CRITERIA

1. Sepsis
2. Malignancy
3. <40 years of age

BONE MARROW HARVESTING PROCEDURE

Autologous cells were extracted from the patient's bone marrow. The right iliac crest puncture was performed under epidural anesthesia in the operating room. A total amount of up to 120 mL of bone marrow was aspirated into 0.1 mL heparin, treated in 20 mL syringes and collected through Harvest Device Technology (Figure 1). Centrifugation was performed according to the protocol *Harvest Tech*. 20 mL of the pellet with total nucleated cells (TNCs) were collected. The medium dose obtained in all patients was 897.000.000 of TNCs counted by Neubauer chamber with trypan blue. The cell viability in all patients was 98%.

PERIARTERIAL BMAC INJECTION

The patient's CT angiography (CTA) was reviewed to guide the subsequent procedure of stem cell implantation. All patients discontinued dual antiplatelet therapy starting from 8 days prior to the stem cell injection. A prophylactic dose of low-molecular-weight heparin (2,500 IU/day) was administered until the night before the intervention. In the operating room, we proceeded to mark 40 points with a sterile marker and we then started to inject stem cells from the last point where the superficial femoral artery appeared patent using Doppler ultrasonography (Figure 2). In the event that superficial femoral artery was completely occluded, the first marking point for the injection site started from the last point where the deep femoral artery appeared patent using Doppler ultrasonography (Figure 3).

During the time required for the centrifugation procedure, patients were laid in the supine position and asepsis was maintained for the stem cell injections. During the initial phase of the procedure, Doppler ultrasonography was used to locate the site where the artery was still patent. Then, multiple BMAC

Figure 1. Harvest Device Technology with Total Nucleated Cells (TNCs).

Figure 2. Procedure employed to mark 40 points with a sterile marker to inject stem cells. Stem cells were injected starting from the last point where the superficial femoral artery appeared patent using Doppler ultrasonography.

injections (1 mL per each injection) were performed using a 22G needle and keeping a 1-cm distance between two adjacent injection sites. Once injections were performed, we placed an elastic bandage along the limb and the patients remained admitted to the hospital for additional 24 hours. On the day following the procedure, patients resumed all the medications

Figure 3. In the event that superficial femoral artery was completely occluded, the first marking point for the injection site started from the last point where the deep femoral artery appeared patent using Doppler ultrasonography.

that were discontinued prior to the hospital admission. The first follow-up visit was scheduled 2 months after the procedure for each patient. In addition, ABI measurement was performed before the intervention and during the subsequent follow-up visit.

ETHICAL APPROVAL AND INFORMED CONSENT

This study was approved by the Medical Ethics Committee of Hospital La Ribera (Alzira City, Valencia, Spain). All patients provided written informed consent for inclusion before they participated in the study. The study was conducted in accordance with the principles of the Declaration of Helsinki (1975).

RESULTS

PATIENT DEMOGRAPHICS AND BASELINE CHARACTERISTICS

A total of 27 patients participated in the study. The mean age was 65.7 years. 25 patients (92.6%) were male and 2 patients were female (7.4%). In total, 11 participants (41%) had type 2 diabetes, 11 (41%) had hypertension, and 12 (44%) had dyslipidemia. The prevalence of cigarette smoking was high (74%; 20 patients). Mean ABI value before intervention was 0.36, indicating the presence of severe PAD (Table 1). Table 2 shows the mean ABI values in relation to age, sex and different comorbidities and risk factors for PAD. Pearson's correlation was used for age, while the non-parametric Mann-Whitney test

Table 1. Patient demographics and baseline characteristics.

N	27
Age (years)	65.7±10.5*
M/F (%)	92.6%/7.4% (25/2)
Type 2 diabetes	41% (11)
Hypertension	41% (11)
Dyslipidemia	44% (12)
Cigarette smoking	74% (20)
ABI value before BMAC injection	0.36±0.08*

Abbreviations: ABI, Ankle brachial index; BMAC, bone marrow aspirate stem cell concentrate; M/F, Male/Female; N, total sample size. *Data expressed as mean ± standard deviation. Absolute numbers are shown in brackets.

Table 2. Mean ABI values in relation to age, sex and different comorbidities and risk factors for PAD.

		ABI*	p-value
Age		-0.062	0.778
Sex	M	0.36±0.08	0.367
	F	0.43±0.11	
Type 2 diabetes	Y	0.36±0.10	0.501
	N	0.37±0.07	
Hypertension	Y	0.34±0.09	0.309
	N	0.38±0.07	
Dyslipidemia	Y	0.38±0.04	0.182
	N	0.34±0.11	
Cigarette smoking	Y	0.36±0.96	0.581
	N	0.37±0.39	

Abbreviations: ABI: Ankle brachial index; M/F, Male/Female; Y/N, Yes/No. *Data expressed as mean ± standard deviation (except for Person's correlation used for age).

was used for the remaining variables. A *p*-value ≤0.05 was considered statistically significant.

FOLLOW-UP DATA

Overall, each procedure lasted approximately 45 minutes and was well-tolerated by all patients. Mean follow-up duration was 12.7±12.9 months. After 1-year follow-up, 4 patients (15%) underwent amputation of the limb affected by CLI. In such subgroup, all patients had a Fontaine stage IV PAD. The only variable that was statistically significant in relation to the need for major amputation was the pre-treatment Fontaine classification stage (Table 3). For this analysis, we used the chi-squared test.

Remarkably, after the intervention, the remaining 23 patients (85%) showed a marked improvement in clinical features and ABI values, without undergoing lower-limb amputation. Changes in ABI values 2 months after periarterial BMAC injection are shown in Table 4. The non-parametric Wilcoxon test was used for paired data.

CTA was performed before and 3 months after the periarterial BMAC injection in all patients: 24

Table 3. Pre-treatment Fontaine stage in relation to the need for major amputation.

		% Amputation (N)	
		No	Yes
Pre-treatment Fontaine stage	III	100% (23)	0% (0)
	IV	0% (0)	100% (4)

p-value = 0.001*

*p-value refers to the comparison between the percentages of patients in the two subgroups (pre-treatment Fontaine stage III and pre-treatment Fontaine stage IV). For this analysis, we used the chi-squared test.

out of 27 participants (89%) showed collateral vessel formation 3 months after the periarterial BMAC injection (Figure 4). In such patients, physical examination of the affected limbs showed improvement in leg temperature, skin color and atrophy at 3 months after BMAC treatment.

DISCUSSION

The results of our study strongly suggest that periarterial injection and implantation of a medium of approximately 800.000.000 autologous BMAC TNCs into the ischemic lower limbs of patients with Fontaine stages III-IV induces a significant and functional neovascularization. Cell therapy may be a valid treatment for patients with CLI who are not susceptible to endovascular or open revascularization. Indeed, cell therapy has already emerged as a promising alternative for the treatment of advanced stages of PAD (Fontaine stages III-IV) during the last decade.

Although medical and surgical treatments are well-established options for the management of CLI, some patients progress towards an advanced-stage disease which can result in the loss of the affected limbs. Our small pilot, open-label, non-randomized, non-controlled study evaluated the safety and feasibility of multiple periarterial BMAC injections among patients with CLI (Fon-

Table 4. Changes in ABI values 2 months after intramuscular BMAC injection.

	Pre-treatment	Post-treatment	Variation	p-value
ABI*	0.36±0.08	0.64±0.11	0.28±0.10	<0.001

Abbreviations: ABI, Ankle brachial index. *Data expressed as mean ± standard deviation.
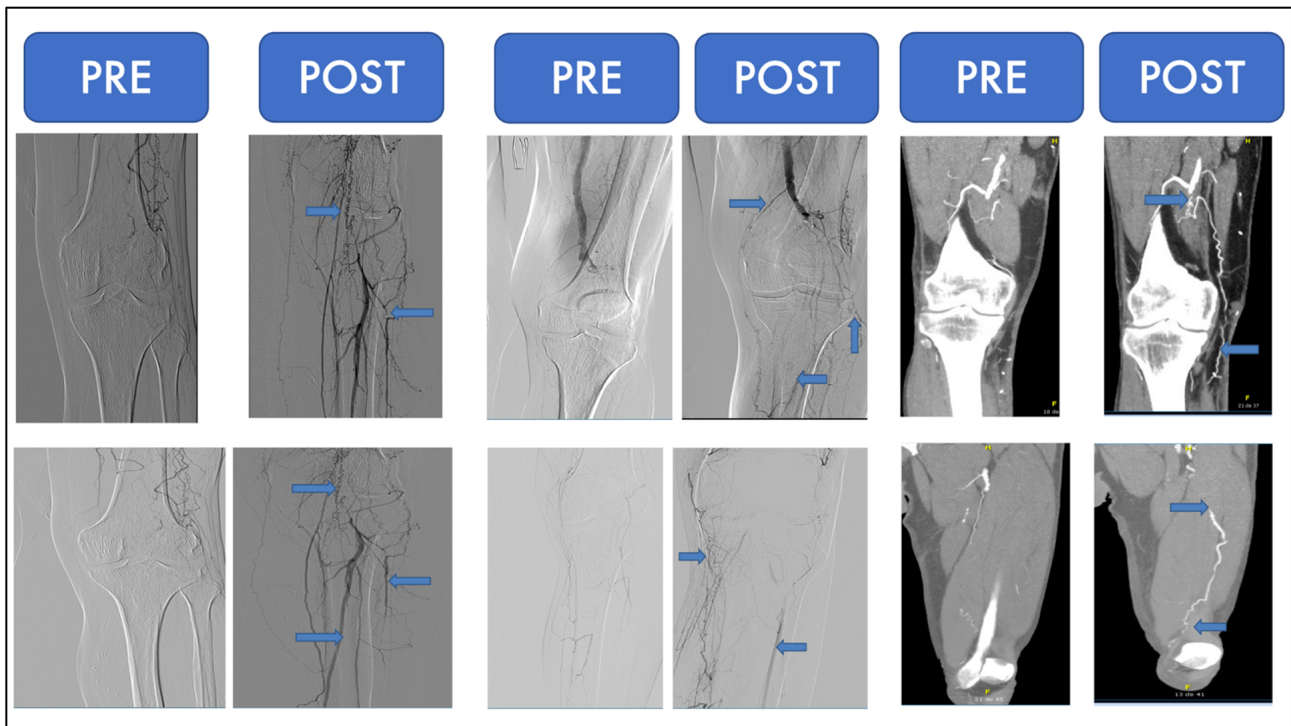
Figure 4. CT angiography (CTA) performed 3 months after the intramuscular BMAC injection showed collateral vessel formation in 24 out of 27 participants (89% of participants).

taine stages III-IV). The study showed that this intervention was safe and effective in improving clinical outcomes (assessed by both clinical examination and imaging tests).

The use of BMAC was initially described by Tateishi-Yuyama et al[2] who demonstrated an increase in ABI values among patients treated with BMAC, as compared to patients treated with peripheral blood MNCs. In our study, there was no control group. Therefore, we could not compare patients treated with BMAC and patients treated with peripheral blood MNCs. However, we documented a significant improvement in ABI values 2 months after the intervention, as compared to baseline ABI values. At 3 months after BMAC treatment, physical examination of the affected limbs also showed improvement in leg temperature, skin color and atrophy. These clinical findings were consistent with CTA findings, which showed increased regional blood flow, as well as the presence of new blood vessels (neovascularization). Similar results have been described in different studies investigating the use of bone marrow-derived MNCs[2,11].

BMAC injection is a commonly used procedure and appears to be safe and effective not only in orthopedic applications[12-14], but also in non-revascu-

larizable PAD. Our group in Argentina have being working since 2004 with these techniques to obtain BMAC for application in cardiovascular diseases, diabetes, chronic obstructive pulmonary disease, Parkinson's disease, autism and knee osteoarthritis[15,16]. BMAC contains multiple cell types, including endothelial cells, MSCs, platelets, nucleated cells such as lymphocytes, and plasma enriched with growth factors. Understanding the difference between cell therapy and stem cell therapy is critical to comprehend which are the main mechanism of actions of such approaches in the field of regenerative medicine. Stem cell therapy usually refers to the use of MSCs, which are expanded for autologous or allogenic use. On the other hand, cell therapy is a generic term referring to the use of different cell types that can exert regenerative properties, although this term does not convey the exact mechanisms of action of such cells (e.g., the direct effects on blood vessel neoformation). Cell therapy also refers to a point-of-care procedure (evaluation and procedure performed during the same day), while stem cell therapy refers to a biological advanced therapy which must be regulated and approved in Spain, as well as in other countries which have specific regulations on advanced therapies.

Ischemia represents the main factor that stimulates the migration of stem cells to injured tissues, since the hypoxic environment favors angiogenesis by inducing the expression of HIF-1a (Hypoxia-inducible factor 1-alpha), which participates in the upregulation of proteins involved in angiogenesis, namely: VEGF (vascular endothelial growth factor), endothelin, endoglin, leptin and TGF-b (transforming growth factor-beta)[17]. In turn, these factors participate in other processes such as maintenance of tissue homeostasis, regulation of immune responses and production of cytokines that favor the migration of bone marrow-derived TNCs[15]. Moreover, it has been reported that injection of bone marrow-derived MNCs into ischemic tissues does not induce cell differentiation into lineages such as fibroblasts, osteoblasts or myogenic cells, as evidenced by the absence of bone formation or fibrosis within the ischemic limbs[1].

CONCLUSIONS

In light of the aforementioned findings, we can draw the following conclusions: i) periarterial BMAC injection in patients with severe PAD leads to the short-term induction of neovascularization; ii) our population showed clinical improvement from baseline, which consisted of increased ABI values, increased leg temperature, and other local signs that are indirect indicators of collateral vessel formation in the affected limbs; iii) periarterial BMAC injection is a feasible and safe procedure, with no adverse events reported after cell therapy performed as a point-of-care procedure in patients with PAD Fontaine stages III-IV. In conclusion, this pilot study opens up the possibility of considering periarterial BMAC injection as a valid therapeutic strategy for treatment of PAD. Future prospective randomized controlled trials are warranted to conclusively ascertain the long-term safety and efficacy of periarterial BMAC injection for treatment of patients with PAD who are not susceptible to endovascular or open revascularization.

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