Therapeutic angiogenesis in Buerger's disease (thromboangiitis obliterans) patients with critical limb ischemia by autologous transplantation of bone marrow mononuclear cells

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Background: **Peripheral arterial disease is a significant problem worldwide. In developing countries such as India, the increased incidence of smoking and other forms of nicotine intake has resulted in a large proportion of young individuals with Buerger's disease. The results of surgical and endovascular treatment for this condition have not been very rewarding. Hence, we focused on providing alternative therapies. Neovascularization by autologous bone marrow mononuclear cell transplantation is being tried as an alternative therapeutic option. We have reviewed our series of patients who underwent autologous bone marrow mononuclear cell transplantation during the last 2 years.**

Methods: **We enrolled 38 patients who were chosen to undergo autologous bone marrow mononuclear cell transplantation for nonreconstructible Buerger's disease. We injected the bone marrow mononuclear cells into the calf muscles of the affected limbs in 36 patients. We monitored ulcer healing, ankle-brachial index (ABI), and transcutaneous oximetry ($TcPo_2$) level.**

Results: **No procedurally related complications occurred, although one injected sample of bone marrow aspirate later revealed infestation with *Strongyloides stercoralis*. Two patients were seropositive on the Venereal Disease Research Laboratory test and were not injected with the bone marrow mononuclear cells. Three patients (12%) underwent major amputations ≤6 months. The others had improvements in their ulcer healing, an increase in the mean ABI of 0.14 (range, 0.1-0.19; $P < .01$), and improvement in the mean $TcPo_2$ of 52 mm Hg (range, 40-68 mm Hg, $P < .01$), with resultant limb salvage in all at 6 months. All patients discontinued smoking during the study period.**

Conclusions: **Use of bone marrow–derived progenitor cell transplantation into ischemic limbs is a relatively safe procedure with no demonstrable side effects at 6 months. These study data support conducting controlled and multicenter trials to evaluate the efficacy of this therapy in preventing amputation in selected patients with Buerger's disease who have critical limb ischemia. (J Vasc Surg 2008;48:53S-60S.)**

Therapeutic angiogenesis represents an attempt to relieve inadequate blood flow by the directed growth and proliferation of blood vessels. Neovascularization is a complex process involving multiple growth factors, receptors, extracellular signaling pathways, and local and bone marrow–derived cells. New blood vessel formation can occur in adult life as a result of two distinct mechanisms. The first is angiogenesis,[1,2] which involves the proliferation of capillaries and hence an increase in the local blood flow. The second is arteriogenesis, wherein the already existing high-resistance collateral vessels increase in size, resulting in a decrease in resistance and an increase in blood flow to the ischemic region.[3]

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Angiogenesis occurs under various conditions, including local ischemia. Local cellular ischemia is said to affect the transcription factor hypoxia-inducible factor 1,[4] which in turn leads to expression of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), VEGF receptors, nitric oxide, and insulin-like growth factors.[5] These local changes result in migration of the endothelial progenitor cells and formation of new thin walled blood vessels.[6-8] The extracellular matrix also plays a role in this process. The sprouting of capillaries forms a new immature capillary network. The maturation of these immature capillaries involves the incorporation of pericytes under the influence of various cytokines and growth factors.[9-11]

Therapeutic angiogenesis can be induced by the implantation of bone marrow mononuclear cells. Kamihata et al[12] and Shintani et al[13] have shown that bone marrow mononuclear cells contain not only endothelial progenitor cells but also angiogenic factors and cytokines and that implantation of bone marrow mononuclear cells into ischemic tissues augments collateral vessel formation. Although CD34 is the most popular marker for stem cell selection for inducing therapeutic angiogenesis, others such as CD133 stem cells[14-16] and CD117 stem cells[17] also are important in therapeutic angiogenesis.

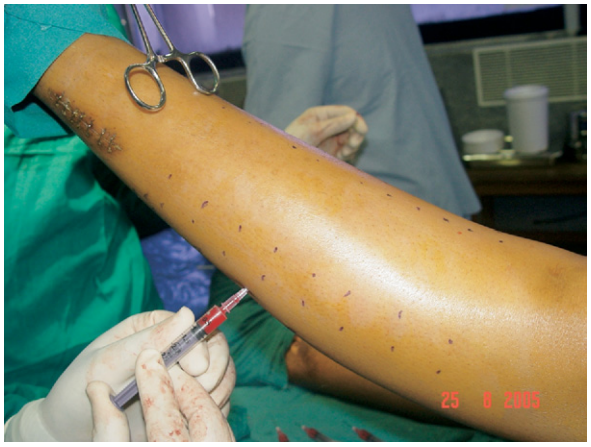

Fig 1. A grid was created over calf muscles and bone marrow mononuclear cells were injected in 30 to 40 sites.

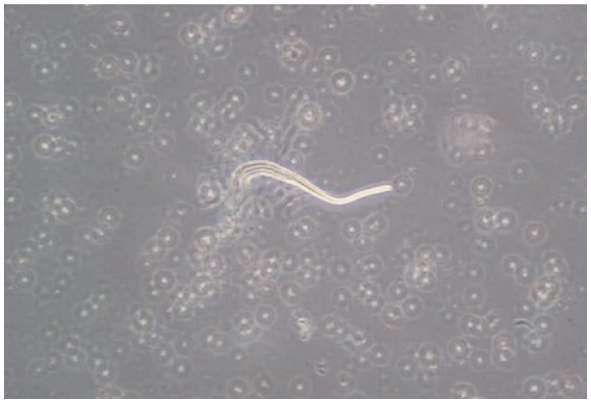

Fig 2. *Strongyloides stercoralis* was found in the bone marrow aspirate of one patient.

Table I. A, Descriptive statistics for ankle-brachial index

Variable	*No.*	*Minimum*	*Maximum*	*Mean*	*SD*	*SE*
Baseline	38	0.12	0.47	0.2637	0.06804	0.0111
1 mon	36	0.27	0.60	0.3681	0.06602	0.01100
6 mon	33	0.30	0.65	0.4173	0.06983	0.01216

Table I. B, Paired samples statistics for ankle-brachial index

Variable	*Mean*	*SD*	*SEM*	P
Baseline-1 mon	.09861	.03735	.00623	<.001
Baseline-6 mon	.14636	.02608	.00454	<.001
1-6 mon	.05030	.02675	.00466	<.001

SD, Standard deviation; *SE,* standard error; *SEM,* standard error of the mean.

Table II. A, Descriptive statistics transcutaneous oximetry

Variable	*N*	*Minimum*	*Maximum*	*Mean*	*SD*	*SE*
Baseline	38	20	51	28.18	5.526	.896
1 mon	36	34	60	48.17	6.134	1.022
6 mon	33	40	68	52.12	5.904	1.028

Table II. B, Paired sample test analysis of transcutaneous oximetry data

Variable	*Mean*	*SD*	*SEM*	P
Baseline-1 mon	19.833	5.940	.990	<.001
Baseline-6 mon	23.697	6.307	1.098	<.001
1-6 mon	3.424	3.666	.638	<.001

SD, Standard deviation; *SE,* standard error; *SEM,* standard error of the mean.

India is experiencing an exponential increase in peripheral arterial disease due to a large population of diabetic patients (42 million in 2007) and unabated smoking. Although the exact data are unavailable, 20% to 40% of these patients are estimated to have Buerger's disease, also known as thromboangiitis obliterans, especially in those who smoke a crude form of rolled tobacco leaves called *beedies* or have other forms of tobacco intake, including chewing of *gutka*, a highly concentrated form of tobacco leaves.

A significant social problem in patients with thromboangiitis obliterans is that they are young, most are from lower socioeconomic strata, and many are single breadwinners for their families and are generally unskilled manual workers. This socioeconomic problem, coupled with the fact that present medical, surgical, and endovascular treatment modalities have not been very successful, has resulted in a dire need for alternative therapies. Thus, investigations have been initiated of the role of bone marrow–derived progenitor cell transplantation into ischemic limbs in these patients, in whom loss of a limb could have serious repercussions on the entire family. Various studies have concluded that transplantation of bone marrow mononuclear cells could contribute to a safe and effective strategy for achievement of therapeutic angiogenesis and hence prevention of amputation.[18,19]

MATERIALS AND METHODS

Patients. Thromboangiitis obliterans was diagnosed by the modified Olin[20] criteria and included smoking history, age of onset <45 years, infrapopliteal arterial occlusive disease, angiographic evidence of medium and small vessel disease, upper limb involvement or phlebitis migrans, absence of atherosclerotic risk factors other than smoking/tobacco use at the time of initial diagnosis, and exclusion of autoimmune diseases, diabetes mellitus, proximal source of emboli, and hypercoagulable states.

Inclusion criteria. Our inclusion criteria for patients qualifying to undergo bone marrow progenitor cells transplantation, as approved by Ethics Committee for Human

Table III. A, Descriptive statistics for the visual analog scale

Variable	*N*	*Minimum*	*Maximum*	*Mean*	*SD*	*SE*
Baseline	38	8	10	8.74	.724	.117
1 mon	36	1	5	3.36	1.046	.174
6 mon	33	0	4	2.45	1.660	.289

Table III. B, Paired samples test analysis of the visual analog scale results

Variable	*Mean*	*SD*	*SEM*	P
Baseline-1 mon	5.361	1.222	.204	<0.001
Baseline-6 mon	6.242	1.696	.295	<0.001
1-6 mon	.879	1.139	.198	<0.001

SD, Standard deviation; *SE,* standard error; *SEM,* standard error of the mean.

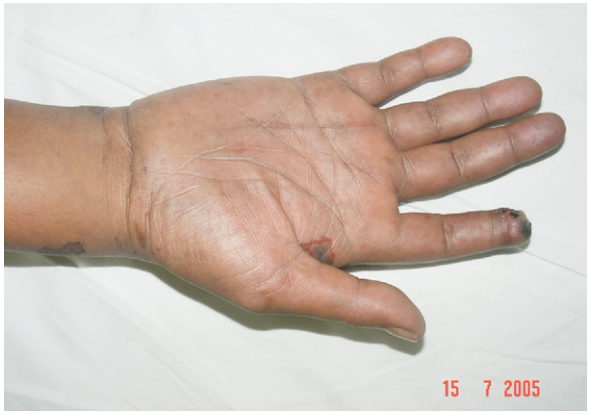

Fig 3. This patient had severe hand ischemia with nonreconstructible thromboangiitis obliterans.

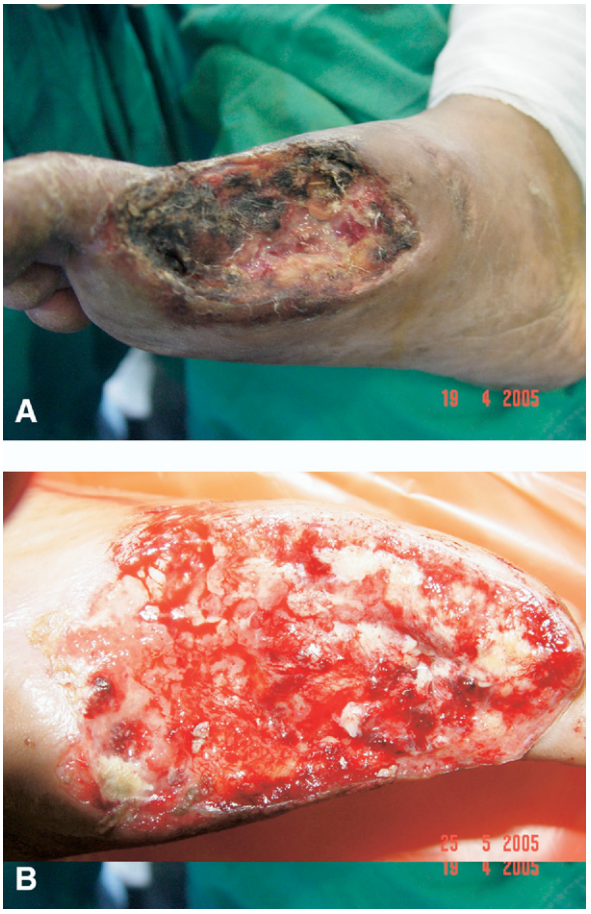

Fig 4. A, Toe amputation site after failed vascular reconstruction and **B,** 5 weeks after therapy.

Research of the hospital and after providing informed consent, were age <45 years, no history of diabetes mellitus, no evidence of malignancy, patients with Buerger's disease, has limb-threatening ischemia, and established methods of treatment were not possible or were tried and were not successful.

Exclusion criteria. Patients with diabetes mellitus, atherosclerosis, thrombotic disease, proximal embolic source, malignancy, or hypercoagulable states were excluded. Those who would require amputation proximal to transmetatarsal level were also excluded.

Procedures. The study enrolled 38 men (38 limbs) with thromboangiitis obliterans. Patients' mean age was 34 years. All 38 patients had nonhealing ischemic wounds of a minimum of 2 months' duration that showed no improvement with conventional therapy. All patients also had rest pain of >2 months' duration.

The baseline evaluation for all patients included a complete blood count, erythrocyte sedimentation rate, blood glucose level, renal and liver function tests, an electrocardiogram, and a chest radiograph. An echocardiogram and tests for connective tissue disorders and hypercoagulable states were not done routinely due to financial constraints in these patients.

An epidural catheter was placed on admission in all patients (except one with upper limb ischemia) for pain control. All were counseled against smoking. Appropriate wound care was commenced and they were prescribed systemic antibiotics. Intra-arterial digital substraction angiography was done for the indexed extremity before the patient was diagnosed as having nonreconstructible disease.

The primary outcome was limb salvage at 6 months. We looked for decrease in rest pain, healing of ulcers, improvements in the ankle-brachial index (ABI) scores, and rise in transcutaneous oxygen pressure ($TcPo_2$) levels in the patients undergoing bone marrow progenitor cell transplantation.

Regional anesthesia was used for the procedure. To increase the yield, bone marrow was aspirated from the posterior iliac crests in different directions using a bone marrow aspiration needle and 20-mL syringes.The average volume of bone marrow aspirated was 400 mL, with min-

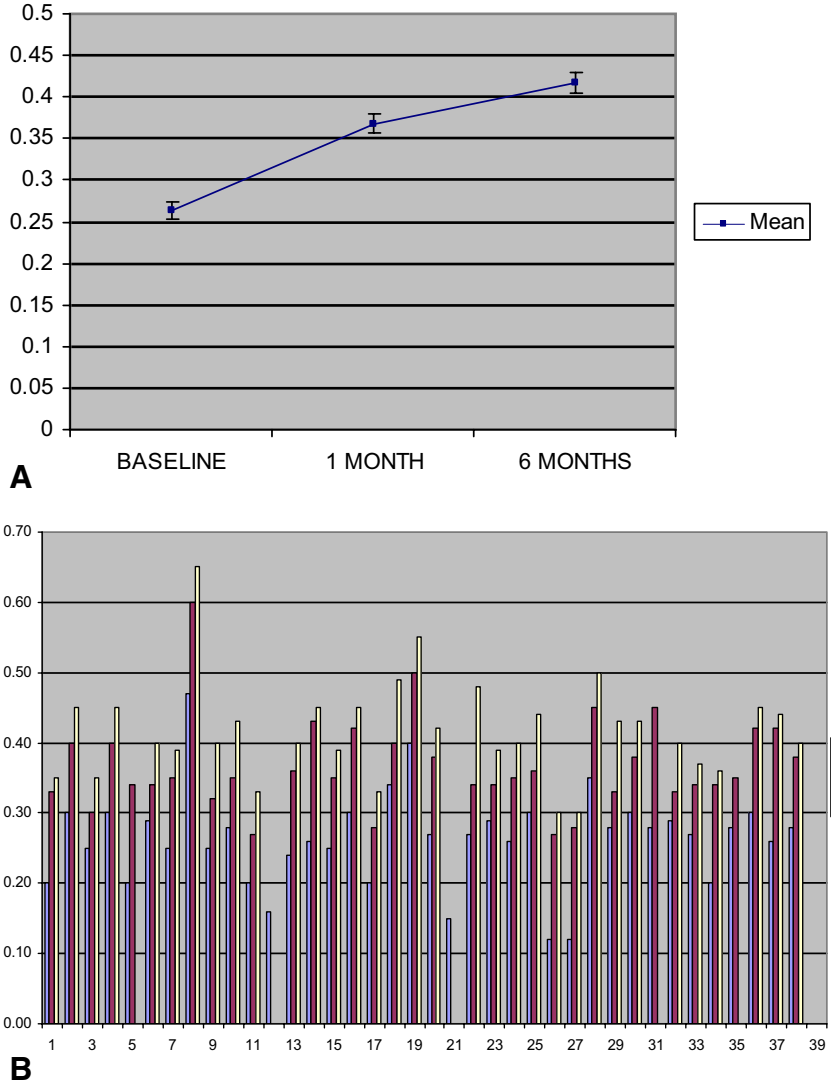

Fig 5. A, Representation of the mean ankle-brachial index and standard error *(bars)*. **B,** Representation of individual subject data for the ankle-brachial index.

imum mixing of sinusoidal blood from each patient. The collection was done in a sterile container.

The marrow was taken to the sterile stem cell processing laboratory where stem cell enrichment took place in cyclic guanosine monophosphate conditions. Mainly, the procedure consisted of red blood cell (RBC) sedimentation by 6% hydroxyethyl starch (HAES) at unit gravity. This resulted in RBC depletion of >90%. The nucleated cells present in the supernatant were concentrated by centrifugation at 1500 rpm. The nucleated cell pellet was reconstituted in sterile 6% HAES, and 0.5 mL of cell suspension was taken for cell count viability and CD34 count by flow cytometry.

After RBC depletion and volume reduction, the nucleated cell count was $5.8 \times 10^7 \pm 4 \times 10^7$/mL. The final volume of the RBC-depleted cell suspension was 45 to 60 mL, which was used for injections into the patient. The total number of CD34 in the cell suspension was $9.8 \pm 9.91 \times 10^6$. Viability was 99%. CD34 count was done using BD Procount (Becton Dickinson, Franklin Lakes, NJ) using International Society of Hematotherapy and Graft Engineering protocol.

The cell suspension was cultured and found to be free of contamination in 36 patients. This was then injected into multiple sites in the gastrocnemius muscle of the ischemic lower limb. The sites marked on the gastrocnemius muscle were 1 cm apart. A 2-mL syringe and a 20-gauge needle were used to inject 0.75 mL into each site intramuscularly (Fig 1). A baseline ABI and $TcPo_2$ were performed preoperatively.

We followed up the patients weekly for 4 weeks and monthly thereafter. Monitoring of ulcer healing, decrease

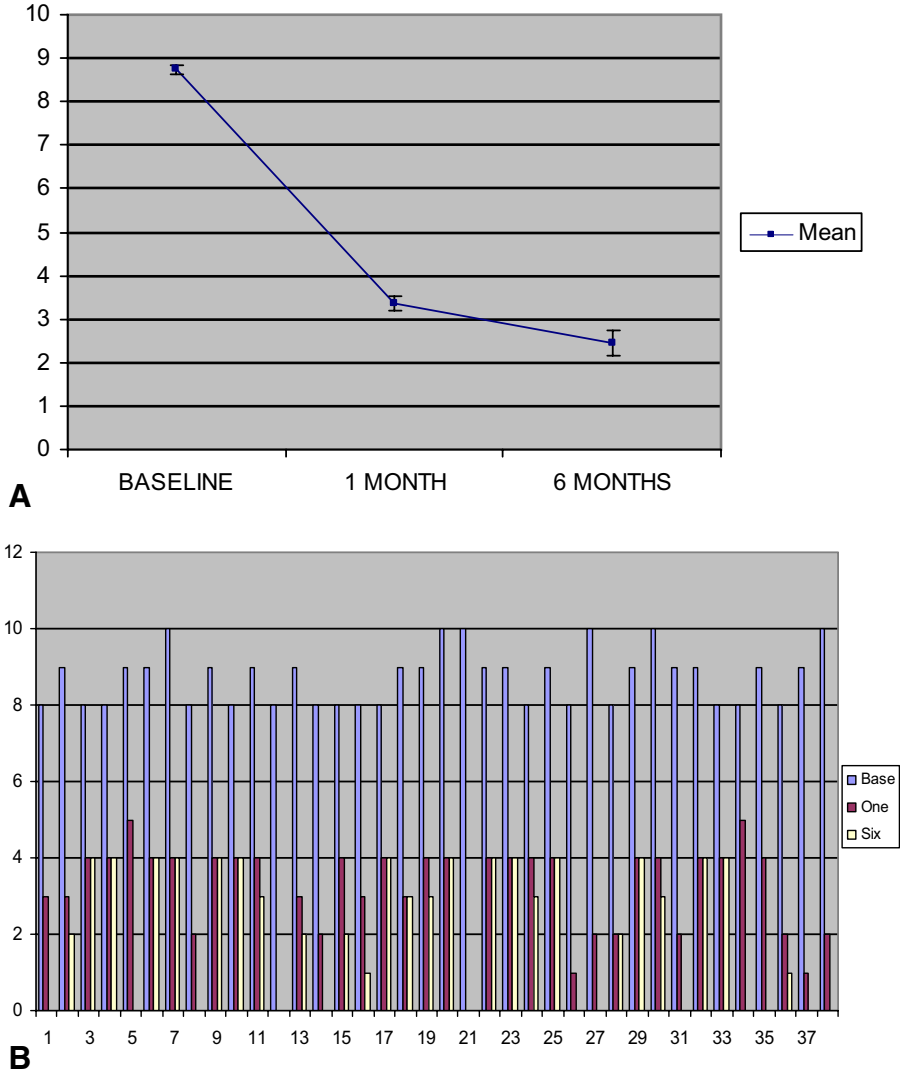

Fig 6. A, Representation of visual analog scale data and standard error *(bars)*. **B,** Representation of individual subject data on the visual analog scale.

in rest pain, ABI and $TcPo_2$ was done at the end of 1 month and 6 months. We judged an increase in ABI of >0.1 as an improvement according to the standard assessment of interventional therapy for peripheral arterial diseases.[21] $TcPo_2$ was measured on the dorsum of the foot using a TCM400 $TcPo_2$ monitoring system (Radiometer Medical APS, Denmark). This report includes 38 patients recruited between November 2005 and 2007. Two bone marrow aspirates were seropositive on the Venereal Disease Research Laboratory test, although the initial test results were negative, and hence were not processed. Three patients required amputation by the 6-month follow-up, which left 33 patients in the study. In addition, one patient was found to have *Strongyloides stercoralis* (Fig 2) in the collection, which we came to know a day after the therapy.

Statistical analysis. The study data were analyzed using SPSS 16.0 software (SPSS Inc, Chicago, Ill). Statistical analysis has been provided for baseline, 1 month, and 6 months for ABI (Table I), $TcPo_2$ (Table II), and visual analog scale scores (Table III). The difference in the baseline and end of study has been compared using the paired-samples *t*-test. The level of significance was fixed at $P = .05$.

RESULTS

We tested the ABI in all study patients before the procedure and also obtained a follow-up ABI measurement in all the patients at 4 weeks and 6 months after the procedure. The ABI improved in all 33 patients (Fig 5). The mean preprocedural ABI was 0.26 (range, 0.12-0.47). An increase in ABI of >0.1 at the end of 1 month and 6 months was noted in 33 patients, with a mean increase of 0.14.

Similarly, $TcPo_2$ was measured before the procedure and again at the end of 4 weeks and 6 months. Compared

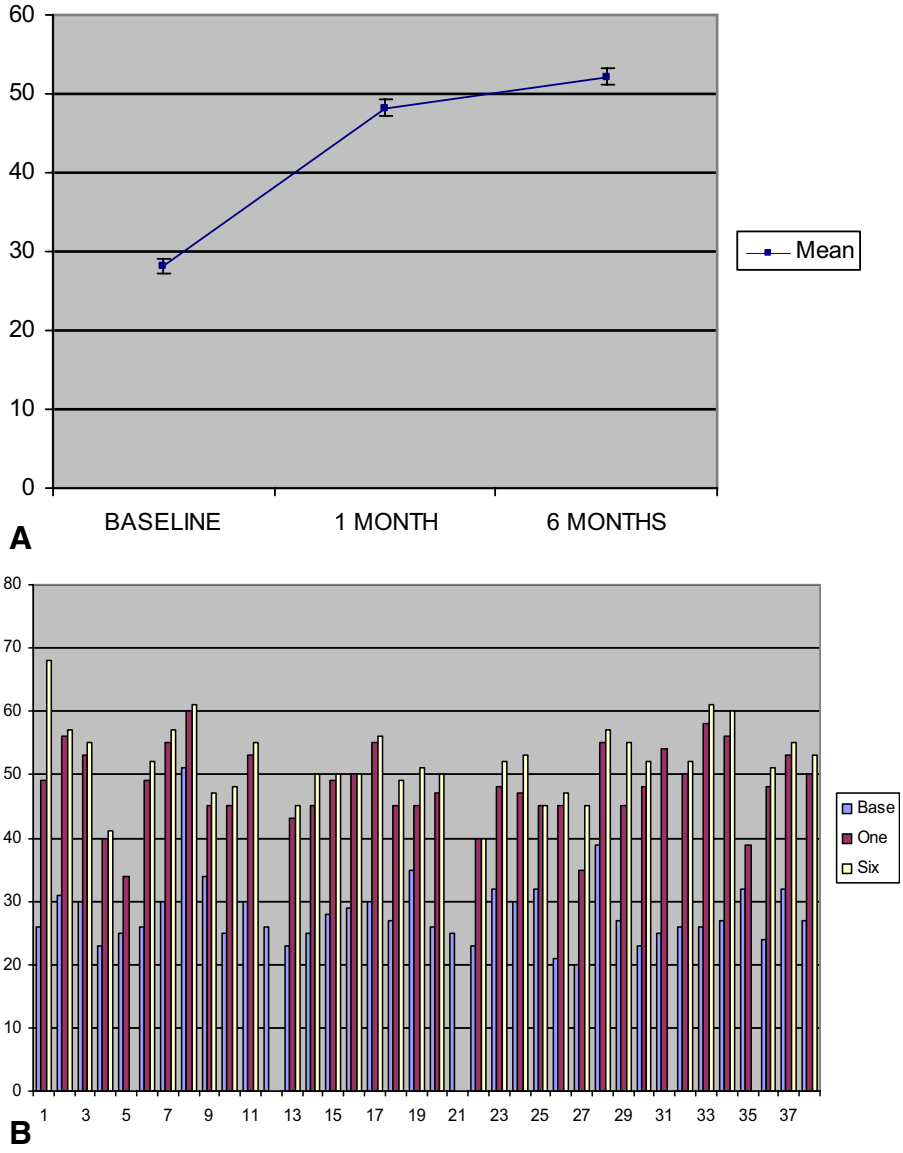

Fig 7. A, Representation of mean transcutaneous oximetry data and the standard error *(bars)*. **B,** Representation of individual subject data for transcutaneous oximetry levels.

with baseline values, the 33 patients showed a significant increase in $TcPo_2$ levels from the mean baseline $TcPo_2$ of 28 mm Hg (range, 20-51 mm Hg). The mean $TcPo_2$ level was 48 mm Hg (range, 34-60 mm Hg) at 4 weeks after the procedure and 52 mm Hg (range, 40-68 mm Hg) at the end of 6 months (Fig 7).

Marrow implantation induced no local inflammatory reaction in the form of redness, pain, erythema, or swelling in any of the study participants. No major adverse events were reported in any of the patients during their hospital stay or in the 6-month follow-up period.

We used bone marrow mononuclear cells containing CD34 cells, and it is postulated that these cell fractions release multiple angiogenic factors to enhance angiogenesis in addition to supplying endothelial progenitor cells. The mean (SD) CD34 count was 9.88 ± 9.91 million (range, 1.62-33.6 million), the total mean mononuclear cell count ($\times 10^8$) was 26.23 ± 17.98 (range, 1.80-64.93), and the mononuclear cell count per mL ($\times 10^7$) was 0.3 to 14.4.

Clinical improvement was shown by decrease in rest pain on a visual analog scale score in 25 patients, with complete regression in eight (Fig 6). Also, ulcer healing was noted in 23 patients, and seven patients had complete healing of their ulcer in a time frame of 1 to 6 months. In three patients the size of the ulcer did not change during the study period. Improvement in rest pain, ulcer healing, ABI, and $TcPo_2$ levels were maintained in all patients during their 6-month follow-up, except in three patients

(12%) in whom an amputation was necessary due to severe rest pain with progression of foot gangrene.

DISCUSSION

We have shown that implantation of bone marrow–derived progenitor cells into the ischemic limbs resulted in an increase in blood flow in the ischemic limbs in 33 patients. This was assessed by documenting an increase in the ABI and in the $TcPo_2$ measurements. Rest pain also decreased in most patients. Similarly on follow-up, we also noted an improved ulcer healing and that seven of the ulcers completely healed (Figs 3 and 4).

Chronic critical limb ischemia is defined as persistent recurring rest pain of >2 weeks' duration not responding to regular, adequate analgesia and an ankle pressure of <50 mm Hg or a toe pressure of <30 mm Hg. The study inclusion criteria were that we enroll patients with Buerger's disease who did not have diabetes and had undergone or were unable to undergo the standard treatment options for their disease. All of the 38 patients in our study had critical limb ischemia despite having undergone earlier surgical treatment in the form of lumbar sympathectomy before they were included in the study.

Angiogenesis[1,22,23] that occurs can result in growth of new capillaries or growth of pre-existing collateral vessels. For newly formed vessels to survive, they must undergo remodeling and acquire a layer of smooth muscle cells. In view of the complex process of vessel formation, treatments that use only one angiogenic factor might result in suboptimal endothelial channels.

The CD34 fraction in bone marrow mononuclear cells reportedly synthesized not only angiogenic growth factors like VEGF and basic FGF but also angiopoietin I,[9] which is important in the maturation and maintenance of the vascular system; hence, resulting in blood vessels with an adequate composition of smooth muscle cells, periendothelial matrix, and pericytes.[24]

Buerger's disease, also known as thromboangiitis obliterans, is characterized by the occurrence of segmental thrombotic occlusions of the medium- and small-sized arteries. This entity is seen in young smokers with no other risk factors and is more common in Asia than in the West. The incidence of major amputation in this patient population according to multiple studies is 12% to 31% and has included both patients with claudication and those with critical limb ischemia.[25]

Because this is probably the first study of this kind in India in patients with ischemic limbs, and also owing to the small number of patients included in the study, the interpretation of the results, which appear promising, are limited. Absence of a control, the small number of patients enrolled, and absence of a postprocedural angiogram are the limitations of this study. A point to be remembered in this discussion is that most of the patients belong to lower socioeconomic strata and are single breadwinners for their families. This procedure, which was done in 36, revealed no adverse effects on any of them and more important, the approximate cost of the total procedure and stay in the hospital was US $200 to $300 compared with peripheral vascular bypass, which would cost approximately US $1000 to $1300. Because these patients belonged to a low-income group, with out any medical insurance, their care was completely funded.

Another factor to be kept in mind is that although all the patients in the study said they did not restart smoking during their 6 month follow-up, no testing for urine cotinine by radioimmunoassay was done. Hence, the contribution of persistent smoking to the disease outcome could not be assessed. We would also like to state in conclusion that this study is a preliminary one to show safety, but that efficacy requires a placebo-controlled study, because other associated treatment, namely smoking cessation, could have substantially influenced the outcome.

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AUTHOR CONTRIBUTIONS

Conception and design: VM
Analysis and interpretation: KR
Data collection: GI
Writing the article: VM
Critical revision of the article: VI
Final approval of the article: KR
Statistical analysis: SU
Obtained funding: SU
Overall responsibility: VM

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DISCUSSION

Dr Henrik Sillesen (Copenhagen, Denmark). This issue has been controversial for many years, and in our part of the world, this is not an option. We just heard from Australia that they are working on the Tamaris project, which is just another option of treatment for these patients who cannot be reconstructed. Would it be possible to do a double-blind trial? I can easily imagine some problems with how to have a placebo injection, but would it be possible?

Dr Vishnu Motukuru. That would be tough. The TAG study was published, we have the first part of the study; it was a double-blind plan in which one group had saline injected and the other had stem cells injected. But in the immune setup, it is not going to be easy. The second thing you need to bear in mind is the whole cost of this procedure, it is probably around $200 to $300, and even this had to be funded in total.

Dr Krish Soundararajan (Philadelphia, Pa). In an era where surgeons seek invasive therapy, it is refreshing to see your work on nonsurgical management of critical limb ischemia. I have two questions. The first one is would the stem cell therapy have any different outcome if you had applied it in ischemia due to atherosclerosis rather than Buerger's disease? Is there any reason why you chose to confine the therapy to Buerger's disease?

The second question is with regards to the cost factor and my understanding that many patients in India may not be able to afford therapies to improve lifestyle in occlusive disease. How do you see stem cell therapy helping an average laborer in India? Would this be a technology which is beyond their means?

Dr Motukuru. Firstly, it is still quite controversial. So we just wanted to apply this, because it is being done for the first time in India, so we wanted to play safe. So that is the reason we excluded diabetics and we offered this only to patients in whom nothing else is possible. Probably in our older patients and patients with atherosclerosis, there are other alternatives which can be tried. But this was offered only to a specific subset of nonclassical Buerger's in whom everything else has been tried. So that is the reason we specifically selected this subset wherein there is no option at all other than an amputation.

Dr Soundararajan. Does that mean if you had a patient with ischemia due to atherosclerosis who did not have any other options you would have included them in this study?

Dr Motukuru. I doubt it, because we were basically looking at people who are young, who are extremely healthy, and who could probably tolerate any complication. So a person with advanced atherosclerosis, your morbidity/mortality would go up given the nature of the disease.

Dr Chris Liapis (Athens, Greece). Most of the times patients with Buerger's disease have lesions in the distal arteries, like digital arteries. Why do you think that injecting the calf area is going to improve the situation in the obstructed distal arteries?

Dr Motukuru. Because this was the first series, we injected all the patients into the calf muscles. But the last five cases, we were also injecting the stem cells into the foot. But because we don't have a 6-month follow-up, I haven't presented those.

Dr Enrico Ascher (Brooklyn, NY). I know of at least three stem cell studies investigating the potential for limb salvage. In our study we agreed to inject the stem cells along the course of tibial vessels. Do you think that this method better promotes angiogenesis?

Dr Motukuru. I am not aware of it, sir. But we started injecting in the foot. But as far as the results are concerned, I have no idea.

Dr Gregorio Sicard (St. Louis, Mo). These patients, obviously, all of them are very heavy smokers. And did you look, even though it is a small number of patients, how many of these patients continued their smoking habits? Because I think that is going to muddy the results.

And the second question, one of the therapies that is being looked at, obviously, in multiple institutions across the world, is rather than obtaining the stem cells from the bone marrow is to use granulocyte stimulation factors or certain factors that promote the recruitment of stem cells from the bone marrow to the site of the ischemia. Have you looked at that in India?

Dr Motukuru. We haven't, sir, because there were a couple of papers which said granulocyte-stimulated factors helped, but then there were a couple of papers that said it doesn't help. And secondly, one of the limitations of the study was we did give them a strong counseling, but we did not do urine creatinine assay levels to really assess whether they were actually smoking or not smoking; and hence, we couldn't really assess the impact of our counseling on the results, purely because of financial constraints.