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The Immunomodulatory Effects of Umbilical Cord Mesenchymal Stem Cell in Critical Limb Ischemia Patients

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Abstract

Transplantation of human umbilical cord mesenchymal stem cells (UC-MSCs) has been shown to be effective in treating critical limb ischemia (CLI). However, the mechanism of MSCs- mediated improvements in CLI patients, especially with respect to the immune- inflammatory aspects of this disease, is still unknown. In this study, we investigated the changes in T-lymphocyte subpopulations and inflammatory mediators (such as IL-6, IL-10 and TNF-α) in PBMCs from CLI patients after UC-MSCs treatment, as well as the correlation between inflammatory mediators and EPCs. TNF-α serum levels increased at 24 h (p=0.017) after treatment and then decreased at 1 month (p=0.031) compared with that before treatment. IL6 serum levels increased at 24 h (p=0.099) after treatment and then decreased at 1 month (p=0.072) compared with that before treatment. The percentages of CD3+ T, CD3+ CD4+ lymphocytes and NK cells were significantly decreased after UC-MSCs treatment (p=0.002, p=0.012 and p=0.029, respectively). TNF-α (r=-0.42, p=0.0004) and IL-6(r=-0.33, P < 0.0001) were shown to be inversely correlated with the number of circulating EPCs. Our studies have shown that UC-MSCs have anti-inflammatory and immunomodulation properties in CLI and promotes healing of non-healing wounds.

Keywords: Critical limb ischemia; Peripheral arterial disease; Umbilical cord mesenchymal stem cell; Immunomodulatory

Abbreviations: UC-MSC: Umbilical Cord Mesenchymal Stem Cells; CLI: Critical Limb Ischemia; PBMC: Peripheral Blood Mononuclear Cell; EPC: Endothelial Progenitor Cell; KDR: Vascular Endothelial Growth Factor Receptor Ii; Pad: Peripheral Artery Disease; RA: Rheumatoid Arthritis; DCs: Dendritic Cells; PDGF: Platelet Derived Growth Factor; MMP: Matrix Metalloproteinase

Introduction

Critical limb ischemia (CLI), defined as rest pain or tissue necrosis with ulcers or gangrene, is the most advanced clinical stage of peripheral arterial disease (PAD). The aims of currently available treatments are to improve distal arterial perfusion by surgical bypass or endovascular procedures. Futhermore, up to one third of patients with PAD, who are unsuitable for revascularization by percutaneous transluminal angioplasty or bypass, still require amputation [1].

CLI results from limb artery occlusion with subsequent drastic reduction in oxygen levels in the affected limb. Following the ischemia, invasion of inflammatory cells and T lymphocytes contribute to secondary injury through production of inflammatory mediators including interleukins (IL) and tumor necrosis factor-α (TNF-α). Since 1997,Asahara et al.[2] described the endothelial progenitor cells (EPCs) isolated from peripheral blood, the interest in EPC biology has been growing continuously. EPCs participate in forming new blood vessels after recruitment and migration into the ischemic tissue. EPCs are usually classified as a subtype of CD34+ hematopoietic stem marker and CD133 and KDR-1, also known as vascular endothelial growth factor receptor-2 (VEGFR2) [3]. Recently, it was reported that patients with CLI have low levels of EPCs and high levels of inflammatory mediators such as IL6 [4,5]. Dopheide et al. also demonstrated that inflammation influences EPC biology [5]. Among the inflammatory mediators, TNF-α and IL-6 may be responsible for the reduction in EPCs.

In many pathological circumstances such as diabetes, the hyper-inflammatory environment inhibits vasculogenesis until the inflammation is controlled to a normal level. Mesenchymal stem cell (MSCs), given their immunomodulatory and angiogenic properties, have been shown to be therapeutically effective in patients with CLI [6,7]. It was reported that MSCs are immunosuppressive, which can suppress several T-lymphocyte and NK cells activities both *in vitro* and *in vivo* [8]. MSCs modulate the inflammatory response by downregulating pro-inflammatory cytokines such as TNF-α [9]. These properties make MSCs therapy a good candidate for CLI patients. In addition, MSCs are multipotent and differentiate into alternate cell types such as bone, cartilage, fat, muscle, endothelial cells and vascular smooth muscle cells. They can be expanded readily to sufficient number of cells of clinical grade MSCs for cell therapy. A phase I/II trial of allogeneic bone marrow derived MSCs for CLI showed the improved ankle brachial pressure index (ABPI) after the treatment. Yang et al. [10] proved that human cord blood-derived MSCs transplantation improved ischemia in patients with ASO/TAO.

However, the underlying mechanism of action of MSC-mediated improvements in CLI patients, especially with respect to the immune-inflammatory aspects of this disease, is still poorly understood. There is little known about the distribution of T-lymphocyte subpopulations and the change of inflammatory mediators in CLI patients before and after MSCs-based treatment. In our study, we treat CLI patients with umbilical cord-derived MSC by intramuscular administration. We aimed to investigate the changes in T-lymphocyte subpopulations and inflammatory mediators (IL-6, IL-10 and TNF-α) in PBMCs from

CLI patients after MSCs-based treatment. We also investigated the correlation between inflammatory mediators and EPCs.

Methods

Patients

Eight patients treated with umbilical cord mesenchymal stem cells (UC-MSCs) transplantation for lower-limb ischemia in the Institute of Hematology and Hospital of Blood, Diseases, Chinese Academy of Medical Sciences and Peking Union of Medical College. Table 1 shows baseline demographics and clinical characteristics of the patients. The 8 patients were selected to undergo UC-MSCs implantation because they were unresponsive to medication or surgical or endovascular procedures were deemed inappropriate. All patients received conventional drug treatment, such as antiplatelet or anticoagulant drugs, before and after transplantation. After approval by the ethical committee board of the Institute of Hematology and Hospital of Blood Diseases, Chinese Academy of Medical Sciences and Peking Union of Medical College, we obtained written informed consent from all patients for the study of cytokine and lymphocyte subsets in serum.

UC-MSCs transplantation

UC-MSCs were provided by the National Engineering Research Center of Cell Products, State Key Laboratory of Experimental Hematology. The immunophenotype of UC-MSCs included positivity for CD13, CD29, CD90, CD44, CD105 (SH2), CD106, CD73 (SH3), CD166, and HLA-ABC but negativity for CD14, CD34, CD38, CD45, CD31, and HLA-DR. CD106 and HLA-ABC were expressed at significantly lower levels than the other markers. In addition, sterility, mycoplasma and endotoxin testing were performed to confirm that the cells were devoid of any microbial contaminants and were sterile. Under general anesthesia, each diseased lower limb was intra-muscularly injected (40 sites, 3×3 cm distance, 1-1.5 cm deep) into the thigh and leg, with 6.29×10^7 /m^2 UC-MSCs.

Blood sampling

Peripheral blood samples patients were taken from drainage veins of the transplant-recipient limb before UC-MSC treatment, and further samples were taken at 24 h, 1 month after UC-MSC treatment. In addition, peripheral blood samples were also taken from 8 healthy volunteers.

Measurement of inflammation/ angiogenesis-related factors and lymphocyte subsets

Serum levels of human IL-6, IL-8, IL-10, TNF-α, VEGF, bFGF, EGF and HGF were measured by ELISA assay. The distributions of CD3+, CD3+CD4+, CD3+CD8+, CD3-CD16/CD56+ cells in PBMCs were measured by flow cytometry.

Assessment of circulating EPCs level

Circulating EPCs are characterized by the expression of immature markers, such asCD34 and CD133, and endothelial markers, such as KDR. To determine the number of EPCs, PBNCs were purified by standard Ficoll-Hypaque density centrifugation. EPC were measured using CD34-FITC, CD133-PE, and KDR-APC(Meltianyi Biotec,GRE). Between 5.0×10^5 and 1.0×10^6 cells were acquired in each analysis. The flow data were collected with a BD FACS Calibur machine (Becton Dickinson) and analyzed by FlowJo software (Tree Star Inc).

Statistical analysis

All experiments were repeated at least three times and differences were determined using Student's t-test. The interaction between the number of EPCs and TNF-α,IL-6 was examined by bivariate correlation. Results were expressed as the mean ± SD, and a value of $^*P < 0.05$, $^{**}P < 0.01$ was considered significant.

Results

Clinical characteristics of the study subjects

The 8 CLI patients had a mean age of 57.75 years (range 21~75) and comprised 7 males and 1 female. 5 of these patients underwent a previous endovascular and/or surgical intervention in the index limb. Patients presented with the following: 5 patients with diabete foot, 2 patients with arteriosclerosis obliterans, 1 patient with Buerger's disease. All patients received intramuscular injections of 6.29×10^7/ m^2 UC-MSCs.8 patients with an average follow-up period of 5.38 months. 8 patients were clinically effective after treatment. All patients were no amputation during follow-up period. The patient characteristics were summarized in Table 1.
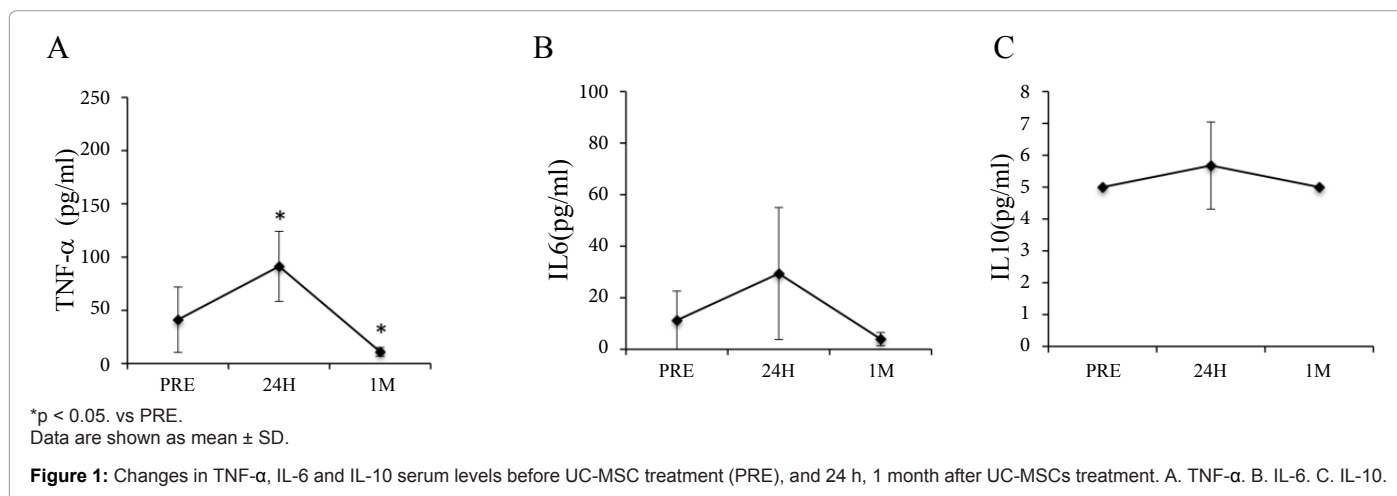
Changes in IL-6, IL-10 and TNF-αserum levels after UC-MSCs treatment

To investigate the effects of UC-MSCs treatment on the inflammatory response, we measured serum cytokine levels before treatment and 24 h, 1 month after treatment. TNF-α and IL-6 are pro- inflammatory cytokines that serve as important serum markers, reflecting the body's inflammatory state. TNF-α (p=0.017; p=0.031) and IL6 (p=0.099; p=0.072) serum levels significantly increased at 24 h after treatment and then decreased at 1 month compared with that before treatment (Figures 1A and 1B). We also examined IL-10 levels, an anti-inflammatory cytokine, that can reduce tissue inflammation

Case	Age	Gender	Diagnosis	Rutherford- Becker	Clinical effect
1	75	M	ASO	6	Skin ulcer↓, Rest pain disappered
2	23	F	DF	5	Skin ulcer↓, Rest pain disappered
3	72	M	ASO	5	Skin ulcer↓, Rest pain relief
4	61	M	DF	6	Skin ulcer↓, Rest pain disappered
5	21	M	Buerger's disease	6	Skin ulcer↓, Rest pain disappered
6	64	M	DF	4	Pain-free walking distance↑
7	72	M	DF	4	Pain-free walking distance↑
8	74	M	DF	6	Skin ulcer↓, Rest pain disappeared

ASO: Arteriosclerosis Obliterans; DF: Diabetic Foot; Rutherford-Becker Categories: Cat 0, Asymptomatic; Cat 1, Mild Claudication; Cat 2, Moderate Claudication; Cat 3 Severe Claudication; Cat 4, Rest Pain; Cat 5, Minor Tissue Loss; Cat 6, Ulceration Or Gangrene.

Table 1: Patient characteristics.

*p < 0.05. vs PRE.
Data are shown as mean ± SD.

Figure 1: Changes in TNF-α, IL-6 and IL-10 serum levels before UC-MSC treatment (PRE), and 24 h, 1 month after UC-MSCs treatment. A. TNF-α. B. IL-6. C. IL-10.

Group	N	CD3+ T cells (%)	CD3+CD4+T cells (%)	CD3+CD8+T cells (%)	Ratio of CD4+/CD8+	CD3-CD16/CD56+ NK cells(%)
CLI patients at baseline	8	73.99 ± 8.55	43.96 ± 7.07	22.49 ± 6.55	2.16 ± 0.81	12.75 ± 4.23
CLI after cells treatment	8	71.78 ± 8.35	41.66 ± 6.72	23.69 ± 7.36	1.96 ± 0.80	10.33 ± 3.78
Healthy controls	8	71.28 ± 7.47	39.27 ± 5.40	24.67 ± 4.14	1.64 ± 0.37	9.8 ± 5.08

Table 2: Distribution of T-lymphocyte subsets (mean ±SD) in CLI patients.

after injury. IL10 slightly increased at 24 h after treatment and then decline to baseline level at 1 month (Figure 1C).

Redistribution of lymphocyte subsets in PBMCs after UC-MSCs treatment

The percentage of CD3+ T lymphocytes was increased in CLI patients (73.99 ± 8.55%) at baseline compared to the healthy controls (71.28 ± 7.47%; p=0.336). The percentage of CD3+ CD4+ T cells was increased (p=0.209), and the percentages of CD3+ CD8+T lymphocytes (p=0.467) was decreased in CLI patients at baseline compared to the healthy controls, resulting in a higher CD4/CD8 ratio. The percentage of CD3- CD16/ CD56+ NK cells was significantly increased in CLI patients (12.75 ± 4.23%) at baseline compared to the healthy controls (9.8 ± 5.08%; p=0.034) (Table 2). However, after UCs-MSC treatment, the percentages of CD3+ T, CD3+ CD4+ lymphocytes and NK cells were significantly decreased (p=0.002, p=0.012 and p=0.029, respectively), whereas the percentages of CD3+ CD8+ lymphocytes were increased (p=0.275) compared to those at baseline (Table 2).

Correlation between pro-inflammatory mediators and EPCs

EPCs are characterized by the coexpression of CD34, CD133 and KDR. The number of circulating EPCs at 24 h after UC-MSCs treatment was lower compared to those at baseline (0.0464 ± 0.0435% vs 0.1051 ± 0.0729%, P=0.214). However, at 1 month after UC-MSCs treatment, the number of circulating EPCs was increased compared to the baseline (0.1939 ± 0.1096% vs 0.1051 ± 0.0729%, P=0.171). Using bivariate analysis, TNF-α(r = -0.42, P=0.0004) and IL-6(r = -0.33, P=0.0001) were shown to be inversely correlated with the number of circulating EPCs (Figure 2).

Discussion

Peripheral artery disease (PAD) is a restriction of blood supply to limb, caused by pathological changes in the vascular system, such as atherosclerosis and inflammation. PAD can cause a wide variety of symptoms including chronic wounds and ulcers, rest pain and tissue necrosis with gangrene. For critical limb ischemia, there is no effective pharmacological treatment. Amputation is inevitable in a third of the PAD cases to solve the unbearable symptoms [1].

It has been reported that immune activation is important at several stages in the development of chronic wounds of CLI, which result from various underlying disorders, including diabetes, vasculitis and vascular insufficiency. The healing process can be disturbed by various factors, including infection, tissue hypoxia, necrosis and hyper-inflammatory cytokines. Tuttolomondo et al. [11] reported that higher plasma levels of IL-6 linking diabetic foot ulcers pathogenesis to microvascular and inflammatory events. Dinh et al. [12] demonstrated that the number of inflammatory cells around vessels in diabetic patients were higher than healthy control subjects through forearm-skin biopsy. IL6, which believed to be secreted from macrophages and lymphocytes, has been shown to have an important role in chronic inflammation disorders. It was reported that IL-6 is highly upregulated in rheumatoid arthritis, and it is an effective treatment for RA with an IL-6 receptor antagonist [13]. TNF-α is another important pro- inflammatory cytokine, which is elevated in many inflammatory diseases. In our study, the average serum levels of IL-6 and TNF-α in patients were above the normal range before UC-MSCs treatment (11.27 ± 11.32 pg/ml vs. < 5.9 pg/ml; 41.22 ± 30.70 pg/ml vs. < 8.1 pg/ml). All the patients Rutherford- Becker categories 4-6,5 of 8 patients underwent bypass surgery. However, their ulcers and rest pain has not been eased. These findings suggest that CLI patients with chronic inflammation state, which impede the improvement in symptoms.

The continuous inflammation phenotype in the wound creates a barrier to treatment in chronic wounds. The infiltration of neutrophils, macrophages, DCs and T cells not only contribute to chronic inflammation but also release the enzyme elastase, which is capable of destroying important healing factors such as PDGF and TGF-β. Dinh et al. [12] also demonstrated that T lymphocytes around blood vessels were higher in diabetic patients. In our study, the percentage of CD3+ T lymphocytes was increased in CLI patients at baseline compared to the healthy controls. In addition, CD4/CD8 ratio is higher compared to the
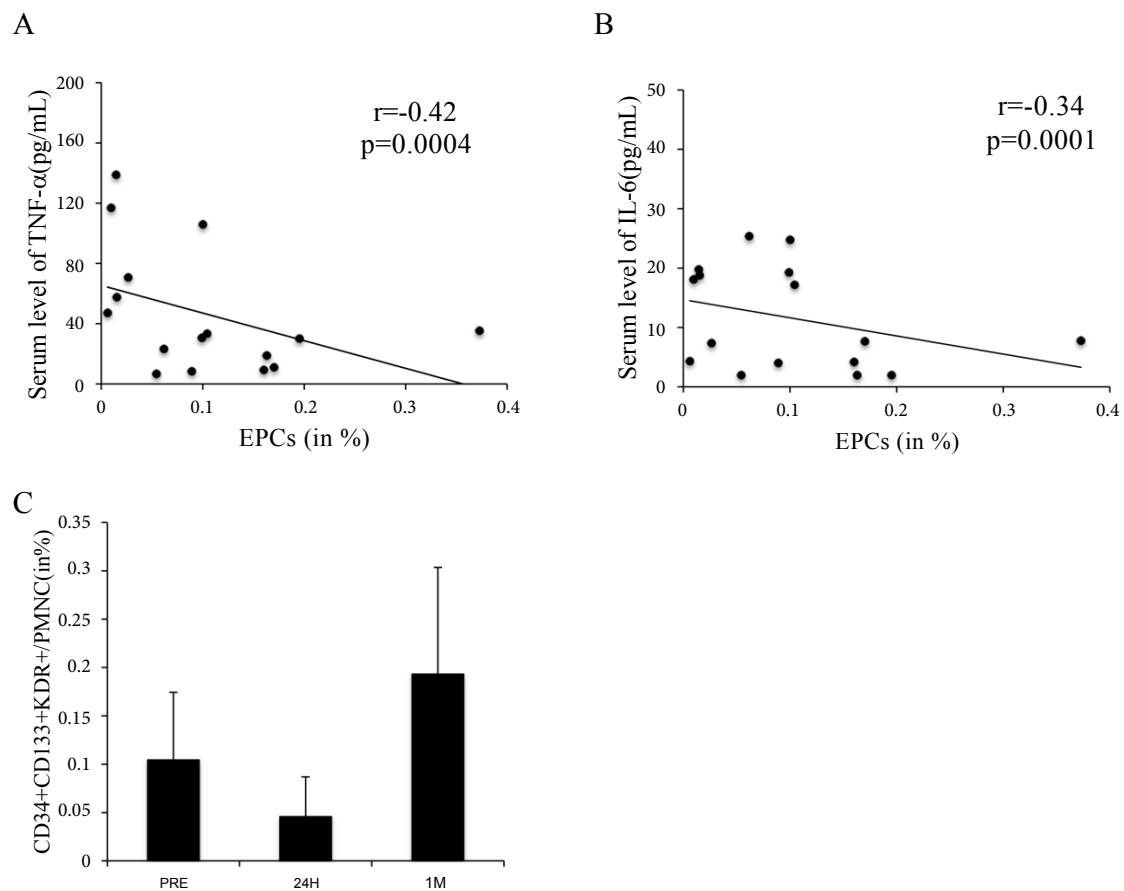
Figure 2: Association of serum TNF-α/IL-6 levels and the relative expression levels of EPCs. A, B There was a negative association between serum TNF- α/IL-6 levels and the relative expression levels of EPCs. Serum TNF-α and IL-6 levels were detected by ELISA, and EPCs was detected using flow cytometry. C shows the time course of circulating EPCs in CLI patients.

healthy controls. It was suggested that the imbalance of T lymphocyte subsets play a vital role in non-healing wound of CLI.

In 1998, Folkman [14] reported the conception of "therapeutic angiogenesis", accumulating evidence supporting cell therapy as new treatment option for CLI. MSCs superior to other cells for low immunogenicity, immunomodulatory and angiogenic properties. One study reported that intramuscular administration of allogeneic MSCs attenuated the local oxidative stress and endothelial inflammation in a mouse model of hind-limb ischemia [15]. In addition, Pinheiro et al. [16] reported in the dystrophin- deficient mice, intramuscular injection of adipose tissue-derived MSCs can reduce local inflammation. It was also reported that Prostaglandin E2 (PGE2) was produced by human MSCs at levels able to suppress IL-6 and TNF-α expression in activated macrophages [17]. However, in our study, TNF-α and IL6 serum levels significantly increased 24 h after UC-MSCs treatment and then decreased 1 month compared with that before treatment. Two reasons might explain the result. The higher levels of pro-inflammatory cytokines was considered to be caused by cell transplantation- induced early inflammatory. It was considered to be a direct action by implanted cells. The serum levels of TNF-α and IL6 decreased 1 month after treatment, which was considered that implanted UC-MSCs secrete anti-inflammatory cytokines, PEG2 and inhibition of T/NK cells proliferation. It was considered to be an indirect paracrine action and immunomodulatory induced by the implanted cells.

The immunomodulatory effects of MSCs, including inhibition of the proliferation of T lymphocytes and NK cells [18]. MSCs inhibit T cell proliferation by arrest at the G0/G1 check point [19]. Ren et al. demonstrated that MSC inhibit T cell proliferation by upregulation of inducible nitric oxide synthase (iNOS), which generates NO can produce such an effect [20]. Chatterjee et al. reported that UC-MSCs could potently suppress NK cell cytotoxicity in overnight cultures via soluble factors prostaglandin (PG)-E2 [21]. MSCs has also been found to inhibit NK via Indoleamine-2,3- dioxygenase expression [22]. In our study, after UC-MSCs treatment, the percentage of CD3+ T, CD3+ CD4+T lymphocytes and NK cells was significantly decreased (p=0.002, p=0.012 and p=0.029, respectively), whereas the percentages of CD3+ CD8+ T lymphocytes were increased (p=0.275) compared to those at baseline. These findings suggested that intramuscular administration of UC-MSCs could inhibit proliferation of T/NK cells and modulate the balance of T lymphocytes subset in CLI.

The correlation between pro- inflammatory mediators and EPCs was also investigated in our study. EPCs generated from bone marrow were believed to participate in endothelial repair and postnatal angiogenesis, either by differentiating into endothelial cells or by secreting factors that stimulate proliferation of resident endothelial cells [23]. Some studies reported that EPCs significantly affected by chronic inflammation [4,24]. The negative correlation between IL-6, TNF-α levels and EPCs were described in RA patients and healthy controls [25,26]. In our study, TNF-α (r = -0.60, P < 0.01) and IL-6 (r = -0.32, P < 0.001) were

shown to be inversely correlated with the number of circulating EPCs. The underlying mechanism might be that proinflammatory-mediated bone marrow suppression or exhaustion [27], resulting in attenuated release of EPC into the circulation. In addition, MMP-9 [28] and SDF-1 [29] play an important role in mobilization of EPC form the BM. Teraa et al. [4] show that MMP-9 levels and activity are reduced in the BM and alterations in the SDF-1a/CXCR4 interaction in CLI contribute to the defective neovascularization response in CLI.

This study has limitations, which are in large part related to the sample size of the study is small. Larger studies should be conducted to identify immunomodulatory effects of UC-MSCs in CLI. The proportion of men and women is unbalanced, which should be improved in a future study. We only assessed pro-inflammatory mediators, but levels of angiogenic factors such VEGF, bFGF, EGF were not detected.

In conclusion we show that chronic hyperinflammation state and the imbalance of lymphocyte subsets play a key role in non- healing wound of CLI. Intramuscular administration of UC-MSCs exert immunomodulatory effects in CLI patients via the decrease in the levels of pro-inflammatory cytokines, including IL-6, TNF-αand reduction of CD3+CD4+T lymphocyte and NK cells. We also show that circulating CD34+CD133+KDR+ EPC was closely correlated with the increased TNF-α,IL-6. In addition, it was considered that UC-MSCs treatment is effective for CLI patients in our study.

Declaration of Interests

All authors declare no competing interests.

Authors' Contributors

W-HG designed the study, did the literature search, interpreted data, and wrote the report. P-PH and S-ZL designed the study, collected, analysed, and interpreted data, and revised the report ,and was responsible for the management of clinical patients.. W-HG,H-ML,J-YY and YG collected blood samples and did experiments. All authors approved the final report.

Acknowledgments

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