Autologous bone marrow mononuclear cell transplantation therapy improved symptoms in patients with refractory diabetic peripheral neuropathy via the mechanisms of paracrine and immunomodulation

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Research

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Abstract

Background: We recently reported that transplantation of autologous bone marrow mononuclear cells (BM-MNCs) may be an effective and promising therapy to treat refractory diabetic peripheral neuropathy (DPN) in patients with type 2 diabetes mellitus (T2DM). This study was designed to investigate the potential mechanisms of BM-MNCs therapy.

Methods: This clinical study recruited 60 patients with DPN, 30 T2DM patients without complications, 30 healthy control participants. All clinical parameters, the levels of inflammatory markers and growth factors in three groups were compared. All patients with DPN received one intramuscular injection of BM-MNCs and clinical follow-ups after the therapy for 2 days, 1, 4, 12, 24, and 48 weeks. Then they were divided into the responder and non-responder groups based on the improvement of nerve conduction velocity. Binary logistic regression was performed to evaluate the corresponding prognostic factors for BM-MNCs treatment.

Results: Patients in DPN group had higher level of tumor necrosis factor-α (TNF-α) and lower level of vascular endothelial growth factor (VEGF) than those in control group. DPN group had the highest level of soluble intercellular adhesion molecule-1 (sICAM-1) among three groups. The level of nerve growth factor (NGF) in DPN group was slightly lower than that in DM group. Neuropathic symptoms were significantly improved after BM-MNCs injection. Thirty five of 54 patients with DPN (64.8%) reached the primary endpoint, and were regarded as the responders. Compared to non-responders, responders were younger, had a longer history of diabetes, and had higher numbers of mobilized CD34+ cells and BM-MNCs. The levels of TNF-α and sICAM-1 decreased just after BM-MNCs injection in both groups and slowly reverted to baseline levels. The durations of downtrend of TNF-α and sICAM-1 in responder group lasted longer than that in non-responder group. Serum level of VEGF in responder group increased immediately after BM-MNC therapy, and reached the highest point after the injection for 12 weeks. On the other hand, VEGF levels in the non-responder group only increased slightly. The number of applied CD34+ cells (OR=1.567, 95% CI 1.106-2.222, p=0.012) and duration of diabetes (OR=0.760, 95% CI 0.597-0.967, p=0.025) were the independent predictors of responding to BM-MNCs therapy. No adverse event associated with the treatment was observed during follow-up observations.

Conclusions: BM-MNCs transplantation is an effective and promising therapeutic strategy to treat refractory DPN. The immune regulation and paracrine function of BM-MNCs may contribute to the improvement of DPN.

Introduction

Diabetic peripheral neuropathy (DPN) is the most common complication of Type 2 Diabetes Mellitus (T_2DM), affecting approximately half of the long-standing T_2DM patients [1]. It is characterized by the symmetrical loss of sensation in the lower limbs, resulting in increased risks of traumatic injuries or chronic pain perceiving a significant reduction of quality of life and high treatment costs [2]. However,

novel strategies of treating DPN are limited to intensive glucose control and pain alleviating, which have marginal therapeutic effect in patients with progressive stage [3]. With poor prognostic outcomes and high mortality, “No-treatment-options” patients are often suffered by recurrent or incurable foot ulcerations and gangrenous infections, eventually leading to limb amputation.

The pathogenic mechanisms of DPN are multifactorial. It is speculated that diabetic neuropathy is secondary to the deficiency of local growth factors, causing a reduction of vasa vasorum and neuronal damage in diabetic peripheral nerves [4]. A number of inflammatory mediators are also involved in the progression of DPN [5]. Stem cell-based therapies aiming at enhancing neovascularization and ameliorating inflammatory cytokines is emerging as a novel treatment strategy for patients with DPN who are not eligible for traditional drug therapy. Recently, bone marrow-derived stem cells based therapies have been applied as a promising strategy for experimental diabetic neuropathy in animal models because of their multipotency to inhibit inflammation and promote angiogenesis and neurotrophy [6, 7].

In our previous study, bone marrow derived mononuclear cells (BM-MNCs) have been applied in 168 patients with refractory DPN, demonstrating that BM-MNCs transplantation is an effective and safe therapeutic strategy [8]. Through regular follow-up and continuous monitoring of clinical indicators, the present study aimed to evaluate the potential mechanisms associated with the therapeutic benefits of BM-MNCs therapy in patients with DPN.

Methods

Ethical approval

This clinical study was undertaken at Central Hospital of Wuhan, Hubei Province, China. All participants were briefly informed about this study. The informed consent was obtained during the study enrollment. This study was approved by the Ethics Committee of the Central Hospital of Wuhan, Wuhan, China.

Study design and participants

In this single-center clinical study, we investigated the clinical effect of BM-MNCs transplantation in 60 patients with refractory DPN recruited from March 2014 to December 2017. The detailed inclusion and exclusion criteria were listed below. All enrolled patients received the autologous BM-MNCs therapy and followed-up after transplantation for 2 days, 1, 4, 12, 24 and 48 weeks.

This clinical study also included 30 healthy control individuals (Control group) and 30 T_2DM patients without complications (DM group). Age, sex, and BMI in all three groups were comparable (all $p>0.05$).

Inclusion criteria of DPN group

1) Age at 40 to 70 years; 2) T_2DM patients defined by 2013 American Diabetes Association (ADA) standards [9]; 3) DPN was defined as the presence of an abnormality of nerve conduction and a symptom or a sign of neuropathy[10]; 4) refractory DPN, defined as no significant relief of the neuropathic symptoms or signs when combine conventional drug therapies for at least one year, which include the antioxidant (a-lipoic acid), aldose reductase inhibitors and transketolase activators (thiamines and allithiamines).

Exclusion criteria of DPN group

1) Severe hepatic and renal dysfunctions; 2) hypercoagulable states or with hematological diseases; 3) foot ulcers and limb deformity; 4) pregnancy; 5) evidence of malignancy during the last 5 years; 6) life expectancy less than 6 months.

Preparation of BM-MNCs

The procedures used for the BM-MNCs treatment have been described in details previously [8]. Briefly, patients were subcutaneously injected $5ug \cdot kg^{-1} \cdot d^{-1}$ recombinant human granulocyte colony-stimulating factor (G-CSF, Qilu Pharmaceutical, China) for 3 consecutive days to mobilize the stem cells in bone marrow. After G-CSF mobilization, approximately 200-300mL bone marrow was harvested from the posterior superior iliac crest under anesthetic conditions in a sterile surgical environment.

The preparation of BM-MNCs was processed in the laminar flow laboratory. Mononuclear stem cells were isolated by Ficoll-Hypaque density–gradient centrifugation. Then, the mononuclear cell layer was harvested and washed 3 times with normal saline and resuspended in 50 mL normal saline. The total concentration of $CD34^+$ cells in the cell suspension comprising mononuclear cells was calculated using flow cytometry.

Transplantation procedures

The prepared BM-MNCs suspensions were injected intramuscularly to both thighs and legs of DPN patients (50 sites, 2 cm*2 cm in intervals, 1-1.5 cm in depth, 1 mL BM-MNCs per site) under continuous monitoring of vital parameters in a sterile surgical environment. Patients were followed-up for at least 24 hours in the intensive care unit after the injection.

Clinical assessment before and after transplantation

Clinical and laboratory data were collected before BM-MNCs administration and at every follow-up visit in DPN group. All patients received similar ordinary treatment throughout the course of this clinical study, including intensive control of blood glucose, blood pressure, and blood lipids. Smoking cessation was

encouraged during the study. Attention was paid during the follow-up visits specifically to any potential adverse effects due to the transplantation.

Measurement of nerve conduction studies (NCS, Viking Quest®, Nicolet Biomedical Inc, WI, USA) was performed by the same experienced technician who was blinded to the patients' clinical information according to validated standards. Routine NCS measurements were performed pre-transplantation and at 12, and 48 weeks post-transplantation. The observation items of NCS included sensory nerve conduction velocity (sNCV), sensory nerve action potential (SNAP) in superficial peroneal and sural nerves, motor nerve conduction velocity (mNCV), and compound muscle action potential (CMAP) in peroneal and posterior tibial nerves.

Neurological evaluations were performed with Toronto Clinical Scoring System (TCSS) before the therapy and at every follow up visit post transplantation.

Blood samples of all the subjects in the study were collected to test the levels of inflammatory markers and growth factors at baseline and at each post-transplantation follow-up visit in DPN group. Venous blood samples were collected from the subjects after a 12-h overnight fast. Serum were separated and stored at -70 ℃.Commercially available enzyme immunoassay (ELISA) kits were used to measure the inflammatory cytokines (IL-6, TNF-α, IL-10, sICAM-1) and growth factors (VEGF and NGF) according to the manufacturer's instruction. All kits were provided by Quantikine, R&D Systems.

Outcome assessment of BM-MNCs transplantation

The primary endpoint of the study was the improvement of NCV. Responder to BM-MNCs therapy was defined as the sensory or motor nerve conduction velocity increased at least by 30% during the follow-up periods. Patients without obvious change of NCV were considered non-responders.

Statistical analysis

Measurement data were expressed as mean ± standard deviation (SD) for continuous variables, interquartile ranges for nonnormal data, and in percentages for discrete variables. Group differences for continuous variable were tested by independent t test or the Mann-Whitney U test. Dichotomous variables were analyzed with Fisher exact test. Multiple group comparisons were performed by analysis of variance (ANOVA). The comparison between baseline and each follow-up visit measurements was performed by employing the paired t test. Subsequently, multiple binary logistic regression analysis was used to study predictors of clinical benefit after BM-MNCs transplantation. Statistical analyses were performed using SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered statistically significant.

Results

Baseline characteristics of the entire study cohort

Baseline characteristics of the entire study cohort are shown in **Table 1**. All groups were matched for age, gender and BMI. The history of smoking and alcohol consumption were similar among three groups. Compared to those without diabetic complications, patients with diabetic neuropathy had a significantly longer duration of diabetes and significantly higher levels of FPG and HbA1c ($p < 0.001$). Systolic and diastolic blood pressure values were lower in the control group but similar in the two diabetic groups ($p < 0.05$). In the aspect of lipid metabolism, the level of TG was lower in the control group compared with the other two diabetic groups, and the level of HDL was higher in control group than DPN group ($p < 0.05$). Compared to control group, a higher proportion of patients in DM group and DPN group had coronary heart disease and experienced stroke ($p < 0.05$). As expected, the treatments with medications of anti-hypertension, lipid-lowering and antiplatelet were more common in two diabetic groups. Regarding the hypoglycemic therapies used in the two diabetic groups, the use of insulin was marginally higher in DPN group compared with DM group. No difference in the remaining diabetic medications was observed.

The measurements of growth factors and inflammatory markers are shown in **Table 1.** The levels of TNF-α and VEGF in DPN group were respectively higher and lower than that in control group ($p < 0.05$). DPN group had higher level of sICAM-1 than the other two groups ($p < 0.05$), and a lower level of NGF than DM group ($p < 0.05$).

Clinical efficacy of transplantation

During the follow-up period, one patient died (36 weeks after BM-MNCs transplantation) because of myocardial infarction, and 5 patients lost contact and did not show in the follow-ups due to various reasons. These patients were excluded from further data analysis.

The primary endpoint of the study was reached in 35 of 54 patients with DPN (64.8%). Nerve conduction study revealed that both sensory and motor nerve conduction velocity notably improved at 12 weeks after transplantation (**Figure 1A, Supplement Table1**). sNCV in responder and non-responder groups increased by 32.5% and 20.0% respectively, whereas the increased levels of mNCV in the two groups were 31.9% and 19.8% respectively. Both groups showed persistent improvement in neuropathic symptoms and signs, which can be reflected from the decline of TCSS **(Figure 1B, Supplement Table2)**.

The characteristics of responder and non-responder groups before BM-MNCs therapy were listed in **Table 1.** Compared to that in non-responder group, patients in responder group were younger ($p<0.01$), and with a longer duration of diabetes ($p<0.01$). BM-MNCs products of responder group were characterized by a higher $CD34^+$ hematopoietic progenitor cell count ($p<0.001$), and a higher number of total BM-MNCs ($p<0.001$) than that of non-responders.

As shown in **Figure 2**, the levels of TNF-α and sICAM-1 decreased just after BM-MNCs transplantation in all groups and reverted to the baseline level at 12 weeks and 24 weeks, respectively. This downtrend

duration lasted longer in responder group than in non-responder group, suggesting that the immune regulation function of BM-MNCs may play a critical part in the treatment of DPN. The serum level of VEGF in responder group was up-regulated immediately after BM-MNC therapy, reaching the highest level at 12 weeks. Whereas we only viewed a slightly increase of VEGF in non-responder group. The baseline levels of other cytokines were similar to that after BM-MNCs therapy.

Procedural safety

No infection, bleeding, or other complications associated with BM-MNCs transplantation were detected. Three patients (5.0%) experienced short-term episodes of slight pain at the injection sites 2 hours after BM-MNCs transplantation, which can be well tolerated with only mild discomfort.

During follow-up, no effect on liver or kidney functions was observed and no case of rejection or malignancy was detected in all participants.

Prognostic factors for BM-MNCs treatment

Prognostic factors are important when deciding on treatment options. We evaluated the clinical outcomes and identified the corresponding prognostic factors for BM-MNCs treatment of patients with DPN. Binary logistic regression was performed to ascertain the effects of age, duration of diabetes, the number of $CD34^+$ cells and BM-MNCs on the likelihood of responding to the cell therapies. The binary logistic regression model was statistically significant (x2(4)=4.596, *p=0.032*). The model explained 46.6% (Nagelkerke R^2) of the variance in treatment outcome and correctly classified 79.6% of cases. The number of applied $CD34^+$ cells (*p=0.012*, Exp(B)=1.567, 95% confidence interval (CI)1.106-2.222) and duration of diabetes (*p=0.025*, Exp(B)=0.760, 95% CI 0.597-0.967) emerged as independent predictors of responding to the cell therapy.

The number of total BM-MNCs positively correlated with an increase of VEGF after 4 weeks (*p=0.023*, r=0.292). A similar but weaker correlation was observed between the absolute number of $CD34^+$ cells and the increase of VEGF after 4 weeks (*p=0.041*, r=0.264) (**Figure 3**).

Discussion

Almost 50% of long-standing T2DM patients are affected by DPN [11], and up to 20% will experience peripheral chronic neuropathic pain [12], resulting in a dramatically decreased quality of life. Currently, no curable therapy is available for patients with progressive stage of DPN. Intensive glucose control is a recommended strategy in ADA guidelines, which may prevent the progression of DPN. However, it cannot cure already established nerve injuries. For patients who experienced abnormal neuropathic symptoms took the maximum dose of symptomatic treatment, the relief is usually incomplete. As a result, the management of effective therapy for refractory DPN is urgently needed.

Our previous clinical study shown that autologous transplantation of BM-MNCs effectively improved neuropathic symptoms and restored the peripheral nerve functions, which demonstrated BM-MNCs transplantation was an effective and safe treatment for refractory DPN. In this follow-up study, firstly, we compared the concentrations of inflammatory cytokines and growth factors in healthy individuals, diabetes mellitus patients without complications and DPN patients. Our present study showed that patients with DPN had relatively higher levels of TNF-α, sICAM-1 and lower levels of VEGF and NGF when compared to healthy control participates and diabetic patients without complications, which indicate the association between varies levels of inflammatory cytokines and growth factors may participate in the development and progression of DPN.

Known pathologies reported in DPN included axonal atrophy, demyelination, nerve fiber loss, and blunted regeneration of nerves. In addition to these pathogeneses, several studies have reported the link between subclinical inflammation and diabetic neuropathy [13, 14]. Diabetic TNF-$\alpha^{-/-}$ mice show no evidence of abnormal nerve function. A single injection of infliximab to suppress the level of TNF-α in diabetic TNF-$\alpha^{+/+}$ mice ameliorates the electrophysiological and biochemical deficits [15]. The involvement of TNF-α in chronic inflammation regulation and immune response may result in various nerve damages. As one of the cell adhesion molecules, sICAM-1 reflects a low-grade vascular inflammation and may be associated with the development of diabetic complication [16]. The level of sICAM-1 is higher in patients with DPN compared to those without complications [17]. The levels of sICAM -1 are associated with nerve conduction velocity and vibration perception thresholds, suggesting a vascular involvement in the development of DPN [18, 19]. VEGF induces angiogenesis by stimulating the proliferation and migration of endothelial cells in ischemic tissues, improving tissue ischemia [20]. Both VEGF and NGF promote neural regeneration and survival of neurons [21]. An increase in inflammatory markers and a decrease in growth factors were in mutual effect with each other and in synergistic effect with the development and progression of DPN.

These findings suggest that a therapeutic method targeting both inflammation regulation and improvement of growth factors may be an effective treatment of DPN. Recently, the transplantation of BM-MNCs has attracted a great deal of attention as a possible therapeutic approach for various clinical targets including ischemic heart disease [22, 23] and peripheral arterial disease [24]. Various animal studies have shown beneficial effects of using BM-MNCs in treating experimental DPN model [7]. Intramuscular injection of BM-MNCs preferentially targets to peripheral nerves, especially around vasa nervorum, and increases expression of angiogenic and neurotrophic factors. In Hasegawa's study, using neutralizing antibody of VEGF suppresses the therapeutic effect of BM-MNCs, which further confirms the mechanism of BM-MNCs' paracrine property [7]. The expression of angiogenic and neurotrophic factors results in the improvement of vasa nervorum functions [25]. NGF releasing from BM-MNCs improves regeneration of sciatic nerve in adult rat and stimulates the proliferation of Schwann and satellite cells [26]. The immune-suppressive property of BM-MNCs to ameliorate inflammatory reaction in peripheral nerve has not been observed in DPN animal models. But in a study including Stsegment Elevation Myocardial Infarction (STEMI) patients, BM-MNCs transplantation was performed via an intracoronary

route. Their results indicated that BM-MNCs transplantation after STEMI affects the balance between proinflammatory and anti-inflammatory cytokines [27]. Additional studies are required to fully understand the immunomodulation effects of BM-MNCs transplantation in treating DPN.

To investigate the mechanisms underlying the therapeutic effect of BM-MNC in patients with DPN, we examined the changes of several growth factors and inflammation cytokines after BM-MNCs transplantation. The serum levels of TNF-α and sICAM-1 decreased at 2 days after BM-MNCs transplantation and this decrement persisted within the next 4 weeks. The level of VEGF moderately increased and reached the maximum at 12 weeks after BM-MNCs transplantation. We divided the DPN patients into the responder and non-responder group based on the improvement of NCV. The degree and duration of trends of VEGF, TNF-α and sICAM-1 variations are more pronounced in responder group than non-responder group. Thus, through our study, we speculated that both paracrine effect of growth factors and immune regulation of BM-MNCs participated in treating DPN. The anti-inflammatory effect may take effect in the early stage of the treatment, and the paracrine effect started later and lasted for a relatively longer time.

The present study investigated prognostic factors predictive of the therapeutic effect of BM-MNCs. Binary logistic regression revealed that the number of applied $CD34^+$ cells and duration of diabetes are the independent predictors of responding to BM-MNCs therapy. The absolute number of applied BM-MNCs and $CD34^+$ were positively correlated with the increase of VEGF after 4 weeks transplantation. It was postulated that the amount of $CD34^+$ cells had a crucial influence to the outcome of BM-MNCs therapy. $CD34^+$, which is broadly accepted as a marker of hematopoietic progenitor cells, also expressed on the surface of endothelial progenitor cells (EPCs). $CD34^+$ cells have the potential for neovascularization in ischemic tissue to restore the microcirculation and improve tissue perfusion [28]. Patients with T_2DM showed impaired mobilization ability of proangiogenic cells and $CD34^+$ cells after G-CSF stimulation [29]. In a meta-analysis of the trails using G-CSF to stimulate bone marrow stem cells in treating patients with cardiovascular disease, there is a strong negative correlation between the prevalence of diabetes and the mobilization of $CD34^+$ cell in response to G-CSF [30]. These reports strongly imply that diabetes may adversely affect the therapeutic potential of autologous BM-MNCs therapy in patients with diabetic neuropathy. Patients with longer diabetic duration may have a worse survival and mobilization potential of bone marrow stem cells. Future clinical studies should consider the timing of bone marrow stem cell therapy and how to improve the mobilization of bone marrow stem cells in patients with diabetes. Several small molecules or growth factors can be used to improve the mobilization ability of stem cells [31-33]. More efforts are needed to transform these experimental findings into clinical applications in the future.

To our knowledge, this is the first clinical study to explore the clinical efficacy, safety and the possible mechanisms of BM-MNCs therapy for patients with refractory DPN. Several limitations of our study must be mentioned. First of all, the low number of participates with DPN and short follow-up duration may decrease the statistical power of the study. The lack of a placebo treated group may lead to a biased effect. Secondly, we used the improvement of NCV as the primary endpoint of our study, instead of the

invasive method of peripheral nerve and cutaneous biopsies, which cannot prove the neovascularization mechanisms of BM-MNCs directly. Thus, future randomized double-blinded controlled trials with larger sample sizes and longer-term follow-ups using biopsy of histological evaluation as the primary endpoint are needed to assess the therapeutic effect of BM-MNCs transplantation. Thirdly, using unfractionated BM-MNCs in present study, we cannot find out which type of cells among the overall BM-MNCs is the most responsible for the beneficial effects to relieve the neurological symptoms. Ultimately, given that DPN is a disease progressing over a long time, a single injection of BM-MNCs may not be enough to maintain the nerve function over a long period of time. The administration route and frequency of BM-MNCs need further investigation.

In conclusion, BM-MNCs therapy may become a potential therapeutic option to treat diabetic neuropathy via the mechanism of paracrine and immunoregulation effect.

Abbreviations

BM-MNCs: bone marrow mononuclear cells; DPN: diabetic peripheral neuropathy; T2DM: type 2 diabetes mellitus; NCV: nerve conduction velocity; TNF-α: tumor necrosis factor-α; sICAM-1: soluble intercellular adhesion molecule; VEGF: vascular endothelial growth factor; NGF: nerve growth factor; IL, interleukin; G-CSF: granulocyte colony-stimulating factor; CI: confidence interval; SD: standard deviation; ADA: American Diabetes Association; sNCV: sensory nerve conduction velocity, SNAP: sensory nerve action potential; mNCV: motor nerve conduction velocity, CMAP: compound muscle action potential; TCSS: Toronto Clinical Scoring System; ELISA: enzyme immunoassay; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HbA1c, glycosylated hemoglobin; TG: triglyceride; TC: total cholesterol; LDL: low density lipoprotein; HDL: high density lipoprotein; Scr, serum creatinine; CAD, cardiovascular disease; DPP-4: dipeptidylpeptidse 4;

Declarations

Ethics approval and consent to participate

This clinical study was approved by the Ethics Committee of the Central Hospital of Wuhan, Wuhan, China (2014-003).

Consent for publication

Written informed consent was obtained from all of the patients included in this study.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflicts of interest

The authors declare that they have no competing interests.

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Author contributions

Wei W and Zhao S contributed to the conception and design of the study. Mao H and Zhao S contributed to funding acquisition. Wei W and Li L acquired and analyzed the data. Wei W wrote and revised the manuscript and contributed to the discussion. Mao H and Zhao S reviewed and edited the manuscript and contributed to the discussion. Dong JJ performed the nerve conduction study. Wang L and Wang HX performed the preparation of BM-MNCs. Deng L, Wang ZJ, Jia T, and Lyu XY performed the procedures of BM-MNCs transplantation. All authors read and approved the final manuscript.

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References

1. Ang L, Jaiswal M, Martin C, Pop-Busui R. Glucose control and diabetic neuropathy: lessons from recent large clinical trials. Curr Diab Rep. 2014; 14(9): 528.
2. Pop-Busui R, Boulton AJ, Feldman EL, Bril V, Freeman R, Malik RA, et al . Diabetic Neuropathy: A Position Statement by the American Diabetes Association. Diabetes Care. 2017; 40(1): 136-154.
3. Javed S, Alam U, Malik RA. Treating Diabetic Neuropathy: Present Strategies and Emerging Solutions. Rev Diabet Stud. 2015; 12(1-2): 63-83.
4. Salis MB, Graiani G, Desortes E, Caldwell RB;Madeddu P;Emanueli C. Nerve growth factor supplementation reverses the impairment, induced by Type 1 diabetes, of hindlimb post-ischaemic recovery in mice. Diabetologia. 2004; 47(6): 1055-1063.
5. Pop-Busui R, Ang L, Holmes C, Gallagher K, Feldman EL. Inflammation as a Therapeutic Target for Diabetic Neuropathies. Curr Diab Rep. 2016; 16(3): 29.

6. Kim H, Park JS, Choi YJ, Kim MO, Huh YH, Kim SW, et al. Bone marrow mononuclear cells have neurovascular tropism and improve diabetic neuropathy. Stem Cells. 2009; 27(7): 1686-1696.
7. Hasegawa T, Kosaki A, Shimizu K,Matsubara H, Mori Y, Masaki H, et al. Amelioration of diabetic peripheral neuropathy by implantation of hematopoietic mononuclear cells in streptozotocin-induced diabetic rats. Exp Neurol. 2006; 199(2): 274-280.
8. Mao H, Wei W, Fu XL, Dong JJ, Lyu XY, Jia T, et al. Efficacy of autologous bone marrow mononuclear cell transplantation therapy in patients with refractory diabetic peripheral neuropathy. Chin Med J (Engl). 2019; 132(1): 11-16.
9. American Diabetes Association. Standards of Medical Care in diabetes--2013. Diabetes Care. 2013; 36(Suppl 1): S11-66
10. Callaghan BC, Cheng HT, Stables CL, Smith AL, Feldman EL. Diabetic neuropathy: clinical manifestations and current treatments. Lancet Neurol. 2012; 11(6): 521-34
11. Pop-Busui R, Lu J, Brooks MM, Albert S, Althouse AD, Escobedo J, et al. Impact of glycemic control strategies on the progression of diabetic peripheral neuropathy in the Bypass Angioplasty Revascularization Investigation 2 Diabetes (BARI 2D) Cohort. Diabetes Care. 2013; 36(10): 3208-3215.
12. Barrett AM, Lucero MA, Le T, Robinson RL, Dworkin RH, Chappell AS. Epidemiology, public health burden, and treatment of diabetic peripheral neuropathic pain: a review. Pain Med. 2007; 8 Suppl 2: S50-62.
13. Herder C, Lankisch M, Ziegler D, Rathmann W, Koenig W, Illig T, et al. Subclinical inflammation and diabetic polyneuropathy: MONICA/KORA Survey F3 (Augsburg, Germany). Diabetes Care. 2009; 2(4): 680-682.
14. Herder C, Bongaerts BWC, Rathmann W, Heier M, Kowall B, Koenig W, et al. Differential association between biomarkers of subclinical inflammation and painful polyneuropathy: results from the KORA F4 study. Diabetes Care. 2015;38(1) :91-96.
15. Yamakawa I, Kojima H, Terashima T, Katagi M, Oi J, Urabe H, et al. Inactivation of TNF-α ameliorates diabetic neuropathy in mice. Am J Physiol Endocrinol Metab.2011; 301 (5): E844-52.
16. Blüher M, Unger R, Rassoul F, Richter V, Paschke R. Relation between glycaemic control, hyperinsulinaemia and plasma concentrations of soluble adhesion molecules in patients with impaired glucose tolerance or Type II diabetes. Diabetologia.2002; 45(2): 210-216.
17. Zakareia FA. Electrophysiological changes, plasma vascular endothelial growth factor, fatty acid synthase, and adhesion molecules in diabetic neuropathy. Neurosciences (Riyadh).2008; 3(4): 374-379.
18. Jude EB, Abbott CA, Young MJ, Anderson SG, Douglas JT, Boulton AJ. The potential role of cell adhesion molecules in the pathogenesis of diabetic neuropathy. Diabetologia.1998; 41(3): 330-336.
19. Hussain MJ, Peakman M, Gallati H, Lo SS, Hawa M, Viberti GC, et al. Elevated serum levels of macrophage-derived cytokines precede and accompany the onset of IDDM. Diabetologia.1996;

39(1): 60-69.

20. Deguchi T, Hashiguchi T, Horinouchi S, Uto T, Oku H, Kimura K, et al. Serum VEGF increases in diabetic polyneuropathy, particularly in the neurologically active symptomatic stage. Diabet. Med.2009; 26(3): 247-252.
21. Yasuda H, Terada M, Maeda K, Kogawa S, Sanada M, Haneda M, et al. Diabetic neuropathy and nerve regeneration. Prog Neurobiol.2003; 69(4): 229-285.
22. Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. Circulation. 2001; 104 (9): 1046-1052.
23. Regueiro A, Cuadrado-Godia E, Bueno-Betí C, Diaz-Ricart M, Oliveras A, Novella S, et al. Mobilization of endothelial progenitor cells in acute cardiovascular events in the PROCELL study: time-course after acute myocardial infarction and stroke. J Mol Cell Cardiol. 2015; 80: 146-155.
24. Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, et al. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. Lancet.2002; 360(9331): 427-435.
25. Han JW, Sin MY, Yoon Y. Cell therapy for diabetic neuropathy using adult stem or progenitor cells. Diabetes Metab J. 2013; 37(2): 91-105.
26. Ribeiro-Resende VT, Pimentel-Coelho PM, Mesentier-Louro LA, Mendez RMB, Mello-Silva JPC, Cabral-da-Silva MC, et al. Trophic activity derived from bone marrow mononuclear cells increases peripheral nerve regeneration by acting on both neuronal and glial cell populations. Neuroscience. 2009; 159(2): 540-549.
27. Alestalo K, Miettinen JA, Vuolteenaho O, Huikuri H, Lehenkari Petri. Bone Marrow Mononuclear Cell Transplantation Restores Inflammatory Balance of Cytokines after ST Segment Elevation Myocardial Infarction. PLoS ONE. 2015; 10(12): e0145094.
28. Fadini GP, Losordo D, Dimmeler S. Critical reevaluation of endothelial progenitor cell phenotypes for therapeutic and diagnostic use. Circ Res.2012; 110(4):624-637.
29. Fadini GP, Albiero M, Vigili de Kreutzenberg S, Boscaro E, Cappellari R, Marescotti M, et al. Diabetes impairs stem cell and proangiogenic cell mobilization in humans. Diabetes Care. 2013; 36(4): 943-949.
30. Fadini GP, Avogaro A. Diabetes impairs mobilization of stem cells for the treatment of cardiovascular disease: a meta-regression analysis. Int J Cardiol.2013; 168(2): 892-897.
31. Fadini GP, Fiala M, Cappellari R, Danna M, Park S, Poncina N, et al. Diabetes Limits Stem Cell Mobilization Following G-CSF but Not Plerixafor. Diabetes. 2015; 64 (8): 2969-2977.
32. Liu F, Chen D, Sun X, Xie HH, Yuan H, Jia WP. Hydrogen sulfide improves wound healing via restoration of endothelial progenitor cell functions and activation of angiopoietin-1 in type 2 diabetes. Diabetes. 2014; 63 (5):1763-1778.

33. Cheng Z, Garikipati VNS, Nickoloff E, Wang C, Polhemus DJ, Zhou J, et al. Restoration of Hydrogen Sulfide Production in Diabetic Mice Improves Reparative Function of Bone Marrow Cells. Circulation. 2016; 134(19): 1467-1483.

Tables

Table1. Clinical characteristics of studied subjects

	DM	Control	DPN	Responders	Non-responders
N	30	30	60	35	19
Gender (F/M)	16/14	15/15	29/31	16/19	12/7
Age(years) [a]	57.6 ± 4.8	58.2 ± 4.6	59.3 ± 5.6	57.3 ± 5.2	62.0 ± 4.8
Diabetic duration (years) [a]	1.7 ± 1.6	/	9.8 ± 5.1	7.1 ± 2.7	11.2 ± 5.4
Smoking (%)	15 (50.0%)	13 (43.3%)	30 (50.0%)	17 (48.6%)	8 (42.1%)
Alcohol consumption (%)	7 (23.3%)	7 (23.3%)	16 (26.7%)	12 (34.3%)	2 (10.5%)
BMI (kg/m2)	23.74 ± 2.81	23.43 ± 2.31	23.20 ± 2.45	22.75 ± 2.32	23.3 ± 2.09
SBP (mmHg) [b]	128.2 ± 15.9	115.0 ± 14.6	130.5 ± 13.0	130.8 ± 13.8	130.8 ± 12.6
DBP (mmHg) [c]	75.9 ± 10.6	71.7 ± 10.4	77.0 ± 10.3	78.1 ± 11.1	75.3 ± 9.3
FPG(mmol/L) [d]	7.78 ± 2.43	4.66 ± 0.42	10.54 ± 3.41	10.50 ± 3.06	10.47 ± 4.31
HbA1c(%) [d]	7.55 ± 1.76	5.01 ± 0.28	9.98 ± 2.52	9.61 ± 2.53	10.54 ± 2.74
TG (mmol/L) [e]	1.78 ± 1.15	1.26 ± 0.70	1.73 ± 0.80	1.78 ± 0.88	1.67 ± 0.75
TC (mmol/L)	4.38 ± 0.99	4.61 ± 0.76	4.59 ± 1.31	4.50 ± 1.38	4.72 ± 1.35
HDL (mmol/L) [f]	1.16 ± 0.39	1.28 ± 0.30	1.09 ± 0.25	1.12 ± 0.22	1.08 ± 0.33
LDL (mmol/L)	2.49 ± 0.78	2.57 ± 0.57	2.79 ± 1.02	2.93 ± 1.11	2.46 ± 0.91
Scr (umol/L)	64.24 ± 12.92	66.68 ± 16.79	71.89 ± 19.96	68.95 ± 20.61	77.10 ± 20.34
Albnmin (g/L)	41.99 ± 2.82	42.42 ± 1.58	41.21 ± 3.13	40.97 ± 3.00	41.55 ± 3.72
History of stroke (%) [f]	5 (16.7%)	0 (0.0%)	14 (23.3%)	7 (20.0%)	7 (36.8%)
History of CAD (%) [g]	5 (16.7%)	0 (0.0%)	26 (43.3%)	12 (34.3%)	11 (57.9%)
Hypertension medication (%) [h]	7 (23.3%)	0 (0.0%)	29 (48.3%)	19 (54.3%)	7 (36.8%)
Lipid-lowering medication (%) [h]	8 (26.7%)	0 (0.0%)	33 (55.0%)	19 (54.3%)	11 (57.9%)
Antiplatelet agents (%) [f]	5 (16.7%)	0 (0.0%)	14 (23.3%)	7 (20.0%)	6 (31.6%)
Diabetic Medications (%)					
Insulin (%)	14 (46.7%)	--	41 (68.3%)	22 (62.9%)	15 (78.9%)
Sulfonylureas (%)	2 (6.7%)	--	12 (20.0%)	8 (22.9%)	3 (15.8%)
Metformin (%)	22 (73.3%)	--	39 (65.0%)	23 (65.7%)	12 (63.2%)
Alpha-glucosidase inhibitors (%)	15 (50.0%)	--	36 (60.0%)	20 (57.1%)	11 (57.9%)
Pioglitazone (%)	5 (16.7%)	--	13 (21.7%)	6 (17.1%)	5 (26.3%)
Glinides (%)	1 (3.3%)	--	3 (5.0%)	2 (5.7%)	1 (5.3%)
DPP-4 inhibitors (%)	9 (30.0%)	--	19 (31.7%)	13 (37.1%)	5 (26.3%)
Biomarkers of cytokines					
IL-6 (pg/mL)	71.26 ± 12.21	70.13 ± 11.17	74.61 ± 10.28	75.30 ± 10.54	73.66 ± 11.26
IL-10 (pg/mL)	653.83 ± 92.99	645.02 ± 79.41	630.81 ± 110.89	637.26 ± 106.05	590.90 ± 106.89
TNF-α (pg/mL) [f]	402.31± 55.47	374.81 ± 63.18	412.90 ± 64.58	416.34 ± 57.89	401.99 ± 74.34
sCIAM-1 (ng/mL) [g]	1342.17 ± 237.54	1308.00 ± 200.94	1477.56 ± 228.00	1501.21 ± 249.15	1439.96 ± 188.28
NGF (pg/mL) [i]	3734.87 ± 647.50	3771.08 ± 655.86	3509.11 ± 438.39	3578.58 ± 499.20	3435.49 ± 345.35
VEGF (pg/mL) [f]	149.62 ± 26.30	157.39 ± 25.11	140.93 ± 24.78	143.21 ± 25.39	134.83 ± 21.65
$CD34^+$ HPC *10^5/L	/	/	16.33 ± 2.71	17.61 ± 2.64	14.79 ± 1.62
Mononuclear Cells *10^8/L	/	/	10.99 ± 2.27	12.05 ± 2.16	9.84 ± 1.53

Data are expressed as mean± SD or number (percentage). DM, diabetes mellitus; DPN, diabetic peripheral neuropathy; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HbA1c, glycosylated hemoglobin; TG: triglyceride; TC: total cholesterol; LDL: low density lipoprotein; HDL: high density lipoprotein; Scr, serum creatinine; CAD, cardiovascular disease; DPP-4: dipeptidylpeptidse 4; IL, interleukin; TNF, tumor necrosis factor; CIAM, intercellular adhesion molecule; NGF, nerve growth factor; VEGF, vascular endothelial growth factor;

[a] Responder *vs.* Non-responder, *P <0.01.*

[b] DPN *vs.* Control, *P <0.0001*; DM *vs.* Control, P *<0.001*.

[c] DPN *vs.* Control, *P <0.05.*

[d] DPN *vs.* Control, *P <0.0001*; DM *vs.* Control, P *<0.0001*; DPN *vs.* DM, *P <0.0001*;

[e] DPN *vs.* Control, *P <0.01;* DM *vs.* Control, P *<0.05*;

[f] DPN *vs.* Control, *P <0.01;*

[g] DPN *vs.* Control, *P <0.001;* DPN *vs.* DM, *P <0.05*;

[h] DPN *vs.* Control, *P <0.0001*; DM *vs.* Control, P *<0.05*; DPN *vs.* DM, *P <0.05*;

[i] DPN *vs.* DM, *P <0.05*;

Figures

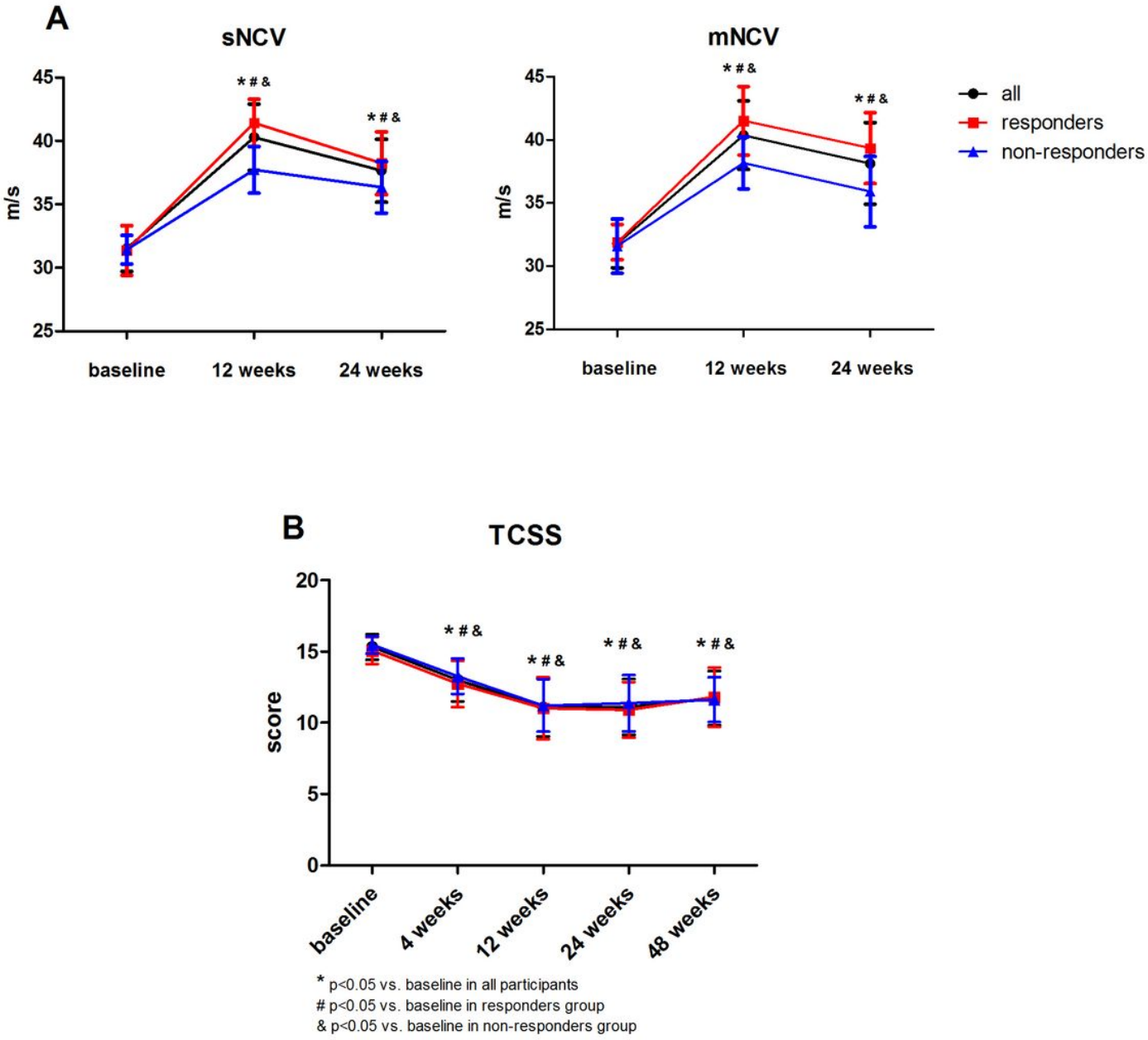

Figure 1

Neurological examinations before and after BM-MNCs therapy sNCV, sensory nerve conductive velocity; mNCV, motor nerve conductive velocity; TCSS, Toronto Clinical Scoring System; A: Both sensory and motor never conduction velocity notably improved at 12 weeks after transplantation. B: The TCSS score decreased after BM-MNCs therapy and remained this downtrend to 48 weeks.

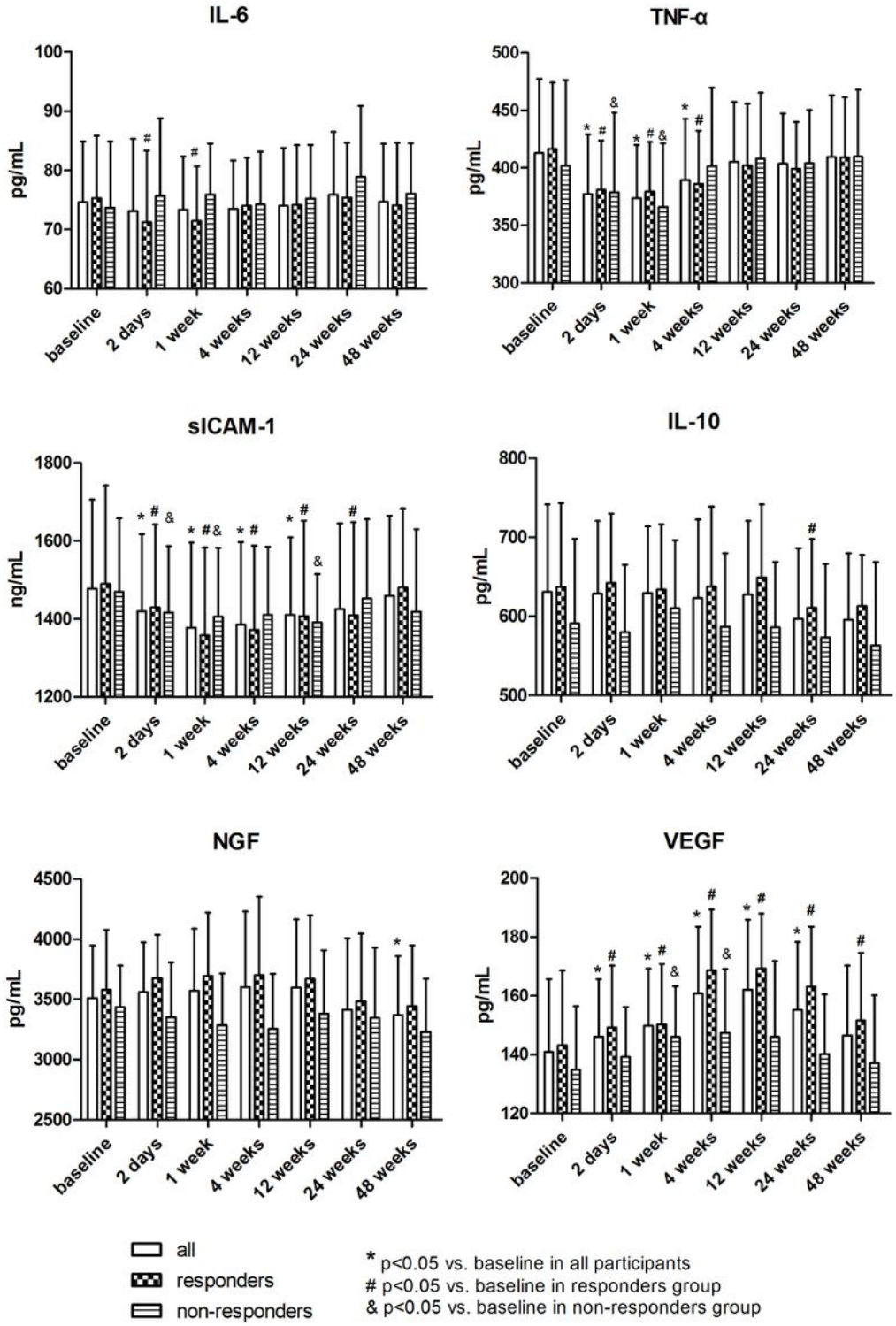

Figure 2

Growth factors and immunological cytokines before and after BM-MNCs therapy

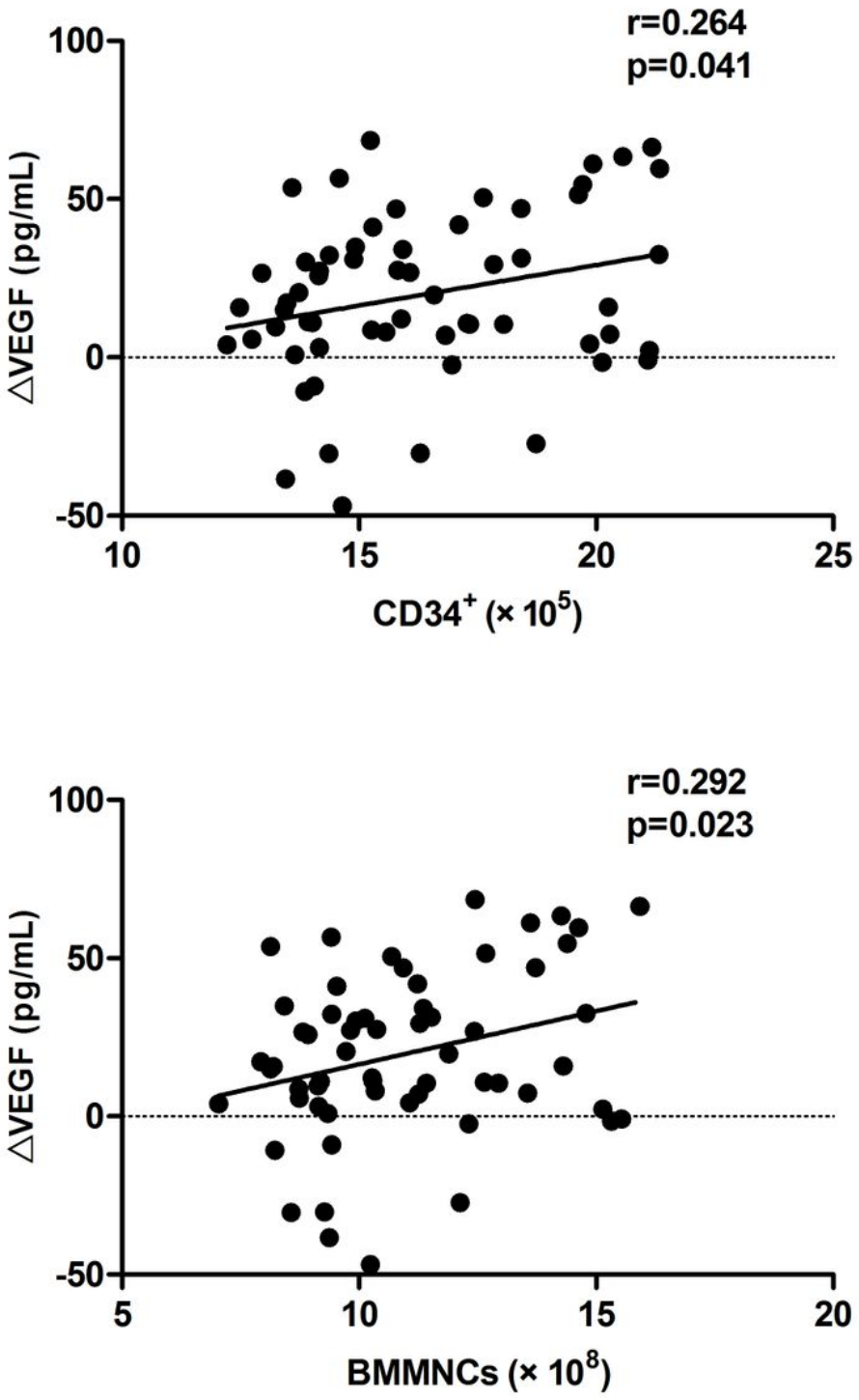

Figure 3

the correlation between an increase of VEGF at 4 weeks post-transplantation and the numbers of CD34+ cells and BM-MNCs

Supplementary Files

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- Supplementmaterials.docx