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Human Bone Marrow Mononuclear Cells Do Not Improve Limb Perfusion in the Hindlimb Ischemia Model

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Effective treatments for chronic limb-threatening ischemia are lacking. (Pre)clinical studies on administration of bone marrow (BM) mononuclear cells (MNCs) and BM-derived mesenchymal stromal cells (MSCs) have shown variable results and no studies have directly compared administration of human BM MNCs and BM MSCs in in vivo models. We studied the effect of intramuscular administration of human BM-derived MNCs and MSCs on limb perfusion in the murine hindlimb ischemia (HLI) model. Human BM MNCs and MSCs were obtained from healthy consenting donors. Both cell types were cryopreserved before use. Twenty-four hours after induction of HLI, nude NMRI mice were randomized to receive intramuscular administration of human BM MNCs ($n=13$), or BM MSCs ($n=14$), or vehicle control ($n=19$) in various doses. Limb perfusion was measured using laser Doppler imaging on day 0, 1, 4, 7, 10, and 14. Intramuscular injection of human BM MNCs did not improve limb perfusion as compared with vehicle over the 2 weeks after cell administration ($P=0.88$, mean relative perfusion for vehicle 0.56 ± 0.04 and 0.53 ± 0.04 for BM MNCs at day 14). Administration of human BM MSCs significantly improved limb perfusion as compared with both BM MNCs and vehicle ($P\leq0.001$, mean relative perfusion at day 14 0.79 ± 0.06). Our data suggest that BM MNCs are less suitable than BM MSCs for cell-based therapy that aims to restore perfusion.

Keywords: bone marrow, cell therapy, peripheral artery disease, hindlimb ischemia

Introduction

Chronic limb-threatening ischemia (CLTI) is a debilitating complication of peripheral artery disease associated with 5-year mortality rates exceeding 50% [1]. CLTI develops as chronic tissue hypoxia due to atherosclerotic vascular occlusion progresses, leading to ischemic pain at rest and tissue loss.

Despite advances in pharmacological management and endovascular and surgical techniques, effective treatments are still lacking for subgroups of CLTI patients [2]. Cell-based therapies have gained interest as a potential treatment to enhance vascularization and prevent amputation in CLTI [3]. Administration of bone marrow (BM) mononuclear cells (BM MNCs) or BM-derived mesenchymal stromal cells (MSCs) may augment neovascularization, either through a direct effect of the administered cells on the vasculature or by secretion of pro-angiogenic factors and modulation of the local immune response [4,5].

It is unclear whether BM MNCs or BM MSCs are more effective, with some clinical trials suggesting that BM MNCs are not effective [6] or BM MSCs may be superior [7]. Only one preclinical study directly compared syngeneic BM MSCs and BM MNCs in the hindlimb ischemia (HLI) model. Iwase et al. showed that syngeneic BM MSC administration caused significantly greater improvement in limb perfusion and capillary density than administration of BM MNCs [8]. However, both BM MNCs as well as BM MSCs from animal donors have very different characteristics compared with BM cells from human donors. No previous studies have compared the effects of human BM MNCs and MSCs on limb perfusion.

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The production of BM MSCs comes at a significant cost, takes time for expansion, and requires more specialized certification and qualification than preparing BM MNCs for clinical administration. However, BM MSCs may be less vulnerable to pre-existing cardiovascular disease than BM MNCs [9].

In this study, we compared the effects of intramuscular administration of human BM MNCs versus human BM MSCs on limb perfusion as measured by laser Doppler imaging in a murine model of HLI.

Materials and Methods

BM cell harvesting

BM was obtained from four healthy patients undergoing elective surgery for thoracic outlet obstruction or hip osteoarthritis (first rib resection or total hip arthroplasty) without any cardiovascular history or risk factors. Participants provided verbal and written informed consent before study procedures. Procedures were approved by the local institutional review board (METC Utrecht) and are in accordance with the Declaration of Helsinki.

Isolation of BM MNCs and expansion of MSCs

BM MNCs were obtained by gradient density centrifugation. Final cryopreservation was in RPMI containing 20% fetal calf serum (FCS) and 10% dimethylsulfoxide (DMSO). Before use, cells were thawed rapidly and washed twice in a chilled centrifuge and kept on ice until use.

BM MSCs were cultured as previously described [10]. MSCs were cryopreserved in MEM alpha containing 20% FCS and 10% DMSO. All experiments were conducted with cells in passage 3 + 1 and were started 48 h after thawing and seeding the cryopreserved cells. Viability before- and after injection was assessed using the trypan blue exclusion principle.

Flow cytometry

After thawing, 50 μL BM MNC suspension was aliquoted and placed on ice until after injections, stained with an anti-CD34 FITC monoclonal antibody (1:50) washed and stained using SYTOX Blue® dead cell stain. Flow cytometry was then performed using a Becton Dickinson Canto II Flow Cytometer.

Animals and housing

Experiments were approved by the local ethical committee in accordance with the Dutch law on animal experimentation (Protocol # 2012.II.04.070). The ARRIVE checklist for this study is available as a Supplementary Data.

Eight-week-old male NMRI Foxn1 nu/nu mice were obtained from Envigo laboratories (Horst, The Netherlands). Animals were housed in individually ventilated cages. They were provided sterile chow and drinking water. Enrichment was provided as extra paper tissues and extra bedding. Mice were allowed a minimum of 1-week acclimatization after arrival at the facility. Cages were randomly distributed over the housing facility.

HLI induction

Mice were anesthetized by intraperitoneal injection of medetomidine/midazolam/fentanyl. HLI was inducted by ligation of the femoral artery of one limb using an electrocauterization device (Bovie) as previously described [10]. Animals received postprocedure analgesia by buprenorphine to reduce pain and distress.

Administration of treatment

Animals were block-randomized according to relative perfusion as measured by laser Doppler perfusion imaging (LDPI) (see the section below) at 1-day post-HLI induction to receive vehicle, BM MNCs or BM MSCs. Animals with a relative perfusion index >50% at day 1 were excluded from the study. Cells were injected in 5 doses of 10 μL cell suspension or vehicle in the adductor muscle at 2 days post-HLI induction. The technician administrating the cells was blinded to the contents of the syringes. Animals received 0.25 to 4.0×10^6 BM MNCs (0.25×10^6: $n = 3$; 1.0×10^6: $n = 7$; 4.0×10^6: $n = 3$), 0.1 to 1.0×10^6 BM MSCs [0.1×10^6, $n = 4$, 0.3×10^6, $n = 4$, 1×10^6, $n = 6$ or vehicle ($n = 19$)]. Part of the MSC cohort was previously reported in [10].

Measurement of tissue perfusion by laser Doppler perfusion imaging

Mice were anesthetized by intraperitoneal injection of medetomidine/midazolam for each measurement to ensure full immobilization. The technician conducting the measurements was blinded to treatment allocation. Measurements were conducted with a Laser Doppler Perfusion Imaging system, (MoorLDI2-HR imager Moor Instruments, United Kingdom) as described previously [10]. Relative perfusion (ischemic limb: nonischemic limb) was measured on day 0, 1, 4, 7, 10, and 14. After measurement, the anesthesia was antagonized using flumazenil and atipamezole.

Humane endpoints and adverse events

In this study, humane endpoints listed in the national code of practice for rodents were used. In addition, hindlimb necrosis extending to more than three toes was considered a humane endpoint.

Statistical analysis

A sample size calculation was performed before the experiments. Based on a previous study by Heeschen et al. [11], a minimum effect size of 0.2 was used. With an alpha of 0.05, a beta of 0.8, the required sample size for a within-factors two-way analysis of variance (ANOVA) that includes 3 groups and 5 time points is 33.

Data are presented as mean ± standard error of the mean. Results of the HLI experiment were analyzed using the area-under-the-curve (AUC) and a one- or two-way-ANOVA or Kruskal–Wallis where appropriate, with multiple testing correction (Dunnett's). A P value of <0.05 was considered significant. GraphPad Prism version 8.4.2 was used for analyses. For statistical analyses, the assessor was blinded.

Results

BM cell viability

After thawing, mean BM MNC viability 71.0 (4.7) for the total population and >95% for $CD34^+$ cells. MSC viability was assessed both before and after injection (in remnants) and was >95% for all preparations.

Induction of HLI

At day 0 of the experiment, mice underwent femoral ligation and at day 1 they received intramuscular injections with the prepared cell products. Average relative perfusion was 17% on day 0 and 31% on day 1 (Fig. 1A). No animals were lost during surgery or excluded due to a relative perfusion >50% on day 1. There were no adverse events due to cell implantation, no limbs were lost due to autoamputation, and there was no premature mortality before the planned termination. Consequently, no animals reached a humane endpoint as described in the methods.

Relative perfusion after cell administration

Relative perfusion of the ligated limb versus the control limb per time point is shown in Fig. 1A. Two-way ANOVA with Dunnett correction for multiple testing showed that at day 14, BM MNCs did not induce better perfusion than vehicle ($P=0.88$, mean relative perfusion for vehicle 0.56 ± 0.04 and 0.53 ± 0.04 for BM MNCs), whereas BM MSCs performed significantly better than both BM MNCs and vehicle ($P\leq0.001$, mean relative perfusion at day 14 0.79 ± 0.06).

To enable subgroup analysis with regard to dose, we aggregated individual relative perfusion/time curves into an AUC per animal. We observed no difference between vehicle and the three assessed doses of BM MNCs ($P>0.1$, Kruskal–Wallis, Fig. 1B). For BM MSCs, the 0.1 and 0.3 M groups were significantly different from vehicle ($P\leq0.01$, Kruskal–Wallis, Fig. 1C), whereas the 1 M group was not different ($P\geq0.1$), suggesting a nonlinear dose–response relationship with an optimum at the lowest dose.

Discussion

We show that intramuscular administration of human BM MNCs does not improve limb perfusion as compared with vehicle in the murine HLI model. This effect is consistent over a range of cell dosages. In contrast, mice that received intramuscular administration of human BM MSCs showed significant increase in limb perfusion over time. Our study is the first to perform a direct head-to-head comparison between human BM MNCs and BM MSCs in an in vivo model.

Our findings seem consistent with a clinical trial that directly compared BM MSCs and BM MNCs. In this study, intramuscular injection of BM MSCs also induced greater improvements in secondary outcomes such as transcutaneous oxygen pressure and ankle-brachial index, compared with BM MNCs [7]. A possible explanation for the greater effects of BM MSCs could be that BM MSCs produce more pro-angiogenic stimuli than BM MNCs, such as vascular endothelial growth factor. BM MSCs also induced tubule formation in in vitro cultured endothelial cells, whereas BM MNCs did not [8].

The lack of efficacy of human BM MNCs in our study seems in contrast with previous preclinical studies. However, the majority of these preclinical studies did not use human BM MNCs, but cells obtained from syngeneic

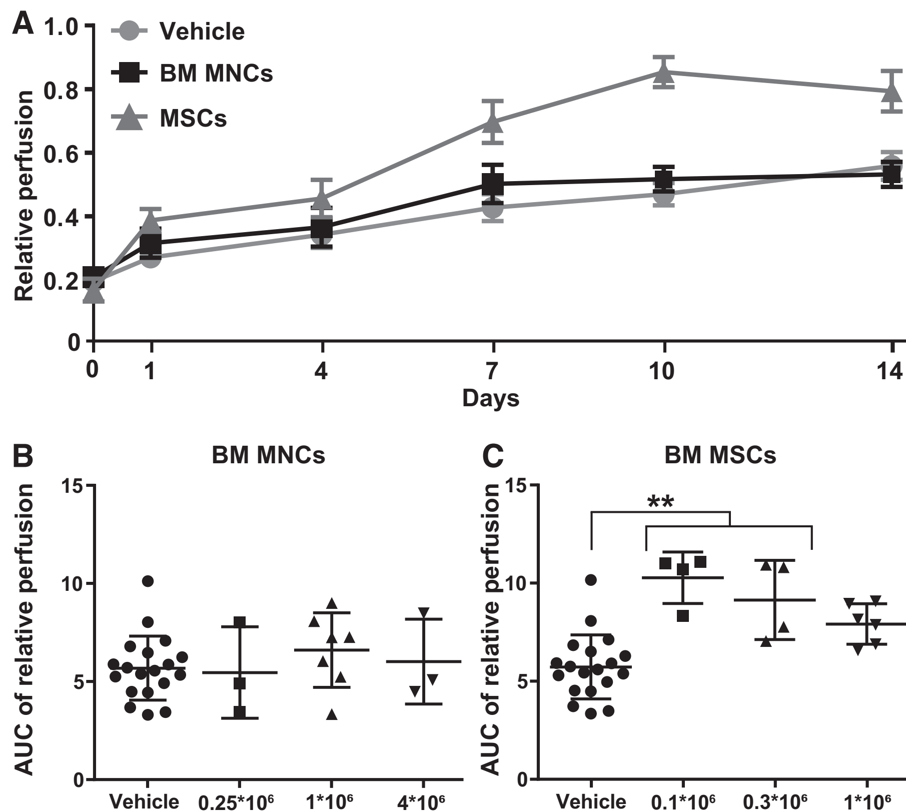

FIG. 1. **(A)** Relative perfusion (ischemic limb vs. nonischemic limb) at various time points in the different treatment groups (dose groups pooled). Overall, BM MNCs do not perform better than vehicle. P values as compared with vehicle (two-way ANOVA with Dunnett correction) on $T=0$: BM MNCs $P=0.96$, BM MSCs $P=0.87$. $T=1$: BM MNCs $P=0.7$, BM MSCs $P=0.11$. $T=4$: BM MNCs $P=0.9$, BM MSCs $P=0.12$. $T=7$: BM MNCs $P=0.4$, BM MSCs $P\leq0.001$. $T=10$: BM MNCs, $P=0.67$ BM MSCs $P\leq0.001$ $T=14$: BM MNCs, $P=0.88$ BM MSCs $P\leq0.001$). **(B/C)** AUC of relative perfusion across different dosages used. **(B)** BM MNCs did not induce a significant change in perfusion compared with vehicle, ($P>0.1$, Kruskal–Wallis). **(C)** BM MSCs induced a significant increase of perfusion in the two lowest dose groups (**$P\leq0.01$, Kruskal–Wallis). ANOVA, analysis of variance; AUC, area-under-the curve; BM, bone marrow; MNCs, mononuclear cells; MSCs, mesenchymal stromal cells.

animal donors, which limits their translational value, as BM progenitor numbers and characteristics are different across species [12]. In addition, the intramuscular administration route may have contributed to the negative result for BM MNCs, as previous studies that did use human-derived BM MNCs all employed the intravascular route [11,13–15]. Furthermore, in our study we used cryopreserved BM MNCs as in clinical settings it is likely that cryopreserved cells will be used [6]. We cannot exclude that BM MNC were negatively affected by cryopreservation, which may explain the discrepancy with the previous studies that used freshly isolated human-derived BM MNCs.

However, cell viability was relatively high, especially of $CD34^+$ cells. We also cryopreserved the MSCs in this study, but re-plated them 48 h before administration. Previous studies have shown that cryopreservation is associated with some dysfunction in MSCs, especially immediately after thawing [16]. Further studies should incorporate a head-to-head comparison of thawed cells without recovery period to better elucidate the cause of the difference in efficacy. It is unlikely that the background of the animals used (nu/nu NMRI) contributed to the lack of efficacy, considering that the previous study has also been done in NMRI mice; therefore, it is unlikely that neovascularization in NMRI nu/nu mice is affected.

Our study has some limitations. The BM donors in this study might not be representative for other donors. Donor variability is a concern for both MSCs [10] and BM MNCs [17]. Particularly in patients with (cardiovascular) comorbidities BM cell function may be impaired. However, as our donors were healthy and did not have comorbidities, it is unlikely that this could explain the lack of effect of BM MNCs. Furthermore, we did not perform histological assessments. However, LDPI measurements generally correlate very well with histological parameters such as the number of capillaries [18].

Conclusion

We show that intramuscular injection of human cryopreserved BM MNCs does not induce perfusion restoration in the murine HLI model, whereas BM MSC administration does have a beneficial effect. BM MNCs may, therefore, be less suitable for therapies aimed at restoring tissue perfusion in ischemic limbs, such as CLTI patients.

Data Availability Statement

Data are available on request.

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Author Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Data

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