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Intravenous administration of mesenchymal stromal cells leads to a dose-dependent coagulopathy and is unable to attenuate acute traumatic coagulopathy in rats

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BACKGROUND: Mesenchymal stromal cells (MSCs) express surface tissue factor (TF), which may affect hemostasis and detract from therapeutic outcomes of MSCs if administered intravenously. In this study, we determine a safe dose of MSCs for intravenous (IV) administration and further demonstrate the impact of IV-MSC on acute traumatic coagulopathy (ATC) in rats.

METHODS: Tissue factor expression of rat bone marrow–derived mesenchymal stromal cell (BMSC) or adipose-derived mesenchymal stromal cell (AMSC) was detected by immunohistochemistry and enzyme-linked immunosorbent assay. The coagulation properties were measured in MSC-treated rat whole blood, and blood samples were collected from rats after IV administration of MSCs. Acute traumatic coagulopathy rats underwent polytrauma and 40% hemorrhage, followed by IV administration of 5 or 10 million/kg BMSCs (BMSC-5, BMSC-10), or vehicle at 1 hour after trauma.

RESULTS: Rat MSCs expressed TF, and incubation of rat BMSCs or AMSCs with whole blood in vitro led to a significantly shortened clotting time. However, a dose-dependent prolongation of prothrombin time with reduction in platelet counts and fibrinogen was found in healthy rat treated with IV-MSCs. Bone marrow–derived mesenchymal stromal cells at 5 million/kg or less led to minimal effect on hemostasis. Mesenchymal stromal cells were not found in circulation but in the lungs after IV administration regardless of the dosage. Acute traumatic coagulopathy with prolonged prothrombin time was not significantly affected by 5 or 10 million/kg BMSCs. Intravenous administration of 10 million/kg BMSCs led to significantly lower fibrinogen and platelet counts, while significantly higher levels of lactate, wet/dry weight ratio, and leukocyte infiltration in the lung were present compared with BMSC-5 or vehicle. No differences were seen in immune or inflammatory profiles with BMSC treatment in ATC rats, at least in the acute timeframe.

CONCLUSION: Intravenous administration of MSCs leads to a risk of coagulopathy associated with a dose-dependent reduction in platelet counts and fibrinogen and is incapable of restoring hemostasis of rats with ATC after polytrauma and hemorrhagic shock. (*J Trauma Acute Care Surg.* 2022;92: 542–552.

KEY WORDS: Cellular therapy; polytrauma; hemorrhagic shock; acute lung injury; rat.

Mesenchymal stromal cells (MSCs) are multipotent nonhematopoietic stem-like cells that are capable of differentiating into adipocytes, chondrocytes, osteocytes, or other cell types when they are cultured in specific differentiation medium in vitro.[1] Mesenchymal stromal cells have been successfully used as a therapeutic approach to attenuate tissue damage and improve tissue regeneration and remodeling when they are delivered at the site of damaged or dysfunctional tissues, such as joint injury or arthritis,[2] myocardial infarction,[3] and type I or II diabetes with dysfunctional pancreatic islet cells.[4] Mesenchymal stromal cells have also been widely investigated for immune modulation by a mechanism of interaction with various types of immune cells or by production of a plethora of bioactive molecules that affect immune cell functionality.[5] Therapeutic effects were previously reported in the treatment of graft versus host disease,[6,7] Crohn's disease,[8,9] and sepsis[10,11] by regulating both innate and adaptive immune responses.[12] Mesenchymal stromal cells can be isolated from various organs and validated using specific culture techniques, clarification biomarkers, and differentiation properties. Mesenchymal stromal cells derived from bone marrow, adipose tissues, and umbilical cord are most commonly administered in clinical trials.[13]

Because of their potential dual functionality in both tissue regeneration and immune modulation, acute intravenous (IV) administration of MSCs could be beneficial in treating severe trauma by playing multifactorial roles of orchestrating the homeostasis of inflammatory and regenerative microenvironments. Results from a previous animal study[14] suggest that acute IV administration of bone marrow–derived mesenchymal stromal cells (BMSCs) reduces endotheliopathy and improves acute lung injury after hemorrhagic shock. These promising results have prompted additional animal studies and subsequent clinical trials to determine

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whether MSCs can be used acutely as a novel cellular therapy to mitigate the morbidity of severe trauma and hemorrhagic shock.

Although many clinical trials using MSCs for a variety of diseases are currently underway, the safety of MSC administration in acute trauma and hemorrhagic shock has not been well characterized. Several recent in vitro studies demonstrate that MSCs are procoagulant and have an innate thrombogenicity owing to constitutive expression of active tissue factor (TF)[15] regardless of their tissue of origin.[16] Similar to the procoagulant functionality of exogenous TF, MSCs induce thrombin generation when they are incubated with human plasma in a dose-dependent manner.[15] Because unexpected thrombosis can become lethal, it is reasonable to take MSC procoagulant activity into account for safety consideration if they are circulated in the blood after being administered intravenously.

Trauma with hemorrhagic shock is a leading cause of death in patients, especially in cases complicated by acute traumatic coagulopathy (ATC).[17] Acute traumatic coagulopathy is defined as prolongation of prothrombin time (PT) or activated partial thromboplastin time (aPTT) before resuscitative therapies, which is frequently exacerbated because of hemodilution after fluid resuscitation.[18] Since an effective therapeutic approach is lacking, mortality and morbidity are particularly high in those subjects who develop ATC shortly after trauma. In a preclinical rat model of polytrauma and hemorrhagic shock, coagulopathy develops acutely after polytrauma and hemorrhage without resuscitation and is characterized by a rise in PT and aPTT, and a decrease in fibrinogen and platelet aggregation.[19] It is unknown whether IV administration of MSCs as a potential cellular therapy in acute trauma can affect hemostasis in ATC.

The current study demonstrates that rat-derived MSCs are procoagulant as measured by in vitro assays, and IV administration of rat MSCs in rats affects systemic hemostasis in a dose-dependent manner. Using a preclinical rat model of ATC, the results also suggest that the benefit of using IV administration of MSCs as a cellular therapy for acute trauma and hemorrhage may be limited by the potential for hemostatic complications unless effective countermeasures are taken.

MATERIALS AND METHODS

The animal study was approved by the Institutional Animal Care and Use Committee of the US Army Institute of Surgical Research and conducted in compliance with the Animal Welfare Act, the implemented Animal Welfare Regulations, and the principles of the "Guide for the Care and Use of Laboratory Animals." The current study was reported following the guidelines of ARRIVE (Animals in Research Reporting In Vivo Experiments, PLoS Bio 8(6), e1000412,2010). Male rats (Sprague-Dawley from Charles River, Houston, Texas, www.criver.com) were used. The light/dark cycle was 12 hours light/12 hours dark. The rats ate Laboratory Rodent Diet 5001 (LabDiet, St. Louis, MO) (www.LabDiet.com). Food and water were given ad libitum. Animal experiments including all surgical procedures were performed under anesthesia using 1.5% to 3% isoflurane (Forane, Baxter, US) with 100% oxygen.

Isolation and Verification of Rat BMSCs and AMSCs

Bone marrow–derived mesenchymal stromal cells[20] and AMSCs[21,22] were isolated from bones (femurs and tibias) and adipose tissues (periremal and epididymal), respectively, in rats after euthanasia as previously described. Bone marrow–derived mesenchymal stromal cells and AMSCs from passages 3 to 5 were used in this study. Both BMSCs and AMSCs were validated as MSCs by measuring negative (CD45) and positive (CD90, CD73, or CD29) superficial makers as described previously.[20]

Measurements of Coagulation

The assays were performed in citrated whole blood (20 mM sodium citrate). The hemostasis analyzer (STart; Stago, Parsippany, NJ) was used to measure PT, aPTT, and fibrinogen in the whole blood. Rotational thromboelastometry (ROTEM delta, Pentapharm GmbH, Munich, Germany) was used to measure coagulation properties of the whole blood with or without TF treatment (tissue factor-triggered extrinsic pathway [EXTEM] or non-activated rotational thromboelastometry [NATEM]), including clotting time (CT), α angle, maximum clot firmness (MCF), and maximum lysis index at 60 minutes (LI60). Impedance aggregometry (Multiplate 5.0 Analyzer; Dynabyte Medical, Munich, Germany) was used to measure platelet aggregation capacity with treatment of adenosine diphosphate (ADP) and collagen. The coagulation assays were followed by the manufacturer's instructions, and the reagents were purchased from the manufacturer as mentioned previously.

Determination of Procoagulant Properties of Rat MSCs

Tissue Factor Expression

The cell lysates of two million BMSCs or AMSCs were collected from five separate culture flasks and resuspended in cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA) followed by centrifugation at 10,000*g* for 10 minutes at 4°C. The TF concentration in each cell lysate was measured in duplicate using rat TF enzyme-linked immunosorbent assay (ELISA) (My BioSource, San Diego, CA) following manufacturer's instruction. The TF expression was also confirmed by immunohistochemistry. Briefly, 6000 BMSCs or AMSCs in growth medium were seeded in each well of eight-well chamber slides (Millipore Sigma, Burlington, MA) and allowed to attach overnight. After removal of culture medium, the adherent cells were washed with phosphate-buffered saline (PBS) three times, fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), and then treated with anti-rat TF polyclonal rabbit antibody (Abcam, Cambridge, MA) followed by Alexa 594 anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA). The slides were costained with DAPI (4′,6-diamidino-2-phenylindole) using ProLong Gold Antifade Mountant with DAPI (ThermoFisher) to identify cell nuclei. The ratio of TF positive cells and cell nuclear counts was calculated to determine the percentage of TF-expressing cells.

Coagulation Properties In Vitro

Rat whole blood (in 20 mM sodium citrate) was collected from anesthetized Sprague-Dawley rats. Bone marrow–derived mesenchymal stromal cells and AMSCs were prepared in various concentrations using normal saline with citrate phosphate dextrose (CPD) at 1:8 ratio before administration. About 20 μL/mL of normal saline (with CPD), BMSCs, or AMSCs was added to whole blood to have final concentration of BMSCs or AMSCs at 0, 0.15, 0.7, 1.5 million/mL. The corresponding

whole blood samples were then collected for measuring PT, coagulation properties (CT, α angle, MCF, LI60 in both EXTEM and NATEM), and platelet aggregation (ADP and collagen). The assays were run in duplicate, and the experiments were repeated three times using the whole blood from three separate rats. Meanwhile, the blood smear was performed to determine TF expression by immunohistochemistry as described above.

Coagulation Properties In Vivo

Sprague-Dawley rats (350–400 g) were anesthetized by 1.5% to 2.5% isoflurane mixed with 100% oxygen, and the femoral vein and artery were cannulated. Bone marrow–derived mesenchymal stromal cells or AMSCs were labeled with green fluorescent chloromethyl derivatives of fluorescein diacetate (CMFDA; ThermoFisher). The cells were washed three times with PBS and resuspended in normal saline mixed with CPD at 1:8 ratio before administration. The rats were randomly treated with BMSCs or AMSCs at doses of 2.5 (n = 4), 5 (n = 4), 10 (n = 6), 20 (n = 4 of BMSC; n = 3 of AMSC), or 40 (n = 4 of BMSC) million/kg through the femoral vein (1 mL of normal saline was used for 2.5 to 20 million/kg MSCs, and 2 mL of normal saline was used for 40 million/kg MSCs). Blood samples were collected at 30 minutes, 1 hour and 3 hours after transfusion to measure coagulation properties, platelet counts (complete blood counts by [ADVIA Siemens Medical Solutions USA, Inc., Malvern, PA]), and blood chemistry (lactate) by iSTAT (Abbot Labs, Abbott Park, IL). The lung, heart, liver, kidney, spleen, brain, and skeletal muscle were taken immediately after euthanasia at 3 hours after cells administration. The CMFDA-labeled MSCs in the circulation or in tissue were detected by green fluorescence using flow cytometry or histology respectively. Cryosections of lung tissue were prepared and stained for platelets (mouse monoclonal anti-rat CD61; BD Biosciences, San Jose, CA), TF, or neutrophil/monocytes (mouse monoclonal anti-rat CD11b (Bio-Rad, Hercules, CA), and costained with DAPI for nucleus, respectively.

BMSC Therapy in Rats With ATC

Rat model of ATC with polytrauma and hemorrhage was previously described.[19,23] Briefly, Sprague-Dawley rats (350–400 g) was anesthetized by 1.5% to 2.5% isoflurane with 100% oxygen. The polytrauma was performed by laparotomy; crush injury at small intestine, liver, and skeletal muscle; and bone fracture at right femur. Immediately after polytrauma, the fixed-volume/pressure-controlled hemorrhage was performed by blood draw through femoral vein to maintain mean arterial blood pressure at 40 mm Hg until 40% of estimated blood volume (6% of body weight + 0.77) was removed. At 1 hour after trauma, the rats were randomly treated of either 1 mL vehicle (normal saline with CPD at 1:8 ratio) or CMFDA-labeled BMSCs (resuspended in 1 mL normal saline with CPD before administration) at 5 million/kg (BMSC-5) or 10 million/kg (BMSC-10) through femoral vein. Blood samples were collected at baseline, and 1 hour and 3 hours after transfusion to measure coagulation properties, platelet counts, and blood chemistry. Lungs were harvested immediately after euthanasia. The CMFDA-labeled MSCs in the circulation or in tissues were detected by flow cytometry and histology, respectively. The entire right interior lobe of the lung was collected, wet weight was measured immediately, and dry weight was determined after 2 weeks in 60°C oven. The wet/dry weight ratio was then calculated. The whole lobe of left lung was processed for histology.

Histology and Immunohistochemistry

All the chemicals were purchased from Millipore Sigma unless stated otherwise. Tissues were collected immediately after euthanization. The tissues samples were immerged in 4% paraformaldehyde overnight, followed by 20% sucrose at 4°C for 24 hours. The tissues were then embedded in Optimal Cutting Temperature Compound (Tissue-Tek; Sakura Finetek USA, Torrance, CA) and frozen in liquid nitrogen for cryosectioning at 5-μm thickness using a cryostat (Leica Biosystems Inc., Buffalo Grove, IL). For immunohistochemistry, tissue sections were fixed in 4% paraformaldehyde for 15 minutes followed by wash with PBS. The sections were blocked with 5% normal donkey serum (Millipore Sigma) for 1 hour at room temperature, and then treated with primary antibody and incubated at 4°C overnight, followed by the treatment with fluorescence conjugated secondary antibody. The slides were then costained with DAPI for nuclear staining and analyzed by fluorescence microscopy (Zeiss, Oberkochen, Germany). The quantification of positive staining was performed by ImageJ software (National Institutes of Health, Rockville, MD).

Assessment of Immune Response

The immune cell phenotype in the whole blood was measured by flow cytometry at baseline, and 1 hour and 3 hours after treatment. Cytokines and chemokines were measured in the plasma at baseline and 3 hours after MSCs administration using multiplex rat cytokine assay (Bio-Plex ProTM rat cytokine; Bio-Rad). The data are presented in supplement.

DATA ANALYSIS

The data analysis was performed by SigmaPlot (Systat Software, Inc. San Jose, CA). All the variables were tested for normality (Shaprio-Wilk test), and equal variation was performed by SigmaPlot. For in vitro study, the one-way repeated analysis of variance (ANOVA) (parametric) or Friedman test (nonparametric) was used to test the mean difference of the coagulation variables among the groups of each given dose of AMSC or BMSC followed by a pairwise comparison with a Tukey's or Dunn's test if applicable; Student *t* test was used for comparison of TF expression between AMSCs and BMSCs. For in vivo study in normal rats, the one-way repeated ANOVA (parametric) or Friedman test (nonparametric) was used to test the mean difference of the variables in coagulation properties among the blood samples from baseline, and 1 hour and 3 hours after MSCs administration at each given dose of AMSC or BMSC followed by a pairwise comparison with a Tukey's or Dunn's test if applicable. For in vivo study in ATC rats, the two-way repeated ANOVA (parametric) was used to test mean differences among vehicle, BMSC-5M and BMSC-10M for the continuous variables over study time points within each group followed by a pairwise comparison with a Tukey method if applicable; one-way ANOVA (parametric) or Kruskal-Wallis (nonparametric) was used to test the mean difference among sham, vehicle, BMSC-5M, and BMSC-10M for the variables at 4 hours followed by a pairwise comparison with a Tukey's or Dunn's test if applicable. Data are presented as means ± SD or a box-whisker plot, and statistical significance is accepted at the $p < 0.05$.

RESULTS

Rat MSC Characterization

Consistent with a previous report,[20] isolated BMSCs and AMSCs were positive for CD90, CD29, and CD73 but negative for CD45 (Supplemental Digital Content, Supplementary Fig. 1, http://links.lww.com/TA/C205) as determined by flow cytometry. Both BMSCs and AMSCs were successfully labeled by CMFDA (99–100%, verified by flow cytometry), and the viability was analyzed by trypan blue staining with >80% viability before use.

Procoagulant Activity

Isolated BMSCs and AMSCs expressed TF under normal culture conditions and were identified by immunohistochemistry on chamber slides using anti-TF antibody and by ELISA measured in whole cell lysate (Supplemental Digital Content, Supplementary Fig. 2, http://links.lww.com/TA/C206). Bone marrow–derived mesenchymal stromal cells had a slightly lower level of TF than AMSCs as shown in both immunohistochemistry and ELISA. Tissue factor expression was retained on the surface of BMSCs after incubation with whole blood in vitro (immunohistochemistry of blood smear slides) (Supplemental Digital Content, Supplementary Fig. 2, http://links.lww.com/TA/C206). The CT as measured by NATEM was significantly shortened in whole blood incubated with BMSCs or AMSCs at 0.15, 0.75, or 1.5 million/mL (equivalent to 8, 40, or 80 million/kg calculated by estimated blood volume of a 400 g rat) in a dose-dependent manner (Fig. 1*A*). However, there was no significant difference in CT between blood incubated with AMSCs and BMSCs. There was no significant change in PT, aPTT, MCF, and LI60 measured in the whole blood treated with either BMSCs or AMSCs (complete data at Supplemental Digital Content, Supplementary Table 1, http://links.lww.com/TA/C211). In vivo, MSCs were not retained in the circulation as tracked by flow cytometry in blood samples taken as early as 30 minutes after IV administration but were found sequestered in the lung instead by histology of the lung tissue collected at 3 hours after administration. Surprisingly, in the lung sections, TF expression was no longer detectable in all MSCs (Supplemental Digital Content, Supplementary Fig. 2, http://links.lww.com/TA/C206). From the whole blood samples taken at 1 hour and 3 hours after MSCs administration, the CT (NATEM) was not shortened but was instead prolonged in rats administered AMSCs at a dose of 10 million/kg or in rats administered BMSCs at a higher dose (40 million/kg) with a significant decline in MCF (Fig. 1*C* and *D*).

Maximum Safe Dose of BMSCs and AMSCs for Hemostasis

At a dose of 20 million/kg, rats treated with BMSCs survived (n = 4 of 4 rats), but rats treated with AMSCs all expired

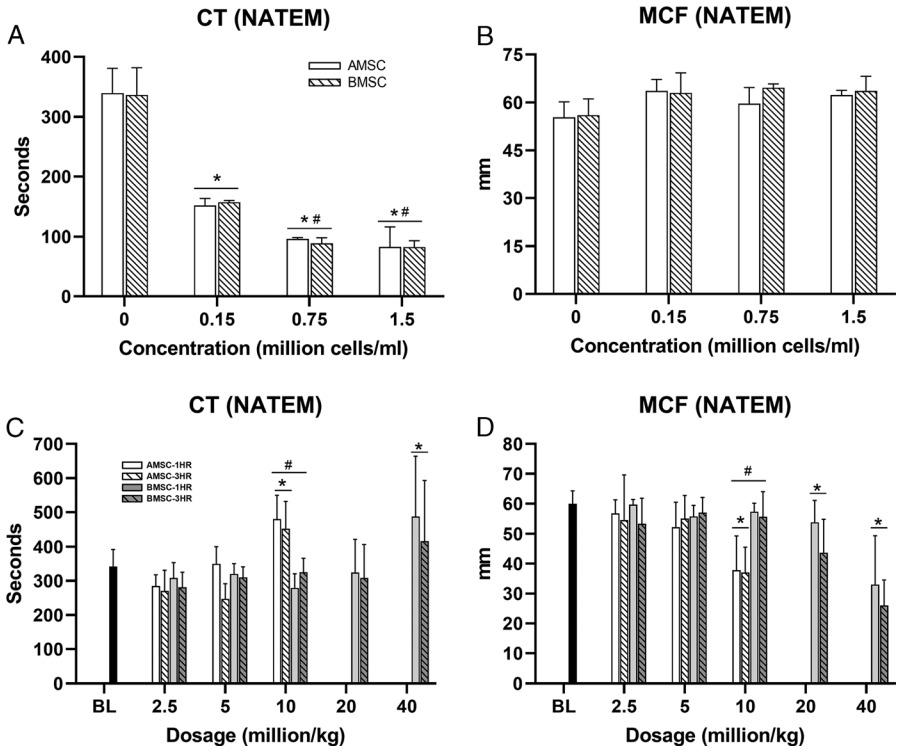

Figure 1. Effects of MSCs on coagulation properties of whole blood. In vitro assay (*A*, *B*): coagulation properties (CT (*A*) and MCF (*B*) as measured by ROTEM [NATEM]) in whole blood at 1 hour after incubation with 0.15, 0.75, or 1.5 million/mL AMSCs (open bar) or BMSCs (cross bar). *$p < 0.05$, Significant difference compared with nontreatment (0); #$p < 0.05$, significant difference compared with 0.15 million/mL. In vivo assay (*C*, *D*): coagulation properties (CT (*C*) and MCF (*D*) as measured by ROTEM [NATEM]) in whole blood samples taken at 1 hour and 3 hours after IV administration of 2.5, 5, or 10 million/kg AMSCs (open bar [1 hour] and cross bar [3 hours]) or 2.5, 5, 10, 20, or 40 million/kg BMSCs (gray solid bar [1 hour] and gray cross bar [3 hours]) in healthy rats. *$p < 0.05$, Significant difference compared with baseline (solid black bar); #$p < 0.05$, significant difference comparing AMSCs and BMSCs at 1 hour or 3 hours after administration. ROTEM, rotational thromboelastometry.

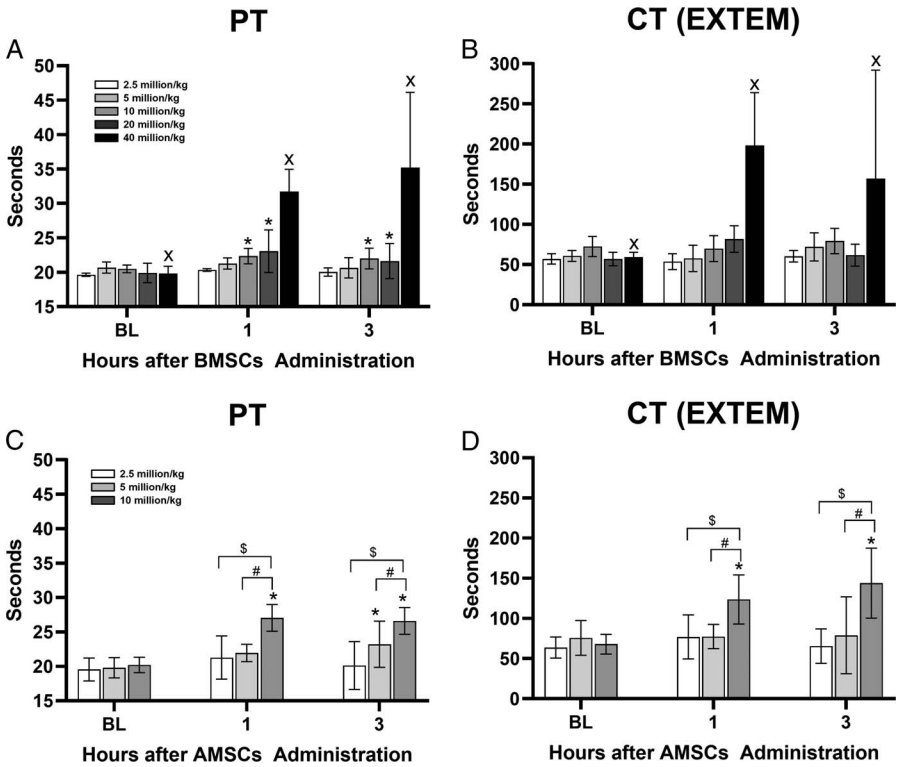

Figure 2. Hemostatic outcomes after IV administration of MSCs in healthy rats. Prothrombin time (*A*, *C*) and CT (EXTEM, *B*, *D*) of rats at 1 hour and 3 hours after IV administration of 2.5, 5, 10, 20, or 40 million/kg BMSCs (*A*, *B*) or 2.5, 5, or 10 million/kg AMSCs (*C*, *D*). x, The groups were not included in the statistical analysis because some variables were undetectable owing to the assay being out of range; *$p < 0.05$, significant difference compared with BL at a corresponding dose of MSC; #$p < 0.05$, significant difference compared with 5 million/kg at 1 hour or 3 hours; \$$p < 0.05$, significant difference compared with 2.5 million/kg at 1 hour or 3 hours. BS, baseline.

(n = 3 of 3 rats) within 3 hours after administration. Three of four rats survived with infusion of BMSCs at the high dose of 40 million/kg. In all survived rats treated with BMSCs or AMSCs at 10 million/kg or above, PT (Fig. 2*A* and *C*) and aPTT were significantly prolonged, and fibrinogen levels significantly declined at 1 hour and 3 hours after infusion (Supplemental Digital Content, Supplementary Table 2, http://links.lww.com/TA/C212). Clotting time (EXTEM) was significantly prolonged (Fig. 2*B* and *D*), and MCF was significantly reduced at 1 hour after infusion of AMSCs at 10 million/kg or BMSCs at 40 million/kg as measured by rotational thromboelastometry (complete data at Supplemental Digital Content, Supplementary Table 3, http://links.lww.com/TA/C213). Fibrinogen deposition in the lungs was significantly elevated after administration of higher doses (Fig. 3*A* and *C*), suggesting that the cause of the decreased circulating fibrinogen (Fig. 3*B* and *D*) was at least partly due to increased deposition of fibrinogen in the lung. Interestingly, the fibrinogen observed in the lungs in the majority of animals did not overlap with the location of MSCs, suggesting that the elevation of fibrinogen in the lung was not solely or directly due to the binding of fibrinogen to the sequestered MSCs. Platelet counts were significantly reduced in a dose-dependent manner after MSC infusion (Fig. 4*A* and *B*). Platelet aggregation (ADP and collagen) significantly declined after infusion of 10 million/kg AMSCs or 20 million/kg BMSCs (Supplemental Digital Content, Supplementary Fig. 3, http://links.lww.com/TA/C207). Platelet aggregation was completely inhibited in rats treated with 40 million/kg BMSC. The binding of MSCs to platelets was shown by immunohistochemistry ($CD61^+$ platelets overlapped with CMFDA-labeled MSCs) in the blood smear from whole blood treated with MSCs (Fig. 4*D*) in vitro. After IV infusion of MSCs, MSCs were sequestered and bound platelet aggregates in the lung (Fig. 4*C*), which was associated with a significant decline in circulating platelet counts in a dose-dependent manner.

Hemostatic Function After IV Administration of BMSCs in Polytrauma and Hemorrhagic Shock

Acute traumatic coagulopathy was induced in the rats with polytrauma/hemorrhage (TH) as characterized previously by an elevation in PT and aPTT and a decline in fibrinogen after polytrauma following by hemorrhage.[19] All rats survived for 3 hours after receiving vehicle or BMSCs at 5 or 10 million/kg at 1 hour after trauma (BMSC-5, BMSC-10). No significant difference was found in PT (Fig. 5*A*) or aPTT among the groups (complete data at Supplemental Digital Content, Supplementary Table 4, http://links.lww.com/TA/C214). Rats in the BMSC-10 group had a significantly lower circulating fibrinogen level as compared with rats in the BMSC-5 and vehicle groups (Fig. 5*B*). Rats in BMSC-treated groups showed higher platelet consumption compared with vehicle-treated rats (Fig. 5*C*) and platelet aggregation at 3 hours after BMSC administration (Fig. 5*D*). There were no significant differences in coagulation properties among the groups as

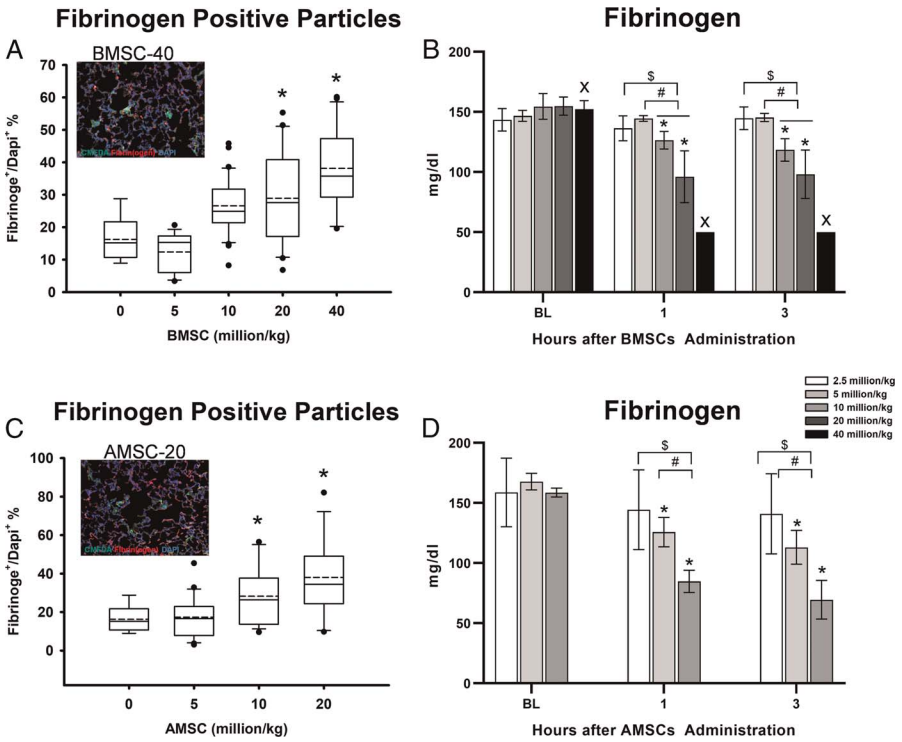

Figure 3. Effects of IV administration of MSCs on fibrinogen levels in healthy rats. (*A, C*) Percentage of positive particles of fibrinogen versus DAPI in the lung sections from rats with IV administration of none, 5, 10, 20, or 40 million/kg BMSCs (*A*), or none, 5, 10, or 20 million/kg AMSC (*C*), and a presentative immunohistochemistry image of fibrinogen (red) and DAPI (blue) costaining in lung sections from rats with IV administration of 40 million/kg CMFDA-labeled BMSCs (green, BMSC-40) or 20 million/kg CMFDA-labeled AMSCs (green, AMSC-20) was provided in A and C, respectively. *$p < 0.05$, Significant difference compared with no-cell treatment. (*B, D*) Fibrinogen levels in blood samples at BL, and 1 hour and 3 hours after IV administration of 2.5, 5, 10, 20, or 40 million/kg BMSCs (*B*), or 2.5, 5, or 10 million/kg AMSCs (*D*). x, The groups were not included in the statistical analysis because some variables were undetectable owing to the assay being out of range; *$p < 0.05$, significant difference compared with BL at a corresponding dose of cells; ***$p < 0.05$, significant difference compared with 5 million/kg at a defined time point; ****$p < 0.05$, significant difference compared with 2.5 million/kg at a defined time point. BL, baseline.

measured by EXTEM (Supplemental Digital Content, Supplementary Table 4, http://links.lww.com/TA/C214). Similar to uninjured rats, BMSCs were found sequestered in the lungs colocalized with platelet aggregates (Supplemental Digital Content, Supplementary Fig. 4, http://links.lww.com/TA/C208) but not detected at the site of trauma (liver) as determined by histology (Supplemental Digital Content, Supplementary Fig. 4, http://links.lww.com/TA/C208). Very few of the infused BMSCs were detected in the circulation at 3 hours postinfusion (Supplemental Digital Content, Supplementary Fig. 4, http://links.lww.com/TA/C208). Platelets and platelet aggregates were significantly increased in the lung after TH and were further increased following administration of BMSCs (Fig. 6*A*). Consistent with previous findings, there was a significant increase in CD11b-positive cells (neutrophils/monocytes) infiltrated in the lungs after TH, and administration of BMSCs at 10 million/kg significantly increased the number of $CD11b^+$ cells compared with vehicle-treated and BMSC-5 animals (Fig. 6*B*). As a result, the lung wet/dry weight ratio was significantly higher (Fig. 6*C*) in BMSC-10 animals compared with that of sham (uninjured), vehicle-infused, and BMSC-5 animals. Compared with the animals treated with a lower dose of BMSC (BMSC-5) or vehicle, infusion of BMSCs at 10 million/kg caused a significant elevation in lactate (Fig. 6*D*).

DISCUSSION

In this study, the procoagulant activity of rat-derived BMSCs and AMSCs was characterized by in vitro assay and showed shortened CT of whole blood treated with MSCs. In contrast to the expected hypercoagulation predicted by those in vitro results, hypocoagulopathy and lethality at higher doses were observed in rats following IV administration of rat MSCs. The maximum safe dose of MSCs for IV administration defined in healthy rats was 5 to 10 million/kg resulting in minimal change in hemostasis after MSC administration. However, IV administration of 5 or 10 million/kg BMSCs in the rats with ATC did not affect prolonged PT and aPTT but potentially intensified the ATC because of additional consumption of platelets and fibrinogen associated with MSC sequestration in the lung. There was no significant reduction in the elevated cytokines/chemokines that occurred in response to trauma and hemorrhage at 3 hours after IV administration of BMSCs (Supplemental Digital Content, Supplementary Fig. 5,

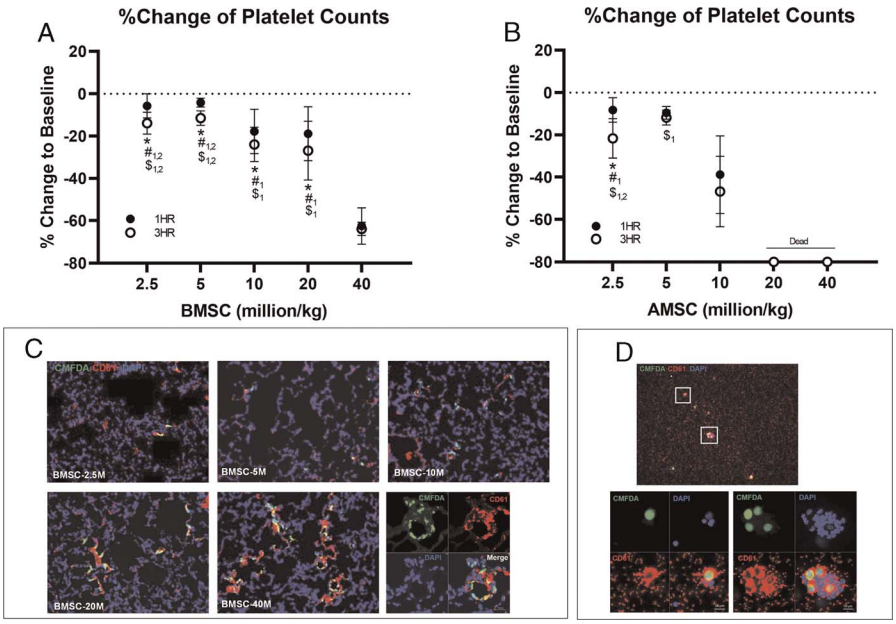

Figure 4. Effects of IV administration of MSCs on platelet counts in healthy rats. (*A, B*) Percentage change of platelet counts at the baseline, and 1 hour and 3 hours after IV administration of 2.5, 5, 10, 20, or 40 million/kg BMSCs (*A*), or 2.5, 5, or 10 million/kg AMSCs (*D*). *$p < 0.05$, Significant difference between 1 hour and 3 hours; #1, $1: $p < 0.05$, significant difference compared with 40 million/kg at 1 hour and 3 hours, respectively; #2, $2: $p < 0.05$, significant difference compared with 20 million/kg at 1 hour and 3 hours, respectively. (*C*) Representative immunohistochemistry images of CD61 (red) and DAPI (blue) costaining of the lung sections from rats received 2.5, 5, 10, 20, or 40 million/kg CMFDA-labeled BMSCs (green, BMSC-2.5M, -5M, -10M, -20M, -40M, respectively). (*D*) Representative immunohistochemistry images of CD61 (red) and DAPI (blue) costaining of the slides from blood smear of whole blood at 1 hour after incubation with BMSCs.

http://links.lww.com/TA/C209, Supplementary Fig. 6, http://links.lww.com/TA/C210), suggesting that there was no acute immunomodulation effect in this early timeframe.

Tissue factor expression has been well characterized in human-derived MSCs,[16] and the procoagulant function of MSCs was identified by in vitro hemostatic assay.[15] Similar to human MSCs, rat BMSCs or AMSCs were procoagulant and accelerated clot formation in whole blood that was incubated with MSCs. The shortened CT of MSC-treated whole blood was only measured by NATEM (induction by calcium) but not EXTEM (induction by calcium and TF) that the coagulation capacity was already maximized by the TF. In contrast to in vitro characteristics of MSCs, hypercoagulation (shortened CT) was not seen in rats after IV administration of any dose of MSCs. Instead, hypocoagulation developed in rats infused with high doses of either BMSC or AMSC as characterized by an elevation in CT, PT, and aPTT and a decline in fibrinogen, platelet counts, and MCF when the dose was at 10 million/kg or higher. Consistent with previous study, MSCs are not found in the circulation for an extended period of time[24] but instead are sequestered in the lung shortly after IV administration. Therefore, the blood samples collected after MSCs administration did not contain any detectable MSCs that could potentially cause clot acceleration as shown in vitro. Surprisingly, TF was not universally expressed in MSCs that were sequestered in the lung at 3 hours after MSC administration, in contrast to stable TF expression in MSCs when they were incubated with whole blood in vitro. This discrepancy provides insight that the potential risk of IV administration of MSCs may not be directly caused by procoagulant activity due to TF expression. However, although we did not find the shortened CT in vivo similar to that seen in vitro, the current study did not provide evidence that TF- or MSC-derived extravascular vesicle could be shed from sequestered MSCs and release into the bloodstream to elicit transient procoagulant activity beyond the current time window of sampling.

The results from our in vivo study suggest that hypocoagulopathy could develop as early as 1 hour after IV administration of MSCs, which was likely associated with a dose-dependent decline in platelet counts and fibrinogen in the blood caused by an increase deposition of platelets and fibrinogen in the lung. The underlying mechanism for this phenomenon is not known. Reassuringly, the labeled MSCs were not found abundantly in tissues such as spleen, liver, kidney, intestine, skeletal muscle (hindlimb), and brain in healthy rats treated with any tested dose of MSCs (data not presented), and we did not observe global thrombogenesis in any organ except in the lung at very high doses of MSC administration. The observation from the lung histology suggested that there was a dramatic increase in platelet aggregates adherent to sequestered MSCs, which likely accounted for the dose-dependent decline in platelet counts. Platelets binding to MSCs or vice versa were also seen in blood smears from whole blood incubated with MSCs. The actual mechanism was not entirely clear. Tissue factor expressed on the surface of MSCs can trigger the extrinsic pathway and lead to thrombin generation, which then activates protease-activated receptor 1 and contributes to the activation and enhancement of platelet aggregation.[25] However, there was no absolute evidence in the current study that platelet-MSC aggregates were formed independently of generating

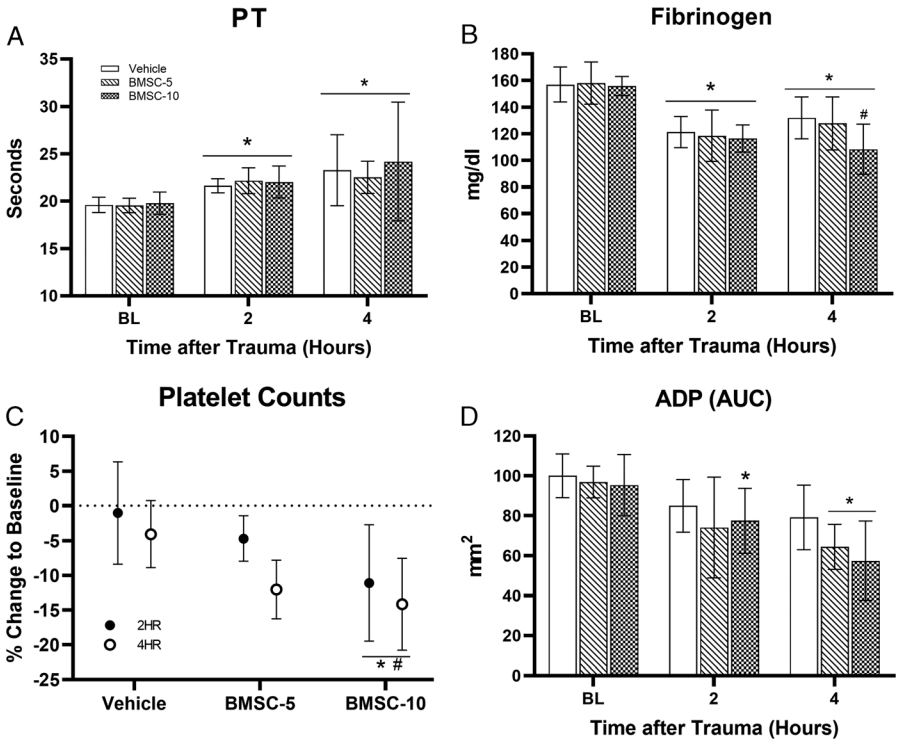

Figure 5. Hemostatic outcomes after IV administration of BMSCs in rats with polytrauma and hemorrhage: PT (*A*), fibrinogen (*B*), platelet counts (*C*), and platelet aggregation (*D*) in ATC rats with vehicle (open bar), 5 million/kg BMSCs (BMSC-5, cross bar), or 10 million/kg BMSCs (BMSC-10, coarse bar). (*A*, *B*, and *D*) *$p < 0.05$, Significant difference compared with BL; #$p < 0.05$, significant difference compared with BMSC-5 and BMSC-10. (*C*) *$p < 0.05$, Significant difference between 1 hour and 3 hours after treatment; #$p < 0.05$, significant difference comparing BMSC-10 to vehicle at 1 hour; \$$p < 0.05$, significant difference comparing BMSC-10 to vehicle at 3 hours. BL, baseline.

thrombin even though the whole blood was treated with an anticoagulant. As TF expression declined in MSCs sequestered in the lung, it is unlikely that platelet-MSCs aggregates were directly caused by TF expression on MSCs. Other adhesion molecules, such as stromal cell–derived factor and CXC chemokine receptor 4, may also contribute to the formation of MSC-platelet aggregates under various conditions.[26] Mesenchymal stromal cell–platelet aggregates may also be formed before MSCs were sequestered in the lung as a protection mechanism to prevent MSC adherence to the vessel wall when infused into the vessel, which may be associated with an activating ligand (podoplanin) for the platelet receptor c-type lectin-like receptor 2.[27] Likewise, we found no direct evidence showing consumption of fibrinogen in other tissues besides in the lung. The increase in MSC-platelet aggregates may suggest thrombogenesis including fibrinogen consumption due to fibrin formation if it was the case. However, as mentioned previously, platelet-MSCs aggregates may form independently of TF expression, which was also suggested by the lack of fibrinogen staining in the cluster of platelet-MSCs aggregates in the lung. Instead, in response to MSCs sequestered in the lung, there was a dramatic and dose-dependent increase in fibrinogen positive cells (other than MSCs) in the lung in either uninjured rats or rats subjected to trauma after MSC administration. CD11b is a component of the integrin receptor macrophage-1 antigen on the surface of leukocytes (mainly neutrophils, monocytes and macrophages) that is capable of binding fibrinogen; this is one of the previously reported mechanisms to induce host inflammatory response.[28,29] Recent study suggests that the immunomodulation outcome after IV administration of MSCs is at least in part associated with phagocytosis of monocytes that eventually mediates, distributes, and transfers the immunomodulatory effect of MSCs.[30] As a result of additional consumption of platelets and fibrinogen in the lung, IV administration of MSCs may not be beneficial in treating ATC and may even risk enhancing ATC, at least in rats, when the single dose is above 5 to 10 million/kg.

Previous studies suggest that TF expression varies between donors and tissue sources for MSC isolation.[16] In rat MSCs, BMSCs have slightly weaker TF expression than AMSCs. In our model, rats were less tolerant of AMSCs (survived only at ≤10 million/kg) than BMSCs (survived at ≤40 million/kg), and rats treated with AMSCs developed more severe coagulopathy than rats treated with BMSCs. However, the current study was not able to provide evidence that the different outcome between BMSCs and AMSCs is largely due to the difference in TF expression. Further investigation is needed to determine whether TF expression or procoagulant activity should be an inclusion criterion for MSC donor or cell source selection for the safety of IV administration. At the very least, this animal study suggests that a single dose at or less than 5 million/kg is safe for IV administration regardless of TF expression or procoagulant activity. Until now, only a few clinical trials have reported dose-response data.[13] There was indeed a limitation of the current study that was conducted in rats, and it is certainly necessary to establish an optimal range of safe IV dose of MSCs

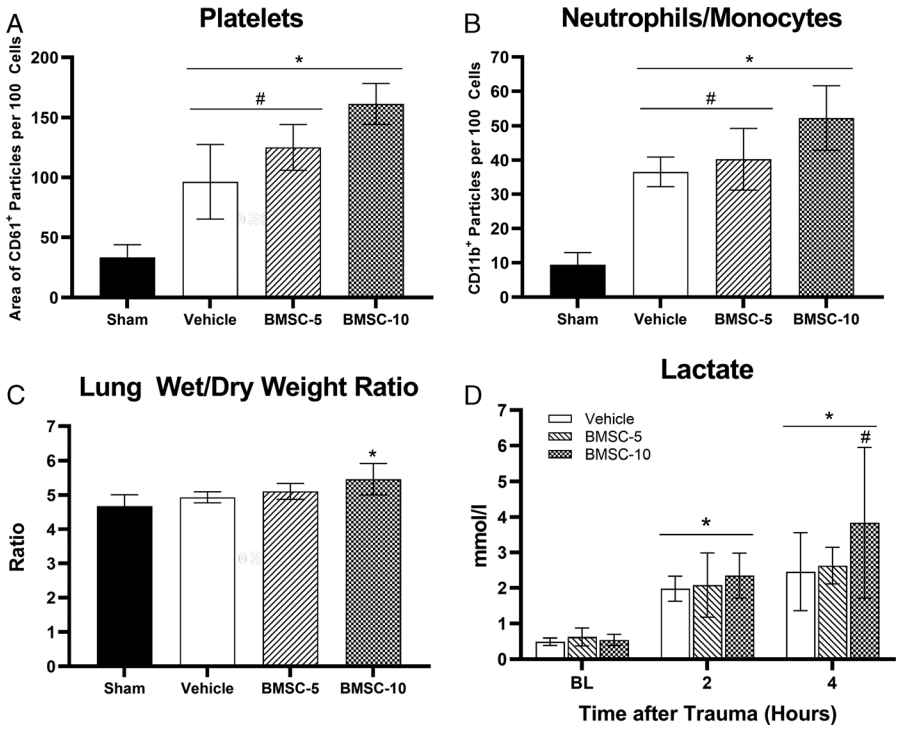

Figure 6. Intravenous administration of BMSCs enhances acute lung injury in rats with polytrauma and hemorrhage. (*A*, *B*) CD61-positive particles (*A*) and CD11b-positive particles (*B*) of immunohistochemistry staining of the lung sections from rats of sham (healthy rats, solid black bar), polytrauma and hemorrhage treated with vehicle (BMSC-V, open bar), 5 million/kg BMSCs (BMSC-5, cross bar), or 10 million/kg BMSCs (BMSC-10, coarse bar). *$p < 0.05$, Significant difference compared with sham; #$p < 0.05$, significant difference compared with BMSC-10. (*C*) Wet/dry weight ratio of the lungs. *$p < 0.05$, Significant difference compared with sham and BMSC-V. (*D*) Lactate levels at BL, and 2 hours and 4 hours after trauma (1 hour and 3 hours after BMSCs administration). *$p < 0.05$, Significant difference compared with BL; #$p < 0.05$, significant difference compared with vehicle and BMSC-5 at 4 hours. BL, baseline.

for clinical trial in the future. Currently, the median dose of MSCs used in clinical trials (although these are not in subjects with acute trauma) is 100 million/patient/dose (1–2 million/kg),[13] which is under the threshold of the toxic dose that was identified in current study. However, the optimal IV dosage of MSC applicable for trauma/hemorrhage has never been investigated. The results of the current study may provide an initial starting range of doses for future clinical trials of MSCs therapy for acute trauma.

In previous studies, ATC was characterized by elevation of PT and aPTT, and a decline in fibrinogen and platelet aggregation (ADP).[19,23] Acute IV administration of a single dose of MSCs did not appear to have an immediate beneficial outcome in the treatment of trauma and hemorrhage shock. First, there was a potential risk to enhance ATC because of increasing consumption of platelets and fibrinogen in the lung. Second, IV administration of BMSCs at either 5 or 10 million/kg did not lead to early immunomodulation by attenuating the rise of cytokines and chemokines in response to trauma and hemorrhagic shock. As MSCs were sequestered in the lung, instead of interacting with leukocytes in the circulation, more leukocytes were trapped or infiltrated into the interstitial space of the lung, and this might contribute to a systemic decline in fibrinogen via an integrin receptor, such as macrophage-1 antigen, as a part of the inflammatory response. Finally, IV administration of BMSCs did not lead to BMSCs homing to the site of injury in our study, so there is no expectation that cell-to-cell contact mechanisms would play a major role to promote tissue regeneration through orchestrating regional inflammation, cell proliferation/differentiation, and angiogenesis as reported in models with MSCs delivered locally to the site of injury. Certainly, this study was not designed to detect a paracrine effect that might occur through the cell secretomes to accelerate wound healing beyond the observation timeframe of the current study. Mesenchymal stromal cell–mediated immunomodulation is governed by production of a plethora of bioactive molecules including indoleamine 2,3-dioxygenase, prostaglandin E2, galectin-9, and hepatocyte growth factor to regulate innate and adaptive immunity. Nevertheless, this study did not observe an acute beneficial outcome within 3 hours after BMSC administration in severe trauma and hemorrhage. Previously, it was demonstrated that rats with polytrauma and hemorrhagic shock developed acute lung injury.[31,32] This study suggests a potential enhancement of acute lung injury within this model with administration of BMSCs at 10 million/kg characterized by increased platelet and leukocyte infiltration and wet/dry weight ratio in the lungs, which at least potentially could lead to a subsequent promotion of acute respiratory distress syndrome. The long-term pathological changes of sequestered MSCs in the lung must be further investigated to assess the risk/benefit for IV administration of MSCs in trauma. Considering that no acute benefit from IV administration of MSCs was shown in the current study, IV administration of MSCs

in acute trauma could render a dose-dependent risk to patients early before any possible delayed benefit could be obtained.

CONCLUSION

This study suggests that IV administration of procoagulant MSCs leads to a risk of coagulopathy associated with a dose-dependent reduction in platelet counts and fibrinogen and is incapable of attenuating ATC in rats. The potential short-term benefits of IV delivery of a single-dose MSCs early after trauma is not suggested in rats with polytrauma and hemorrhagic shock, which could be offset by pathological responses to sequestered MSCs in the lungs. The results from the current study can at least provide an initial starting range of doses for systemic administration of MSCs in future clinical applications of trauma.

AUTHORSHIP

X.W. contributed in the literature search, study design, animal experiment, data analysis, data interpretation, writing, and critical revision. D. N.D. contributed in the literature search, data analysis, and data interpretation. J.A.B. contributed in the study design and data interpretation. A.P. C. contributed in the study design, data interpretation, and critical revision. C.L.S. contributed in the animal experiment. J.D.K. contributed in the animal experiment, and assay and data collection. B.L. contributed in the assay and data collection. B.A.C. contributed in the data interpretation and critical revision.

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DISCLOSURE

The authors declare no conflicts of interest.
The views expressed in this article (book, speech, etc.) are those of the author(s) and do not reflect the official policy or position of the US Army Medical Department, Department of the Army, Department of Defense, or the US Government.
Research was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare regulations, and the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council. The facility's Institutional Animal Care and Use Committee approved all research conducted in this study. The facility where this research was conducted is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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