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Clonidine Reduces Norepinephrine and Improves Bone Marrow Function in a Rodent Model of Lung Contusion, Hemorrhagic Shock and Chronic Stress

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Abstract

Background—Propranolol has been shown previously to restore bone marrow function and improve anemia after lung contusion/hemorrhagic shock. We hypothesized that daily clonidine administration would inhibit central sympathetic outflow and restore bone marrow function in our rodent model of lung contusion/hemorrhagic shock with chronic stress.

Methods—Male Sprague-Dawley rats underwent six days of restraint stress after lung contusion/hemorrhagic shock during which the animals received clonidine (75µg/kg) after the restraint stress. On post-injury day seven, we assessed urine norepinephrine, blood hemoglobin, plasma granulocyte colony stimulating factor (G-CSF), and peripheral blood mobilization of hematopoietic progenitor cells (HPC), as well as bone marrow cellularity and erythroid progenitor cell growth.

Results—The addition of clonidine to lung contusion/hemorrhagic shock with chronic restraint stress, significantly decreased urine norepinephrine levels, improved bone marrow cellularity, restored erythroid progenitor colony growth, and improved hemoglobin (14.1±0.6 vs. 10.8±0.6 g/dL). The addition of clonidine to lung contusion/hemorrhagic shock with chronic restraint stress significantly decreased HPC mobilization and restored G-CSF levels.

Conclusions—After lung contusion/hemorrhagic shock with chronic restraint stress, daily administration of clonidine restored bone marrow function and improved anemia. Alleviating chronic stress and decreasing norepinephrine is a key therapeutic target to improve bone marrow function after severe injury.

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Keywords

trauma; G-CSF; erythroid progenitor cells; anemia; rodent

Introduction

Severe injury induces bone marrow dysfunction in rodents and humans, which is associated with a hypercatecholamine state¹⁻⁴. After trauma, the initial catecholamine surge is a necessary and appropriate response to injury, but this surge can be detrimental when prolonged. A prolonged hypercatecholaminemia is associated with pathophysiologic changes, including cardiac, metabolic and psychiatric disorders⁵⁻⁷. The molecular pathways by which hypercatecholaminemia leads to the manifestations of these diseases is poorly understood and the loss of autonomic balance alters homeostasis in the host⁷. Previous work has demonstrated that increased concentrations of norepinephrine (NE) levels after injury can persist up to six days in rodents and up to 10 days in humans^{1, 8-10}. We have demonstrated in rodents after a model of lung contusion/hemorrhagic shock, that hypercatecholaminemia, particularly an increase in NE, was associated with decreased bone marrow cellularity, decreased growth of an erythroid progenitor cell colony, and prolonged mobilization of hematopoietic progenitor cells⁸.

Previous investigation of the role of NE in bone marrow dysfunction after trauma focused on propranolol, a non-selective beta blocker, that competitively blocks NE from binding to its receptors. Use of propranolol after lung contusion/hemorrhagic shock with chronic restraint stress in a rodent model decreased plasma granulocyte-colony stimulating factor (G-CSF) and decreased mobilization of hematopoietic progenitor cells (HPC)¹¹⁻¹³. Currently, there are no data examining the suppression of NE within the central nervous system and its' effect on bone marrow function. Clonidine, an alpha 2-adrenergic agonist, acts as a central sympatholytic that specifically inhibits the release of NE but not epinephrine or dopamine¹⁴⁻¹⁸. In hypertensive patients, treatment with clonidine decreases urine NE levels up to 90%¹⁹. If NE is a key mediator of prolonged bone marrow dysfunction after trauma, then clonidine administration could be a cost-effective intervention to improve post-injury outcomes. This study investigated the effects of daily clonidine administration in a rodent model of lung contusion/hemorrhagic shock with chronic restraint stress that better attempts to recapitulate the human condition. NE levels, mobilization of hematopoietic progenitor cells, bone marrow erythroid function, and hemoglobin levels were determined as part of the post-injury evaluation in these animals. Using our model, we hypothesized that daily clonidine administration would inhibit central sympathetic outflow and restore bone marrow function.

Material and methods

Animals

Male Sprague-Dawley rats (Charles River, Wilmington, MA), weighing 300 to 315g (n=6-8/group) were housed in pairs with free access to food and water during daily night/day cycles of 12 hours each. Female animals were excluded due to estrous cycle variability and its

impact after hemorrhagic shock. The animals were housed and handled in accordance with the Guide for the Care and Use of Laboratory Animals and the protocol had been approved by the Institutional Animal Care and Use Committee prior to initiation (Protocol #201408271).

Experimental Design

Animals were allocated randomly into one of seven groups (Table 1). The groups (n=6–8/group) included: 1) naïve control; 2) chronic restraint stress (CS) alone; 3) CS with administered clonidine (CS+clonidine); 4) lung contusion followed by hemorrhagic shock (LCHS); 5) LCHS with administered clonidine (LCHS + clonidine), 6) LCHS followed by CS (LCHS/CS); and 7) LCHS/CS with administered clonidine (LCHS/CS + clonidine). In the LCHS + clonidine group, clonidine (Letco Medical, Decatur, AL#684754), 75µg/kg was injected intraperitoneally 10 minutes after LCHS and daily for six days. In the groups subject to CS or LCHS/CS, clonidine was given immediately after the CS period. Naïve control animals underwent daily handling. All groups were sacrificed on day seven after LCHS or CS and peripheral blood, urine, and bone marrow were harvested.

Tissue Injury and Hemorrhagic Shock

As described previously, after intraperitoneal administration of 50 mg/kg pentobarbital, a unilateral lung contusion was created with a percussive nail gun (Sears Brand, Chicago, IL) applied directly to a 12 mm metal plate that was placed in the right axilla of the rat⁸. Ten min after lung contusion, the right femoral artery and internal jugular vein were exposed and cannulated with Polyethylene 50 tubing (PE-50) (BD Biosciences, San Jose, CA). Both cannulas secured using 4–0 silk sutures placed proximal and distal to the insertion site were flushed with heparinized saline (10units/ml). The femoral artery tubing was then connected to a continuous blood pressure monitoring device (BP-2 Digital Blood Pressure Monitor; Columbus Instruments, Columbus, Ohio) for measurement of mean arterial pressure and heart rate. Blood was then withdrawn from the internal jugular to maintain a mean arterial pressure of 30–35 mmHg for 45 min. After the period of shock animals were reinfused their shed blood, and the cannulas were removed.

Chronic Restraint Stress (CS)

CS was performed daily for six days and consisted of two h of restraint in a nose cone rodent cylinder (Kent Scientific Corporation, Torrington, CT, USA) that restricted their movements. Every 30 min during the two h of restraint, loud, ringing, beeping, constant alarms were played for two min, and the animals were repositioned to prevent habituation.

Norepinephrine and G-CSF Measurements

Urine NE levels were determined using a quantitative ELISA kit (IBL America, Minneapolis, MN), and plasma G-CSF levels were assessed using a standard ELISA (R&D Systems, Minneapolis, MN, USA). Samples were processed in duplicate according to the manufacturer's instructions.

Flow Cytometry

Peripheral blood collected on day seven was used to identify HPC that were counted by flow cytometry using CD-71 and c-kit antibodies. CD-71, also known as transferrin receptor-1, is a marker for one of the earliest precursors of erythroid progenitor cells, burst-forming unit-erythroid (BFU-E) cells.²⁰ C-kit, also known as CD 117, is a tyrosine protein kinase receptor also expressed on early progenitor cells, such as colony-forming units-granulocyte-, erythrocyte-, monocyte- megakaryocyte (CFU-GEMM), BFU-E, and colony-forming unit-erythroid (CFU-E).²¹ Briefly, whole blood was incubated with mouse anti-rat CD71 (BD Biosciences, San Jose, CA) antibody conjugated with fluorescein isothiocyanate and rat anti-mouse CD117 (c-kit) (Southern Biotech, Birmingham, AL, USA) antibody conjugated to phycoerythrin (BD Biosciences). After washing and lysing the red blood cells, the samples were run on BD LSR II flow cytometer equipped with FACSDiva software (BD Biosciences) to quantitate CD117 and CD71 positive cells. CD117 and CD71-positive cells included CFU-GEMM, BFU-E, and CFU-E.

Bone Marrow cellularity

Bone marrow was flushed from the left femur using a 19-gauge needle with a syringe containing 1ml Iscove's Modified Dulbecco's Medium (IMDM) (Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA, USA). Cell viability was determined by 0.4% Trypan blue staining, and cellularity was assessed on a hemocytometer.

Assays of Bone Marrow Erythroid Progenitor Colonies

Growth of bone marrow erythroid progenitor colonies was determined for colony-forming units-granulocyte-, erythrocyte-, monocyte- megakaryocyte (CFU-GEMM), burst-forming unit-erythroid (BFU-E), and colony-forming unit-erythroid (CFU-E) as described previously^{13, 22}. Briefly, bone marrow mononuclear cells in a concentration of 1×10^6 cells/mL of IMDM were plated in duplicate with Methocult media (Stemcell, Vancouver, BC, Canada). Plates were supplemented with 1.3 U/mL rhEpo and 6 U/mL rhIL-3 for BFU-E and CFU-E colonies or 3 U/mL rhGM-CSF for CFU-GEMM. Plates were then incubated at 37°C in 5% CO₂ incubator. A blinded reader assessed colony growth of CFU-E on day seven, BFU-E colonies on day 14, and CFU-GEMM on day 17.

Hemoglobin Measurements

Heparinized blood samples were obtained via cardiac puncture and used for the analysis of hemoglobin (VetScan HM5, Abaxis, CA, USA). The levels were measured on day seven.

Statistical Analysis

The data were analyzed statistically using Graph Pad Prism Version 6 (GraphPad Software, Inc, San Diego, CA, USA) as t-tests followed by one-way analysis of variance (ANOVA) and Tukey-Kramer for multiple comparisons. The data are expressed as mean \pm standard deviation. Significance was defined as * $p < 0.05$ vs. naïve controls ** $p < 0.05$ vs. untreated counterpart.

Results

The Effect of Clonidine on Urine NE Levels

CS alone, LCHS, and LCHS/CS all resulted in a significant increase in urine NE levels on day seven as compared to naïve controls (Figure 1) (* $p=0.006$, * $p=0.0369$, and * $p=0.0357$). This illustrated that CS, as well as tissue injury and shock, are significant stimulators of NE. When clonidine was administered to all three rodent models, only CS and LCHS/CS resulted in suppressed urine NE levels versus their untreated counterpart (Figure 1) (* $p=0.0144$ and * $p=0.0281$). In contrast, LCHS+clonidine did not change urine NE levels compared to LCHS (Figure 1).

The Impact of Clonidine on Mobilization of Hematopoietic Progenitor Cells

Both LCHS and LCHS/CS increased plasma G-CSF levels by 51 and 58% (* $p<0.04$ each) while CS alone increased plasma G-CSF levels by 23% (* $p=0.006$) when compared to naïve controls (Figure 2A). In contrast, the addition of clonidine to all three rodent models resulted in the suppression of plasma G-CSF levels. When the LCHS/CS animals received clonidine daily, the plasma G-CSF levels were decreased by 44% (** $p<0.004$) compared to LCHS/CS (Figure 2A). The addition of clonidine to CS alone and LCHS resulted in a trend of decreased plasma G-CSF levels by 41 and 34%, respectively (Figure 2A) ($p=0.0961$ and $p=0.0995$).

After severe trauma, NE and G-CSF have been demonstrated to be associated with prolonged HPC mobilization^{1, 8, 12, 23–25}. Seven days after CS alone, HPC mobilization was significantly prolonged (Figure 2B). Similar to previous work, the addition of CS to LCHS increased HPC mobilization by 84% (* $p=0.003$) compared to naïve (Figure 2B)⁸. When CS and LCHS/CS animals received daily clonidine, the prolonged mobilization of HPC was markedly attenuated by 66 and 84% (** $p=0.0416$ and ** $p=0.0057$) compared to CS and LCHS/CS (Figure 2B and 2C). Not surprisingly, daily use of clonidine after LCHS without chronic stress had no impact on HPC mobilization, because HPC mobilization was not prolonged after LCHS alone (Figure 2B).

The Effects of Clonidine on Bone Marrow Erythroid Function

Bone marrow cellularity and erythroid progenitor colony growth were determined to evaluate bone marrow function. Seven days after CS and LCHS, bone marrow cellularity was decreased by 19 and 21% (* $p=0.0111$ and * $p=0.0084$). Seven days after LCHS/CS, there was a 30% suppression (* $p=0.0009$) in bone marrow cellularity compared to naïve (Figure 3A). When CS, LCHS, and LCHS/CS animals received daily clonidine, bone marrow cellularity improved by 21, 27, and 31% (** $p=0.0081$, 0.0012, <0.0001) as compared to their untreated counterparts (Figure 3A). The addition of clonidine to CS, LCHS, and LCHS/CS animals also restored bone marrow cellularity to that of naïve animals (Figure 3A).

On day seven, CS, LCHS, and LCHS/CS resulted in decreased growth of bone marrow CFU-GEMM, BFU-E, and CFU-E erythroid progenitor colonies (Figure 3B–D). In contrast, when CS, LCHS, and LCHS/CS animals were administered daily clonidine, bone marrow

CFU-GEMM, BFU-E, and CFU-E colony growth improved by 10–39% (** $p < 0.0001$ to 0.0086) when compared to their untreated counterparts (Figure 3B–D). The addition of clonidine to CS, LCHS, and LCHS/CS animals restored BFU-E and CFU-E colony growth to that of naïve animals (Figure 3C, D).

The Impact of Daily Clonidine on Hemoglobin

Seven days after CS alone, there was no significant impact on hemoglobin when compared to naïve controls (13.7 ± 1.0 vs. 14.3 ± 0.3 g/dL). After LCHS alone, there was a mild persistent anemia compared to naïve controls (12.0 ± 0.9 vs. 14.3 ± 0.3 g/dL) (* $p = 0.0006$) (Figure 4). As shown previously, LCHS/CS was associated with a persistent, injury-associated anemia with a hemoglobin of 10.8 ± 0.8 g/dL(* $p < 0.0001$)⁸.

Daily clonidine administration after CS had no significant effect on hemoglobin. After LCHS, the use of daily clonidine improved hemoglobin levels (LCHS+clonidine: 13.1 ± 0.8 vs. LCHS: 12.0 ± 0.9 g/dL)(** $p = 0.0492$). Daily administration of clonidine after LCHS/CS resulted in the greatest improvement in hemoglobin (LCHS/CS+clonidine: 14.1 ± 0.6 ** vs. LCHS/CS: 10.8 ± 0.6 g/dL)(** $p = 0.0001$). Seven days of clonidine administration after LCHS/CS resolved the persistent, injury-associated anemia.

Discussion

After severe injury, trauma patients experience substantial bone marrow dysfunction that is associated with a hypercatecholamine state and prolonged HPC mobilization^{1, 2, 4, 11, 26}. Clinically, this bone marrow dysfunction is important because it manifests as a persistent, injury-associated anemia that can last for several weeks after trauma^{2, 26, 27}. Our previous work revealed that urine NE levels in severely injured trauma patients remained increased for two weeks after injury¹. This persistent hypercatecholamine state was associated with prolonged HPC mobilization to the peripheral blood in humans²⁵. In addition, increased NE levels attenuate growth of bone marrow erythroid progenitor cells, both in vitro and in vivo^{1, 5, 8, 28}. By blocking beta-adrenergic receptors, daily propranolol use prevented prolonged HPC mobilization and improved the growth of bone marrow erythroid progenitors^{12, 13, 22, 26, 26}. Our study examined the effects of clonidine on bone marrow function after trauma. Our study demonstrated that the daily use of clonidine after lung contusion/hemorrhagic shock with CS, a clinically relevant rodent model of persistent anemia, prevented prolonged HPC mobilization, improved bone marrow function, and thus restored hemoglobin levels.

Severe trauma triggers a persistent hypercatecholamine state that is associated with increases in plasma levels of G-CSF and mobilization of bone marrow HPCs which disrupts function of the homeostatic bone marrow^{1, 5, 8, 12, 23}. In three different injury models (CS, LCHS, and LCHS/CS), urine NE levels increased three to six fold. These findings correlate with previous rodent work by Bible et al.⁸, where the addition of CS to LCHS injury increased urine NE levels six days post injury. Clonidine has been shown to decrease urinary NE levels, and our work has verified this result after both CS and LCHS/CS^{15, 19, 21}. In the absence of chronic stress, clonidine administration after LCHS had no effect on urinary NE levels. Daily chronic stress, whether alone or in addition to LCHS, appears to be the driving

force behind the effects of NE-mediated hypercatecholaminemia. We hypothesize that clonidine best works in environment of constant stimulation of sympathetic outflow such as CS and LCHS/CS models of rodents. This concept would explain why there was no decrease in NE levels after LCHS alone. One mechanism through which clonidine suppresses NE output is at the level of the central nervous system by targeting alpha-2 adrenergic receptors, located in the locus coeruleus within the medial prefrontal cortex¹⁷⁻¹⁹. Kubota et al.¹⁷ demonstrated that when rodents were treated with clonidine, there was a suppression of basal rates of NE release from the brain by binding to alpha-2 adrenergic receptors in the central nervous system. In addition, Martin et al.¹⁵ and Dorman et al.³⁰ demonstrated that clonidine decreased plasma and urine NE levels by 54–90% one day after administration in patients with alcohol amnesic disorder and in patients undergoing major abdominal surgery. It is likely that a decrease in central NE outflow was responsible for some of the NE-mediated effects seen in the bone marrow of our rodent model of traumatic lung injury model with CS.

HPC mobilization has been shown to be mediated by both NE and G-CSF²³. In steady-state hematopoiesis, G-CSF regulates the level of neutrophils in the bone marrow and the blood, but G-CSF is not essential for erythroid differentiation and growth in the bone marrow³¹. In our rodent model, the lung contusion is a stimulus for the mobilization of HPC to the injured tissue to aid in repair. Seven days after LCHS and CS, our previous rodent data showed that plasma G-CSF remained persistently increased and that there was a prolonged HPC mobilization¹². These results were confirmed, and in the absence of injury in the CS alone model, there was no increase in plasma G-CSF. Those animals who received daily clonidine after LCHS/CS had a decrease in plasma G-CSF levels. This is different from previous work, where daily propranolol administration decreased plasma G-CSF levels in LCHS in addition to LCHS/CS¹². This observation can be explained in part by the work of Katayama et al.²³ who demonstrated that when mice received G-CSF directly into central nervous system via intracerebroventricular infusion versus when given systemically by subcutaneous injection, HPC mobilization was less after central nervous system administration rather than systemic injection. The G-CSF-induced HPC mobilization can also be terminated with NE depletion in mice treated with 6-hydroxydopamine²³. These findings indicate that G-CSF release may be dependent on NE release for HPC mobilization to occur and that the effects of clonidine on G-CSF are mediated by the degree that clonidine suppresses NE release. Once clonidine depleted the central outflow of NE from the brain, G-CSF-induced HPC mobilization after LCHS/CS was decreased. Thus, by decreasing plasma levels of G-CSF with clonidine use, the continued retention of hematopoietic progenitor cells in the bone marrow allows these cells to differentiate along the erythroid pathway and restore growth of erythroid colonies.

Both in rodents and humans, severe traumatic injury suppresses the growth of erythroid progenitor cell and induces anemia^{1-3, 5, 8, 13, 32}. Previous injury models in rodents have shown that although hemorrhagic shock and injury decrease growth of bone marrow erythroid colonies, the presence of high NE or chronic stress continue to persistently suppress bone marrow function^{1, 8, 13, 28}. In our study, CS alone decreased bone marrow cellularity and growth of bone marrow erythroid progenitor colonies. The use of daily clonidine after CS not only restored bone marrow cellularity but also preserved growth of

bone marrow erythroid progenitor colonies. The benefit of daily clonidine administration was most important in the combined LCHS/CS model, which had improved bone marrow cellularity and growth of bone marrow erythroid progenitor cells. This observation is supported by Baranski et al.²² and Mohr et al.¹⁷, who showed similar results with the use of propranolol after LCHS.

In the LCHS/CS model, the decrease in bone marrow cellularity and growth of bone marrow erythroid progenitor colonies along with the prolonged HPC mobilization is associated with persistent, injury-associated anemia. Similarly after critical illness, recovery of erythropoiesis is impaired for up to six months after discharge from the intensive care unit²⁷. Our study demonstrated that daily clonidine administration after LCHS/CS improved hemoglobin, similar to our previous work where daily propranolol treatment after LCHS improved hemoglobin¹³. Ours is the first study to describe clonidine as a potential therapy for persistent, injury-associated anemia after LCHS/CS. In both rodent studies, propranolol and clonidine decreased the hypercatecholamine state through different mechanisms but were able to improve bone marrow function. Peripherally, propranolol competitively blocks NE from binding to bone marrow beta-adrenergic receptors. Centrally, clonidine depletes NE release from the brain by competitively binding to alpha -2 adrenergic receptors, decreasing NE outflow. Improved bone marrow function was manifested by improved bone marrow cellularity, improved growth of bone marrow erythroid progenitor cells and decreased HPC mobilization which leads to an improvement in hemoglobin. A decrease in NE is hypothesized to improve bone marrow function by both decreasing plasma levels of G-CSF and allowing HPCs to remain in the bone marrow to differentiate along the erythroid pathway as well as preventing the dose-related, adverse effects of NE on growth of bone marrow erythroid colonies.

In summary, this study confirms the important role NE plays in bone marrow dysfunction after traumatic injury and chronic stress. Using a clinically relevant rodent model of lung contusion/hemorrhagic shock, and chronic stress, the use of daily clonidine depleted the central outflow of NE and maintained bone marrow function. Future therapeutic goals should focus on decreasing the prolonged hypercatecholamine state associated with chronic stress that is seen after severe traumatic injury to improve bone marrow function and decrease anemia.

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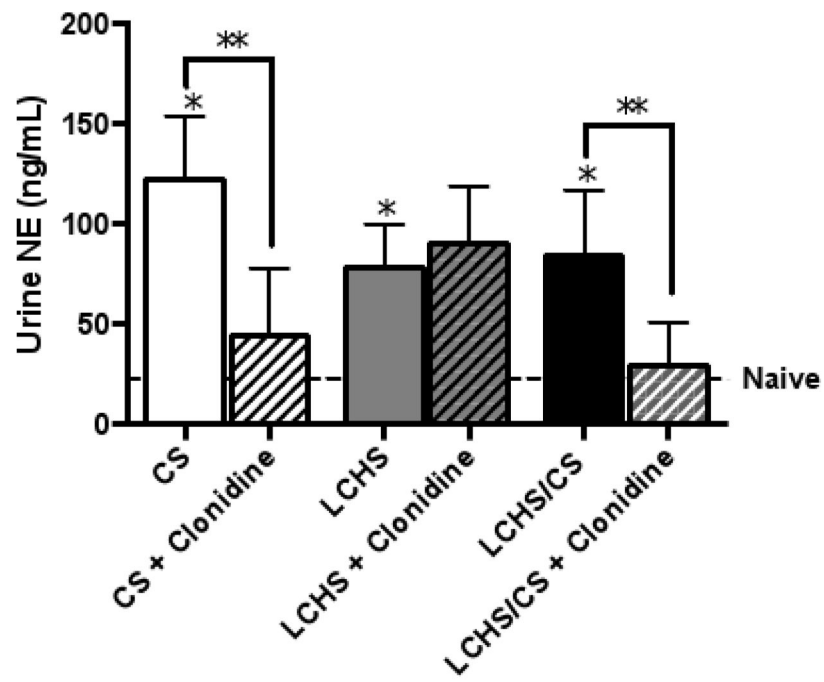


Figure 1.

Daily clonidine administration decreased urine norepinephrine (NE) levels seven days after CS and LCHS/CS. CS= chronic restraint stress; LCHS= lung contusion and hemorrhagic shock; LCHS/CS= lung contusion, hemorrhagic shock, and chronic restraint stress; * $p < 0.05$ vs. naïve and ** $p < 0.05$ vs. untreated counterpart. (n=6–8/group).

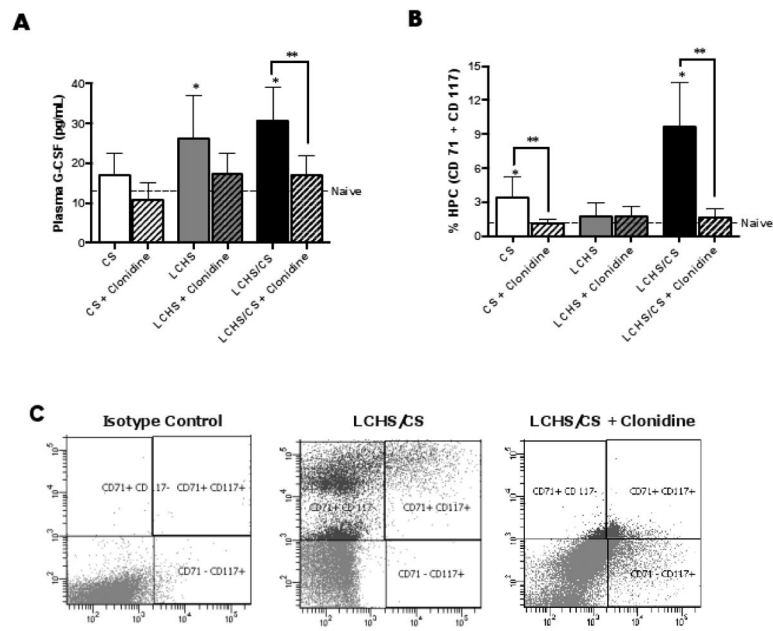


Figure 2.

Figure 2A–B. Seven days of clonidine administration decreased hematopoietic progenitor cell mobilization after CS and LCHS/CS. 2A. Clonidine administration significantly decreased plasma G-CSF levels after LCHS/CS. 2B. Clonidine administration significantly decreased peripheral blood mobilization of CD71⁺ and CD117⁺ progenitor cells seven days after CS and LCHS/CS. 2C. Representative flow cytometry analysis of the number of progenitor cells (CD 71+/CD 117+) in the peripheral blood seven days after isotype, LCHS/CS, LCHS/CS + clonidine in one rodent. CS= chronic restraint stress; LCHS= lung contusion and hemorrhagic shock; LCHS/CS= lung contusion, hemorrhagic shock and chronic restraint stress; *p < 0.05 vs. naïve and **p < 0.05 vs. untreated counterpart. (n=6–8/group)

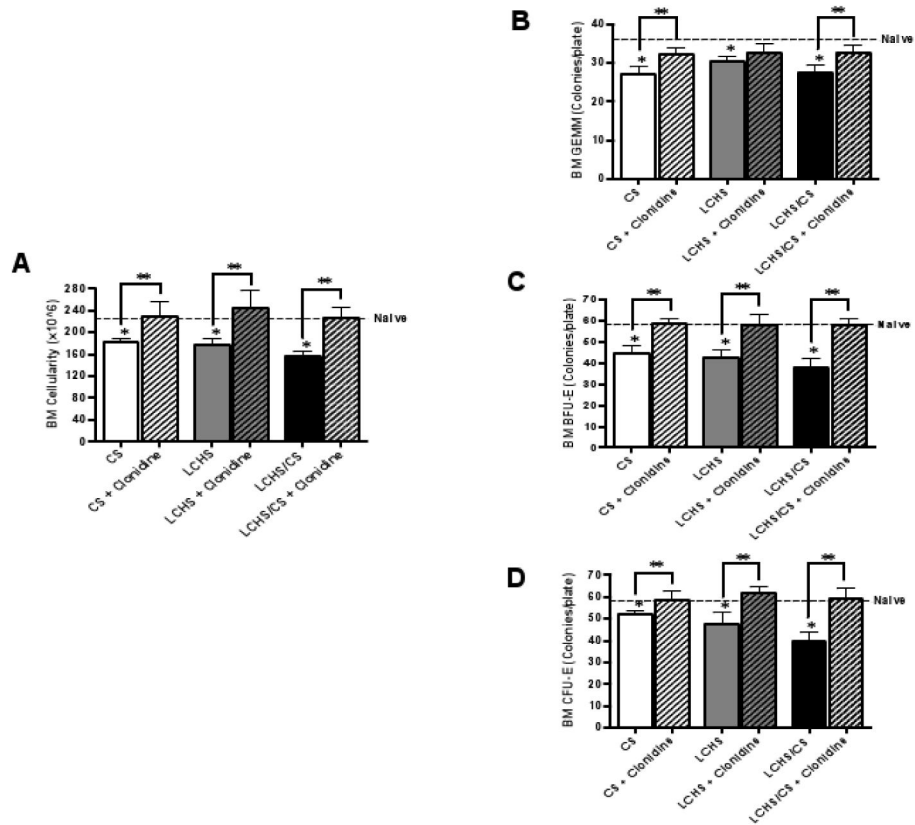


Figure 3.

Figure 3A–D. Seven days of clonidine administration restored bone marrow function after CS, LCHS and LCHS/CS. **3A.** Clonidine administration significantly improved bone marrow cellularity in all three rodent models. **3B–D.** The addition of clonidine restored bone marrow CFU-GEMM, BFU-E, and CFU-E colony growth after CS, LCHS, and LCHS/CS. BM=bone marrow; CS= chronic restraint stress; LCHS= lung contusion and hemorrhagic shock; LCHS/CS= lung contusion, hemorrhagic shock and chronic restraint stress; * $p < 0.05$ vs. naïve and ** $p < 0.05$ vs. untreated counterpart. (n=6–8/group)

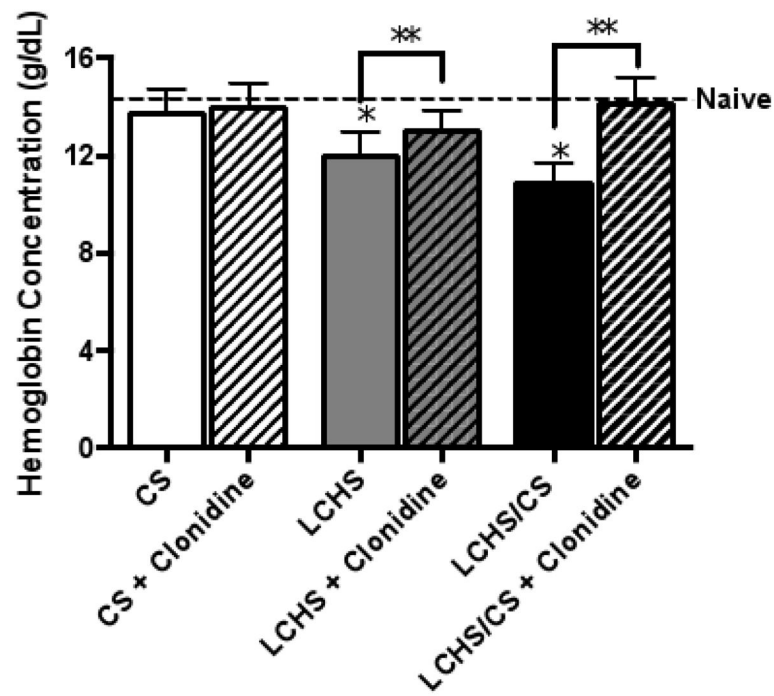


Figure 4. Seven days of daily clonidine administration improved hemoglobin levels after LCHS and LCHS/CS. CS= chronic restraint stress; LCHS= lung contusion and hemorrhagic shock; LCHS/CS= lung contusion, hemorrhagic shock and chronic restraint stress; * $p < 0.05$ vs. naïve and ** $p < 0.05$ vs. untreated counterpart. (n=6–8/group)

Experimental groups defined. CS= chronic restraint stress; LCHS= lung contusion and hemorrhagic shock; LCHS/CS= lung contusion, hemorrhagic shock, and chronic restraint stress.

Table 1

	N	Lung contusion	Hemorrhagic shock	Chronic restraint stress	Clonidine administration
Naïve	6	-	-	-	-
CS	6	-	-	+	-
CS+clonidine	6	-	-	+	+
LCHS	8	+	+	-	-
LCHS+clonidine	6	+	+	-	+
LCHS/CS	8	+	+	+	-
LCHS/CS+clonidine	6	+	+	+	+