The American Journal of Surgery 220 (2020) 1312–1318

Contents lists available at ScienceDirect

The American Journal of Surgery

journal homepage: www.americanjournalofsurgery.com

The effects of selective beta-adrenergic blockade on bone marrow dysfunction following severe trauma and chronic stress

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ARTICLE INFO

Article history:
Received 15 February 2020
Received in revised form
19 April 2020
Accepted 25 June 2020

Keywords:
Trauma
Anemia
Selective beta blockade
CFU-E
BFU-E

ABSTRACT

Introduction: Propranolol has been shown to improve erythroid progenitor cell growth and anemia following trauma and this study sought to investigate the mechanisms involved by evaluating the effects of selective beta blockade.
Methods: Male Sprague-Dawley rats were subjected to lung contusion, hemorrhagic shock and chronic stress (LCHS/CS) ± daily selective beta-1, beta-2, or beta-3 blockade (B1B, B2B, B3B). Bone marrow cellularity and growth of erythroid progenitor colonies, hemoglobin, plasma granulocyte colony-stimulating factor (G-CSF), hematopoietic progenitor cell mobilization, and daily weight were assessed.
Results: Selective beta-2 and beta-3 blockade improved bone marrow cellularity, erythroid progenitor colony growth and hemoglobin levels, while decreasing plasma G-CSF, progenitor cell mobilization and weight loss following LCHS/CS.
Conclusions: Attenuating the neuroendocrine stress response with the use of selective beta-2 and 3 adrenergic blockade may be an alternative to improve bone marrow erythroid function following trauma.

Introduction

Severe traumatic injury is often associated with anemia that persists for weeks to months despite the use of blood transfusion and cessation of bleeding.[1,2] Persistent injury-associated anemia is increasingly important given that advances in critical care have improved survival following traumatic injury. Patients who survive their initial injury and are critically ill in the intensive care unit continue to be transfused an average of one unit of packed red blood cells per week after the first week of their injury despite use of restrictive transfusion strategies and control of bleeding.[1,2] Despite the risks, blood transfusion continues to be the mainstay of treatment for anemia while critically ill. Both anemia and transfusion are associated with morbidity and mortality.[1,2] Therefore, characterization of mechanisms involved in persistent injury-associated anemia can lead to the development of novel treatment strategies that can improve anemia and reduce the number of blood transfusions.

Prior studies have demonstrated that persistent injury-associated anemia is correlated with a prolonged hyperadrenergic state, inflammation, and bone marrow dysfunction which can impact long-term functional recovery.[3–11] In particular, bone marrow dysfunction following trauma is associated with decreased erythroid progenitor colony growth, prolonged hematopoietic progenitor cell mobilization to the peripheral blood, erythroid to myeloid reprioritization, impaired functional iron homeostasis, and hematopoietic progenitor cell sequestration at the site of injury.[3,6,11–16]

In previous studies using a rodent trauma model, as well as a prospective trial in trauma patients, the use of daily propranolol, a non-selective beta adrenergic blocker, was found to increase bone marrow erythroid progenitor colony growth, reduce hematopoietic progenitor cell mobilization, improve iron homeostasis and redirect bone marrow hematopoietic commitment toward erythropoiesis.[3–6,11,14,15] The exact mechanism of improved bone marrow function following trauma with the use of a non-selective beta blockade remains unknown.

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https://doi.org/10.1016/j.amjsurg.2020.06.058
0002-9610/

Beta-1, beta-2 and beta-3 adrenergic receptors are found throughout the body. Beta-1 receptors are most predominant on cardiac tissue, but they are also found in the kidneys, adipose tissue, skeletal muscle and immune cells.[17] Beta-2 adrenergic receptors are more widespread, being found in hematopoietic progenitor cells, osteoblasts, glia cells, fibroblasts, endothelial cells, smooth muscle, skeletal muscle, liver and gastrointestinal tract.[18–20] Beta-3 adrenergic receptors are found in adipose tissue, urinary bladder, gallbladder and bone marrow stromal cells.[17–19] The possible effects of beta-3 adrenergic receptors have had little investigation due to their low expression levels. All beta-adrenergic receptors are involved in the catecholamine-induced activation of adenylate cyclase to form cyclic adenosine monophosphate through the action of G proteins.

The objective of this study was to identify the mechanism by which beta blockade impacts persistent injury-associated anemia. Using selective beta blockade in a rodent model of lung contusion, hemorrhagic shock and chronic stress, the effects of beta-1, beta-2 and beta-3 adrenergic receptors in bone marrow function will be evaluated.

Methods

Animals

Male Sprague-Dawley rats aged 8–9 weeks (Charles River, Wilmington, MA) were housed in pairs under barrier-sustained conditions with a 12-h light/dark cycle with free access to chow and water. They were given at least a 5-day acclimation period before surgery was initiated. On day seven, the rats were anesthetized using a rodent cocktail consisting of ketamine (80–100 mg/kg) and (xylazine 5–10 mg/kg), then underwent sacrifice exsanguination via cardiac puncture. Female rodents were excluded from this study due to possible estrous cycle effect on systemic stress state as well as evidence of sex-related differences in leukocyte genomic response during severe injury and hormonal differences accounting for improved survival in sepsis.[21] All animal care was conducted in accordance with the University of Florida Institutional Animal Care and Use Committee.

Rodents were randomly assigned to one of five groups ($n = 6–11$/group). The experimental groups consisted of: 1) naïve control; 2) lung contusion followed by hemorrhagic shock and chronic stress (LCHS/CS); 3) LCHS/CS followed by administration of beta-1 adrenergic blockade (LCHS/CS + B1B); 4) LCHS/CS followed by administration of beta-2 adrenergic blockade (LCHS/CS + B2B); 5) LCHS/CS followed by administration of beta-3 adrenergic blockade (LCHS/CS + B3B). In groups given beta blockade, atenolol was used for beta-1 adrenergic blockade (10 mg/kg), butoxamine was used for beta-2 adrenergic blockade (10 mg/kg) and SR59230A was used for beta-3 adrenergic blockade (10 mg/kg). These selective antagonists have been successfully used in previous murine hypermetabolism studies.[17] Dosing was based on previous work demonstrating propranolol dosing that reduced heart rate 10–20% without hemodynamically significant hypotension.[5] Beta blockade was given via intraperitoneal (IP) injection daily for six days following restraint stress. Blood and bone marrow were collected on day seven.

Trauma rodent model

Following anesthesia with IP injection of pentobarbital (50 mg/kg), a right lung contusion (LC) was performed using a percussive nail gun (PowerShot Model 5700 M, Saddle Brook, NJ) applied to a 12-mm metal plate placed on the right axilla of the rat. Approximately 10 min following lung contusion, hemorrhagic shock was initiated. The right internal jugular vein and femoral artery were cannulated using polyethylene tubing containing 10 units/ml heparinized saline. The femoral artery tubing was connected to a blood pressure monitoring device (BP-2 Digital Blood Pressure Monitor; Columbus Instruments, Columbus, OH) to measure the mean arterial pressure (MAP) and the heart rate. Venous blood was withdrawn from the internal jugular vein until a MAP of 30–35 mmHg was achieved. MAP was kept in this range for 45 min. Then shed blood was reinfused at 1 ml/min.

Chronic stress consisted of 2 h of restraint stress daily for six days. The rodents were placed in a nose cone animal cylinder (Kent Scientific Corporation, Torrington, CT) which was adjusted to restrict its movements. During the 2-h period, the restraint cylinder was rotated 180° every 30 min, and alarms (80–85 dB) were sounded for 2 min at the time of each rotation. At the end of 2 h, the rats were placed back in their housing where they had free access to food and water.

Bone marrow cellularity and erythroid progenitor colonies

Bone marrow was harvested from the left femur using a 19-gauge needle and erythroid progenitor colony growth assays were performed, including colony-forming unit granulocyte, erythrocyte, monocyte megakaryocyte (CFU-GEMM), burst-forming unit erythroid (BFU-E), and colony forming unit erythroid (CFU-E). The femur was flushed with Iscove's Modified Dulbecco's Medium (IMDM) + 2% Fetal Bovine Serum (FBS) (Stem Cell) and a 1:100 dilution of this cell suspension was made in IMDM+2%FBS and the cells are counted in a TC-20 Automated Cell Counter (Bio Rad). Diluted cells were mixed with 10 μl of trypan blue dye and placed in the cell counter to determine the percentage of live cells. 50,000 bone marrow cells in 1 ml methocult (SF M3436 for CFU-E and BFU-E from Stem Cell, and HSC012 from R&D Systems for CFU-GEMM) were added to 35 mm petri dish. Cells were incubated in a CO_2 incubator maintained at 36 °C, 5% CO_2 in air and ≥95% humidity. A blinded reader assessed colony growth for CFU-E on day seven, BFU-E on day 14, and CFU-GEMM on day 17.

Hematopoietic progenitor cell mobilization to the peripheral blood

Peripheral whole blood from cardiac puncture on the day of sacrifice was used to determine hematopoietic progenitor cell mobilization by flow cytometry. Markers CD-71 and CD-117 (c-kit) were used to identify hematopoietic progenitor cells. CD-71 is a marker for BFU-E, one of the earliest erythroid progenitors.[22] CD-117 is expressed on progenitor cells such as CFU-GEMM, BFU-E, and CFU-E.[23] Briefly, 100 μl of whole blood was incubated with mouse anti-rat CD-71 antibody conjugated with fluorescein isothiocyanate (BD Pharmingen) and rat anti-mouse CD117 antibody conjugated with allophycocyanin (Southern BioTech). 2 ml of BD Phosflow Lyse/Fix Buffer was added then the solution was spun at 400 g for 6 min. The procedure of adding PBS, spinning the solution and decanting the fluid was repeated until it was clear. Then the final fluid was aspirated and 500 ml of Stain Buffer was added. The blood sample was then run on BD LSR II flow cytometer equipped with FACSDiva software (BD Biosciences) to enumerate $CD71^{+}117^{+}$ cells.

Plasma measurements

Granulocyte colony stimulating factor (G-CSF) was measured in the plasma on day seven by enzyme linked immunosorbent assay (MyBioSource, Inc). All samples were run following the manufacturer's protocol.

On the day of sacrifice, heparinized whole blood was obtained

by cardiac puncture. Blood was then used for hemoglobin analysis using VetScan (HM5, Abaxis, CA).

Statistical analysis

Statistical analysis and figure production were performed using GraphPad Prism Version 6.05 (GraphPad Software, La Jolla, CA) to calculate one-way analysis of variance with Dunnett's, Bonferroni's, Sidak's or Tukey's multiple comparisons test with a single pooled variance. Significance was set at $\alpha = 0.05$ and data were reported as mean ± standard deviation.

Results

The impact of selective beta blockade on hemoglobin

Lung contusion, hemorrhagic shock and chronic stress (LCHS/CS) alone decreased rodent hemoglobin by 7% compared to naïve rodents (Fig. 1). The use of beta-2 and beta-3 adrenergic blockade following LCHS/CS significantly increased the hemoglobin compared to LCHS/CS by 12% ($p = 0.01$) and 10% ($p = 0.04$) respectively (Fig. 1). The use of beta-1 adrenergic blockade following LCHS/CS had no impact on hemoglobin.

The effects of selective beta blockade on the bone marrow

Seven days after LCHS/CS alone, there was a 20% decrease in bone marrow cellularity compared to naïve rodents (Fig. 2A). The daily use of beta-2 and beta-3 adrenergic blockade following LCHS/CS led to a significant increase in bone marrow cellularity compared to LCHS/CS alone, with a respective 45% ($p < 0.01$) and 33% ($p = 0.03$) increase (Fig. 2A). The use of beta-1 adrenergic blockade following LCHS/CS had no impact on bone marrow cellularity.

The impact on erythroid progenitor cell was assessed by examining blast forming unit-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E) growth. LCHS/CS alone significantly decreased BFU-E growth by 28% as compared to naïve rodents ($p < 0.01$) (Fig. 2B). The use of beta-1 adrenergic blockade following LCHS/CS did not improve BFU-E growth, but instead BFU-E growth remained significantly suppressed compared to naïve (Fig. 2B). The use of daily beta-2 and beta-3 blockade following LCHS/CS had a significant improvement in BFU-E colony growth compared to LCHS/CS, with a respective 27% ($p < 0.01$) and 24% ($p = 0.01$) increase (Fig. 2B). Similarly, CFU-E growth was significantly suppressed by 30% following LCHS/CS alone when compared to naïve rodents ($p < 0.01$) (Fig. 2C). The use of beta-1 and beta-2 adrenergic blockade did not alter CFU-E growth and CFU-E remained

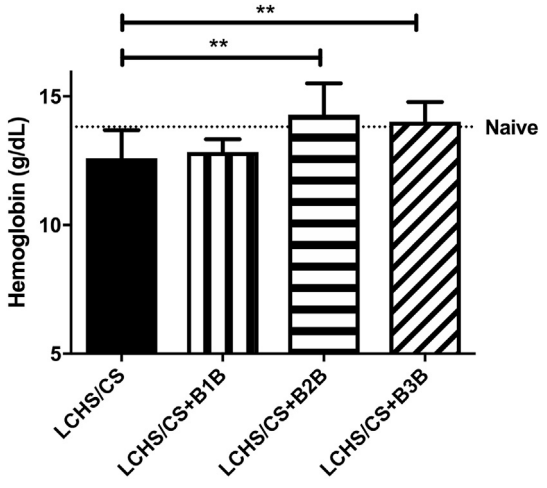

Fig. 1. The impact of selective beta blockade on post-injury anemia. Beta-2 and beta-3 blockade significantly increased hemoglobin compared to LCHS/CS. LCHS/CS = lung contusion hemorrhagic shock/chronic stress; B1B = selective beta-1 blockade; B2B = selective beta-2 blockade; B3B = selective beta-3 blockade; **p < 0.05 treatment group vs. LCHS/CS.

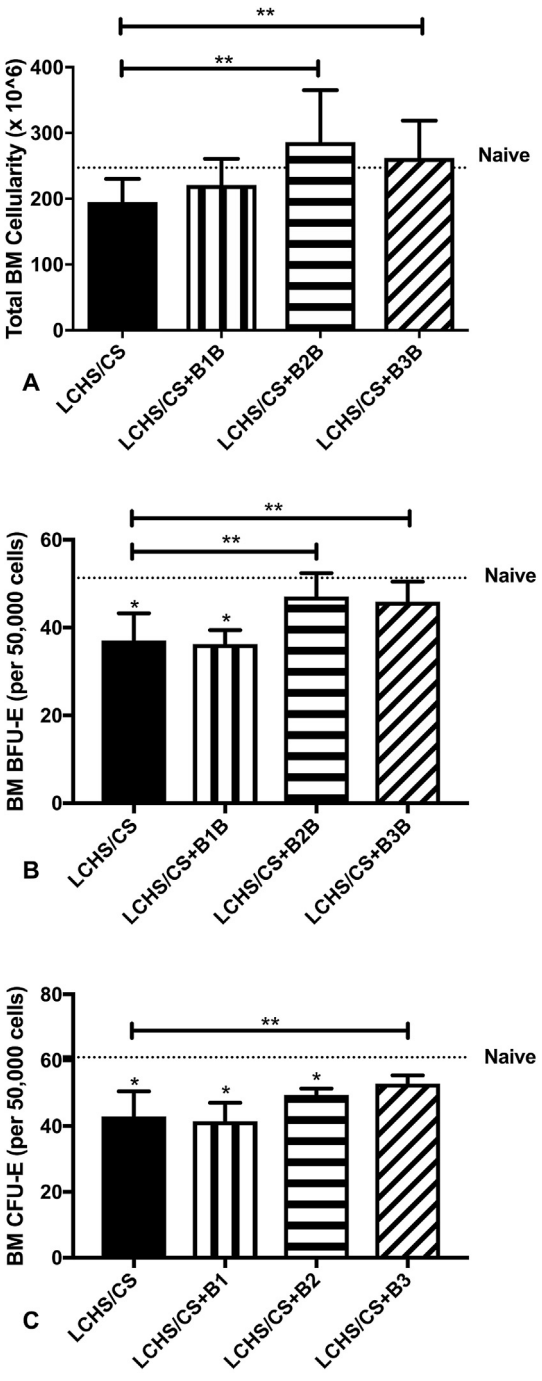

Fig. 2. A-C. The effect of selective beta blockade on post-injury bone marrow cellularity and erythroid colony growth. 2A. Beta-2 and beta-3 blockade significantly increased bone marrow cellularity compared to LCHS/CS. **2B.** Beta-2 and beta-3 blockade significantly increased BFU-E growth compared to LCHS/CS. **2C.** Beta-3 blockade significantly increased CFU-E growth compared to LCHS/CS. BM = bone marrow; LCHS/CS = lung contusion hemorrhagic shock/chronic stress; B1B = selective beta-1 blockade; B2B = selective beta-2 blockade; B3B = selective beta-3 blockade; *p < 0.05 vs naïve; **p < 0.05 treatment group vs LCHS/CS.

significantly suppressed compared to naïve (Fig. 2C). The use of daily beta-3 blockade following LCHS/CS led to significant improvement in CFU-E colony growth compared to LCHS/CS, with a 23% increase ($p < 0.01$) (Fig. 2C).

The effects of selective beta blockade on hematopoietic progenitor cell mobilization

LCHS/CS alone significantly increased the percentage of hematopoietic progenitor cells in the peripheral blood 1000-fold compared to naïve rodents ($p < 0.01$) (Fig. 3A). The use of daily beta-2 adrenergic blockade following LCHS/CS led to a significant decrease in hematopoietic progenitor cell mobilization by 54% compared to LCHS/CS alone ($p = 0.03$) (Fig. 3A). The use of beta-3 adrenergic blockade following LCHS/CS led to a 42% decrease in hematopoietic progenitor cell mobilization.

The elevation in plasma G-CSF following LCHS/CS alone correlated with the increase in hematopoietic progenitor cell mobilization (Fig. 3B). The use of selective beta-adrenergic blockade following LCHS/CS significantly decreased plasma G-CSF concentration in all groups (Fig. 3B). Compared to LCHS/CS alone, beta-1 adrenergic use decreased plasma G-CSF by 89% ($p = 0.02$), beta-2 adrenergic use decreased plasma G-CSF by 95% ($p = 0.01$) and beta-3 adrenergic use decreased plasma G-CSF by 83% ($p = 0.02$) (Fig. 3B).

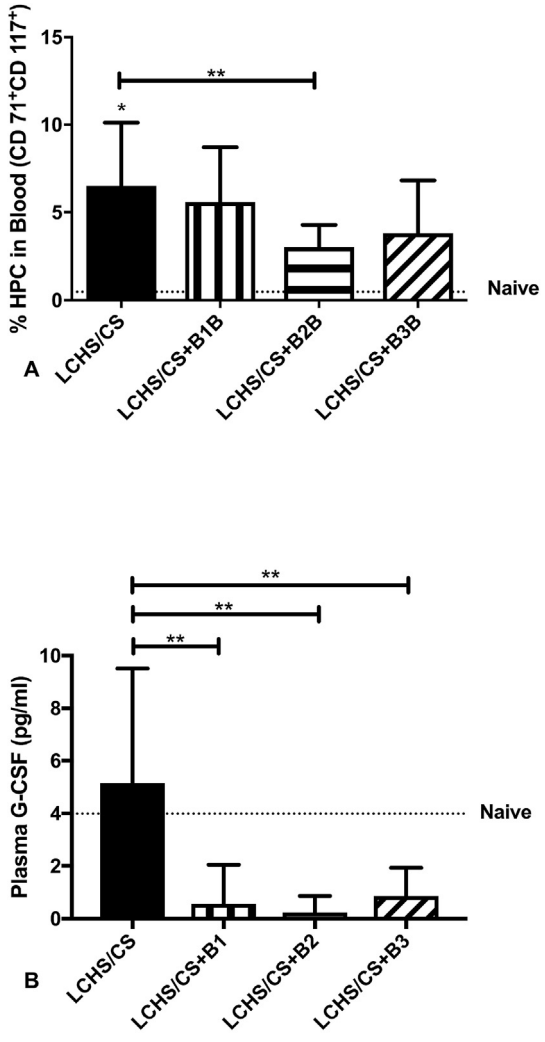

Fig. 3. A-B. The impact of selective beta blockade on post-injury hematopoietic cell mobilization. 3A. Beta-2 blockade significantly suppressed HPC mobilization compared to LCHS/CS. **3B.** All three beta-blockade treatment groups significantly decreased plasma G-CSF concentration following LCHS/CS. HPC = hematopoietic progenitor cell mobilization; LCHS/CS = lung contusion hemorrhagic shock/chronic stress; B1B = selective beta-1 blockade; B2B = selective beta-2 blockade; B3B = selective beta-3 blockade; G-CSF = granulocyte-colony stimulating factor; *$p < 0.05$ vs naïve; **$p < 0.05$ treatment group vs LCHS/CS.

The influence of selective beta blockade on weight gain

Naïve rodents gained weight at an average of 8.0 g/day. LCHS/CS rodents lost the most weight over the first two days at a rate of 7.0 g/day, which was expressed as the slope of a best-fit line, that was significantly different from a slope of zero ($p = 0.01$) (Fig. 4A). The use of daily beta-1 blockade following LCHS/CS led to weight loss at a significant rate of 7.1 g/day ($p = 0.03$) (Fig. 4B). The daily use of beta-2 and beta-3 blockade following LCHS/CS led to a loss of weight at a slower rate of 3.3 g/day and 4.6 g/day (Fig. 4C and D).

Discussion

As previously demonstrated, severe traumatic injury has been shown to lead to persistent anemia seven days from injury in both rats and humans while propranolol attenuated this anemia via non-selective beta blockade.[3–10,12,15,24–26] In the present study, it was found that beta-2 and beta-3 adrenergic blockade most significantly improved hemoglobin levels, bone marrow cellularity, erythroid progenitor colony growth, hematopoietic progenitor cell mobilization and mitigated weight loss following traumatic injury and chronic stress. In addition, beta-1 adrenergic blockade had no impact on bone marrow function following lung contusion, hemorrhagic shock and chronic stress.

The use of beta-2 and beta-3 adrenergic blockade following LCHS/CS significantly increased hemoglobin compared to LCHS/CS alone. Hanoun et al.[18] identified that beta-2 adrenergic receptors are located on hematopoietic stem cells. These hematopoietic stem cells are found in the beginning of the erythroid development commitment stage.[17] Muthu et al.[27] demonstrated in a mouse study that the beta-2 adrenergic receptor is located on the pluripotent hematopoietic stem cell that is lineage^{neg}Sca1^{+}CD117^{high} (uncommitted, lineage negative, SCA-1+ with high cell surface expression of CD117+, an early progenitor marker) and remains on the progenitor cells as they become CD34^{+}CD117^{+} multipotent cells. In addition, alpha-1 and alpha-2 adrenergic receptors are found on these cells as well.[27] Hasan et al.[16] in a mouse burn injury model utilized selective beta blockade to demonstrate that the post-injury erythroid to myeloid shift in commitment is beta-2 adrenergic receptor dependent via MafB, a myeloid-specific transcription factor. In addition, beta-3 adrenergic blockade improved late stage erythropoiesis but beta-2 and beta-3 adrenergic blockade significantly improved peripheral blood hemoglobin and red blood cells (RBCs).[17] Staining of the bone marrow post-burn injury demonstrated in control animals a decrease in reticulocytes and red blood cells with an increase in myeloid cells, while treatment with beta-2 adrenergic blockade increased late erythroblasts and treatment with beta-3 adrenergic blockade increased reticulocytes and RBCs.[17] These studies establish that the beta-2 and beta-3 adrenergic receptors are involved in both early and late stage erythropoiesis, and that their antagonism during times of chronic stress and hypercatecholaminemia can improve anemia.

Our study also demonstrated that LCHS/CS inhibited bone marrow cellularity and BFU-E growth compared to naive while beta-2 and beta-3 adrenergic blockade significantly improved bone marrow cellularity and BFU-E growth compared to LCHS/CS alone. Beta-3 adrenergic blockade significantly improved CFU-E growth while beta-1 and beta-2 adrenergic blockade had no effect. Fonseca

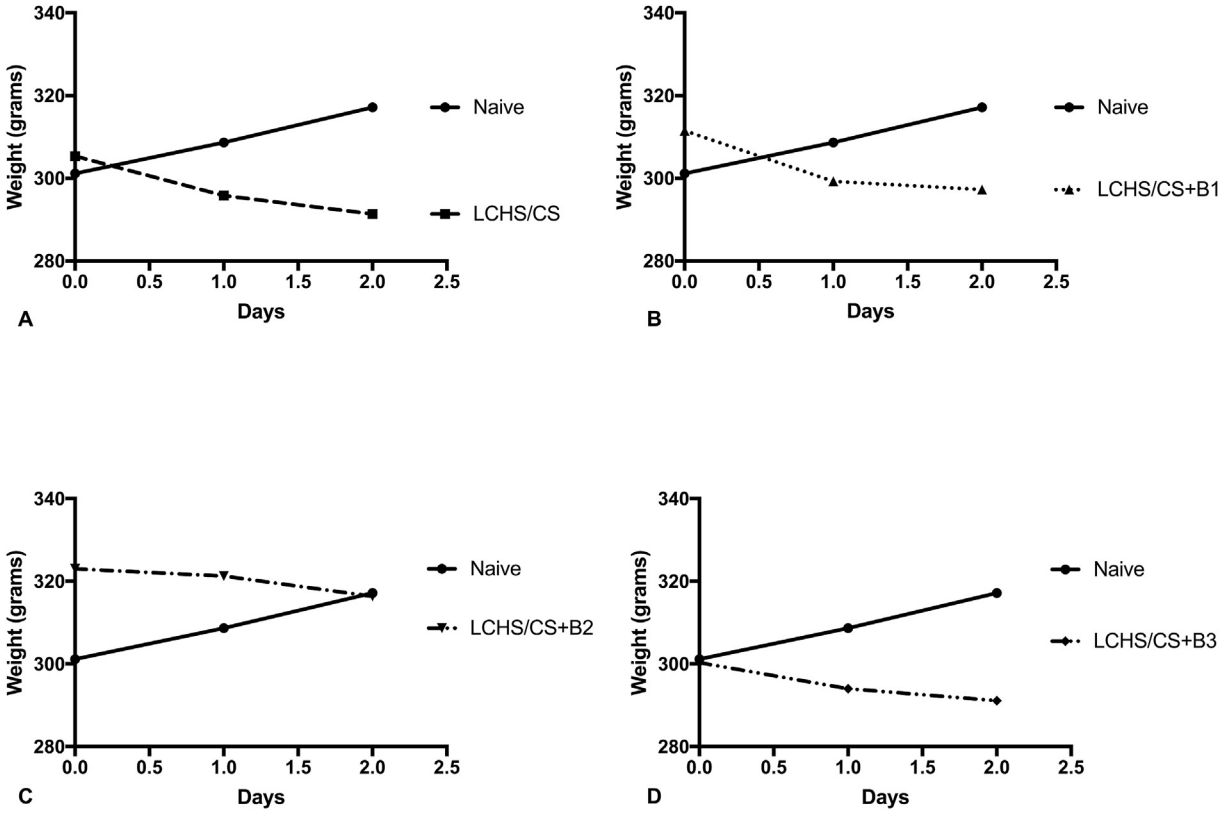

Fig. 4. D. The effects of selective beta blockade on weight loss following traumatic injury. 4A. Naïve rodents gained weight over the first two days while LCHS/CS rodents lost weight at a significant rate. **4B.** Rodents treated with beta-1 blockade lost weight at a similar weight as LCHS/CS. **4C.** Rodents treated with beta-2 blockade lost weight at a slower rate than LCHS/CS or beta-1 groups. **4D.** Rodents treated with beta-3 blockade lost weight at a slower rate than LCHS/CS or beta-1 groups. LCHS/CS = lung contusion hemorrhagic shock/chronic stress; B1B = selective beta-1 blockade; B2B = selective beta-2 blockade; B3B = selective beta-3 blockade.

et al.[7,8] has previously shown that pathologically high doses of norepinephrine suppresses bone marrow BFU-E and CFU-E growth, and propranolol treatment attenuated these effects. Our findings are supported by Muthu et al.[27] who demonstrated that the early commitment stage of erythropoiesis (BFU-E growth) is beta-2 receptor dependent and Beckman et al.[28] who found that BFU-E colonies were more sensitive to beta-2 selective medication than CFU-E colonies. In addition, only beta-3 adrenergic blockade improved late stage erythropoiesis which correlates with our finding that only beta-3 adrenergic blockade improved CFU-E growth.[27] Similarly, Beiermeister et al.[11] utilized a rodent lung injury model to demonstrate that selective beta-2 and beta-3 adrenergic blockade improved both BFU-E and CFU-E growth following lung contusion alone.

The use of beta-1, beta-2 and beta-3 adrenergic blockade following LCHS/CS significantly decreased plasma G-CSF, while only beta-2 adrenergic blockade significantly reduced hematopoietic progenitor cell mobilization. Several studies have shown that increased plasma levels of G-CSF led to increased hematopoietic progenitor cell mobilization from the bone marrow to the peripheral blood.[13,29,30] More focused work into the mechanism of this association has found that increased G-CSF led to the degradation of transcription factor Sp1, which is associated with down-regulation of CXCL-12 and hematopoietic progenitor cell mobilization.[19] Méndez-Ferrer et al.[19] found that mobilization is cooperatively mediated by beta-2 adrenergic receptors on osteoblasts and beta-3 adrenergic receptors on stromal cells. In absence of beta-2 and beta-3 adrenergic receptors, there is significantly compromised mobilization to the bloodstream.[19] However, norepinephrine and epinephrine mediated activation of only beta-2 adrenergic receptors on human $CD34^{+}$ hematopoietic progenitors which promoted their migration, proliferation and mobilization.[19] Although all selective antagonists decreased plasma G-CSF in the current study, only beta-2 antagonism reduced actual hematopoietic progenitor cell mobilization which is supported by Hasan et al.[17] findings that hematopoietic progenitor cells are beta-2 receptor dependent. Therefore, both beta-2 and beta-3 adrenergic receptors likely participate in hematopoietic progenitor cell mobilization, but it is likely there are separate beta-2 and beta-3 adrenergic receptor pathways that result in distinct of biologic functions.

As expected, naïve rodents gained weight daily, while LCHS/CS and beta-1 adrenergic blockade groups lost weight at a significant rate following injury. The use of beta-2 and beta-3 adrenergic blockade following LCHS/CS led to a slower rate of weight loss. The hypercatecholamine and hypermetabolic state correlated with persistent injury-associated anemia is also associated with catabolism and weight loss.[31] Douris et al.[32] negated the hypercatecholamine effect by using mice lacking all beta receptors, that ultimately had increased adipose tissue and weight gain. Douris et al.[33] also demonstrated that mice who ate a low carbohydrate diet had an increased metabolic rate associated with weight loss and that fibroblast growth factor 21 mediated these changes by activating the sympathetic nervous system. In a review of post-burn hypermetabolism pharmacology, the use of propranolol titrated to 20% reduction in baseline heartrate in severely burned patients, improved lean body mass leading to a positive net balance of protein.[34] While the current study is not specific in determining which beta adrenergic receptors are associated with weight changes, Mersmann et al.[35] found that more than 85% of the beta

adrenergic receptors in rat adipose tissue are beta-3 adrenergic receptors. This suggests that it is the beta-3 adrenergic receptor that plays a key role in weight change.

There are limitations of the present study. This study only examined the effects of selective adrenergic blockade on day seven after injury. Previously, the authors have studied the use of propranolol, a non-selective beta-blocker, and while no hypotension was found with its use, they are some concerns with its use early after resuscitation.[5] Also, adaptive mechanisms of chronic beta-adrenergic blockade such as potential desensitization of receptors were not captured in this study. Also, similar studies in beta-1, beta-2 and beta-3 knockout mice would aid in further investigation of bone marrow dysfunction following trauma. One other limitation is that SR590230A is not approved for human use which impedes further translational study of this drug.

Conclusion

In summary, selective beta-2 and beta-3 adrenergic blockade appear to be most effective in improving persistent injury-associated anemia, erythroid colony growth, bone marrow cellularity and reducing hematopoietic progenitor cell mobilization following trauma and chronic stress. Beta-2 and beta-3 receptors are expressed at opposite ends of the erythropoiesis timeline, and in varying stromal cells and tissues. Attenuating the neuroendocrine response with selective beta-2 and beta-3 adrenergic blockade may be a safer alternative relative to propranolol to improve bone marrow dysfunction following severe traumatic injury given that selective blockade may have less hemodynamic side effect as compared to propranolol. Further research needs to be done regarding the effects of selective beta blockade on hematopoiesis during homeostasis and times of stress.

Funding

The authors were supported in part by grants R01 GM113945-01 (PAE), R01 GM105893-01A1 (AMM) and P50 GM111152–01 (PAE and AMM) awarded by the National Institute of General Medical Sciences (NIGMS). ESM was supported by a post-graduate training grant (T32 GM-008721) in burns, trauma and perioperative injury by NIGMS.

Declaration of competing interest

All authors contributed significantly to all aspects of manuscript production and the authors have no conflicts to disclose. ESM, CGA, KBK, PAE and AMM contributed to the literature review and study design. ESM, CGA, ZMF and KBK contributed to data collection. ESM, CGA, ZMF and AMM contributed to data analysis. ESM, AMM drafted the manuscript. PAE and AMM provided critical revisions.

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