

DIMETHYL SULFOXIDE INDUCES HEMOLYSIS AND PULMONARY HYPERTENSION

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ABSTRACT

Vascular and lung injury are well established complications associated with hemolytic disorders, and hemolysis associated pulmonary hypertension (PH) has emerged as the most serious complication of sickle cell disease. The causal relationship between intravascular hemolysis and the development of PH is still under investigation. Previously we have shown that repetitive administration of hemolyzed autologous blood causes PH in rats. Dimethyl sulfoxide (DMSO), a widely used solvent and anti-inflammatory agent, induces hemolysis in vivo. We hypothesized that repetitive administration of DMSO would induce PH in rats. We also examined hemolysis-induced release of adenosine deaminase (ADA) and arginase from red blood cells, which may amplify hemolysis-mediated vascular injury. Acute administration of DMSO (1.5ml/30 min into the right atrium) induced intravascular hemolysis and pulmonary vasoconstriction. DMSO-induced increase in right ventricular peak systolic pressure (RVPSP) was associated with increased release of ADA. Notably, the acute increase in RVPSP was attenuated by administration of an adenosine A2A receptor agonist or by pretreatment of animals with ADA inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA). Repetitive administration of DMSO for 10 days produced anemia, hemoglobinuria, hemoglobinemia, splenomegaly, and development of PH. Histopathological analysis revealed pulmonary vascular remodeling. The presented data describe a new model of hemolysis induced PH, suggesting that hemolysis is mechanistically related to pulmonary hypertension, and pointing to a potential pathogenic role that adenosine deaminase and accelerated adenosine metabolism may play in hemolysis associated pulmonary hypertension.

Keywords: hemolysis, pulmonary hypertension, adenosine, adenosine deaminase

INTRODUCTION

Vascular and lung injury are well-established complications of chronic hemolytic disorders

[1–4]; one such complication is pulmonary vascular injury and the remodeling which lead to

the development of pulmonary hypertension (PH). Importantly, PH has emerged as the most serious complication of hemolytic disease, including sickle cell disease (SCD), thalassemia, hereditary spherocytosis and paroxysmal nocturnal hemoglobinuria [5–8]. Recent studies report a 6–10 % prevalence of PH in SCD patients [9–11] and 2.1 % [12] in patients with thalassemia, compared to 0.25% in the general population [13]; moreover, in adult patients with hemolytic disorders, development of PH is associated with poor prognosis and worsened mortality. Compared to patients with idiopathic PH, patients with hemolysis-associated PH develop less severe elevation of pulmonary arterial pressure; yet, in SCD patients, this form of PH, even when of mild or moderate severity, has been identified as the single greatest risk factor for death [5, 9–11, 14]. Finally, because SCD and thalassemia are the most common monogenetic diseases worldwide [15], hemolytic anemia is emerging as a major cause of PH.

The increasing number of studies in animals and humans suggest that hemolysis is directly involved in the pathophysiology of PH in patients with hemolytic disease. In a mouse model of SCD, all animals develop PH even in the absence of in situ thrombosis or vaso-occlusive events [16]. In patients with SCD, elevated pulmonary pressure closely correlates with all markers of hemolytic anemia [5]. Similarly, in patients with idiopathic PH, levels of free hemoglobin are significantly elevated, compared to healthy controls, and they significantly correlate with pulmonary arterial

pressure, pulmonary arterial resistance, cardiac index, and functional class [17]. Furthermore, SCD patients who experience hyperhemolytic syndrome develop PH more frequently and die sooner than patients with less extensive hemolytic events [18]. These data suggest that hemolysis is mechanistically related to PH.

Several mechanisms could potentially contribute to hemolysis-induced PH. During intravascular hemolysis, hemoglobin and arginase from red blood cells (RBCs) are released into plasma. The cell-free hemoglobin reacts with and inactivates endothelium-derived NO, limiting vascular NO bioavailability [19], whereas free arginase shifts arginine metabolism toward ornithine and polyamine production, decreasing the amount of arginine available for NO synthesis [20]. These two events lead to subsequent severe NO deficiency, rapid endothelial barrier dysfunction [21], oxidative injury, and vascular inflammation and proliferation, eventually resulting in the development of PH and proliferative vasculopathy in adulthood [3]. In addition to being rich in hemoglobin and arginase, RBCs are also richly endowed with adenosine deaminase (ADA) [22] and therefore hemolysis would be expected to release significant amounts of ADA from injured RBCs into plasma. A relatively small molecule [23], ADA can pass between endothelial cells, i.e., escape from the circulation, and reach the extracellular space and perivascular tissue. Adenosine deaminase converts adenosine (ADO) to inosine, and therefore, could accelerate ADO metabolism and

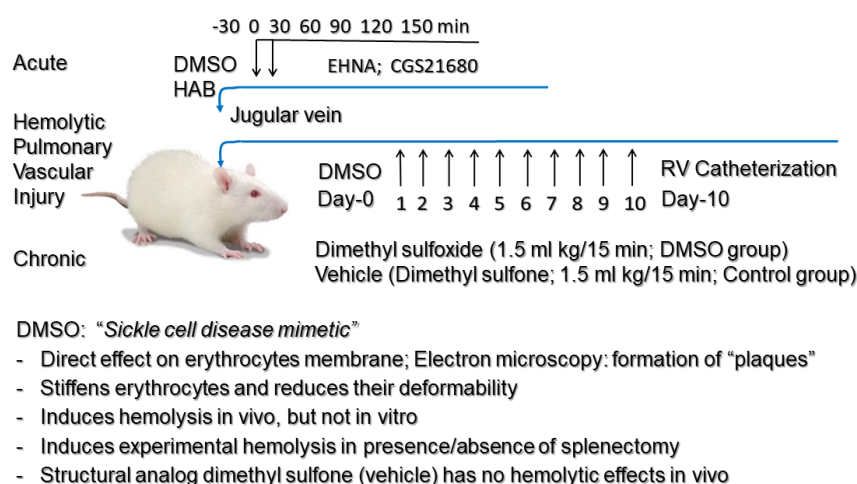


Figure 1. Outline of experimental protocols and relevant pharmacological properties of dimethyl sulfoxide (DMSO); HAB- hemolyzed autologous blood; EHNA - erythro-9-(2-hydroxy-3-nonyl) adenine, an adenosine deaminase inhibitor; CGS21680 – adenosine A_{2A} receptor agonist.

thereby reduce extracellular ADO levels in diseases involving intravascular hemolysis. Indeed, our previous work suggests that injured RBCs release ADA [24–27]. Furthermore, in both rats receiving hemolyzed autologous blood and in SCD patients, we detected an increased ADA activity and accelerated adenosine metabolism [28]. Adenosine, by interacting with cell surface receptors, induces myriad biological effects that in aggregate may produce vasoprotective and anti-occlusive effects [24, 26].

Dimethyl sulfoxide (DMSO) is a common biological solvent and drug vehicle [29]. Studies from the 1960s [30] showed that when administered intravenously, DMSO is a potent hemolytic agent. A more recent study has reported on the toxic effects of DMSO on endothelial and red blood cells and platelets [31, 32], all three key players in the chain of cellular events in SCD that ultimately lead to vaso-occlusion and multiple organ damage. Given its effects on RBCs [24–27], [29–32], DMSO could be viewed as a “sickle cell disease-mimetic” (Figure 1).

In the present study, we used the hemolytic agent DMSO to determine whether hemolysis is mechanistically related to PH and pulmonary vasculopathy in rats. In addition to testing the hypothesis that hemolysis directly causes PH, we also investigated the ADA-adenosine pathway that could be involved in hemolysis-induced PH by determining whether hemolysis is associated with release of ADA from RBCs and whether it correlates with changes in extracellular adenosine levels. Finally, we examined the effects of hemolysis on the aforementioned “arginase pathway”, a major pathogenic pathway involved in hemolytic vasculopathy and SCD.

MATERIAL AND METHODS

Animals

A total of 57 male, 10-12-week-old Sprague Dawley rats were purchased from Charles River (Wilmington, MA). The animals were allowed to acclimatize for 1 week before inclusion into the study. All experimental protocols were approved by the University of Pittsburgh Animal Care and Use Committee, and all experiments were conducted in accordance with the university guidelines for animal welfare.

Protocol 1: DMSO- and Hemolysis- Induced Acute Pulmonary Vasoconstriction

For acute administration of DMSO, animals were anesthetized with pentobarbital (45 mg/kg intraperitoneal) and placed on a Deltaphase Isothermal pad (Braintree Scientific, Braintree, MA) to maintain body temperature at $37 \pm 0.5^\circ\text{C}$. A PE-240 polyethylene catheter was inserted into the trachea to facilitate breathing, and a PE-50 catheter was inserted into the left carotid artery and connected to a digital blood pressure analyzer (BPA, Micro-Med. Inc., Louisville, KY) for continuous measurements of systolic, diastolic and mean arterial blood pressure and heart rate. The left jugular vein was exposed and cannulated with a PE-50 catheter for delivery of DMSO. Next, the right jugular vein was exposed and a miniature pressure transducer catheter (SPR-513, Millar Instruments, Houston, TX) was connected to a digital heart performance analyzer (PowerLab 400, ADInstruments, Colorado Springs, CO). This was advanced into the right ventricular cavity via the right jugular vein and right atrium. A 30-minute stabilization period was permitted before a 150-minute recording of right ventricular peak systolic pressure (RVSP) was made. DMSO infusion (50 $\mu\text{l}/\text{min}$) was initiated at 30 min and stopped at 60 min. Some animals were pretreated with ADA inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA; 10mg/kg). In order to confirm that the effects of DMSO are due to hemolysis, and assess the relative hemolytic potency of DMSO, a subset of animals received hemolyzed autologous blood (HAB; 1.5ml/30 min). The acute effects of DMSO and HAB on plasma free hemoglobin and pulmonary and systemic circulation were compared. Some of the DMSO or HAB animals were infused with CGS 21680 (0.5mg/min), a selective and stable adenosine A2A receptor agonist.

Protocol 2: DMSO-Induced Pulmonary Hypertension

For chronic administration of DMSO, under isoflurane anesthesia, the right jugular vein was exposed, completely dissected from surrounding tissue, and a PE-50 catheter that was filled with 10% heparin solution was inserted and advanced to the right atrium. The catheter was tunneled subcutaneously to the back of the neck. After the rats regained consciousness, they could move freely in the cage and had free access to food and tap water. Animals were allowed to recover

for 24 hours, before beginning daily infusions of DMSO. For ten days, under isoflurane anesthesia, animals received twice-daily infusions of DMSO (0.5ml/15 minutes). One day after the last DMSO infusion, animals were anesthetized (pentobarbital, 45 mg/kg i.p.) and instrumented for open-chest measurement of RVPSP, as described previously. [33] Briefly, a PE-240 polyethylene catheter was inserted into the trachea to facilitate breathing. A PE-50 catheter was inserted into the left carotid artery and connected to a digital blood pressure analyzer (BPA, Micro-Med. Inc., Louisville, KY) for continuous measurements of blood pressure and heart rate. The rats were then mechanically ventilated (Harvard Rodent Ventilator, Model 683, Harvard Apparatus, MA) using a constant breath rate (50/min) and tidal volume (1.0 ml). The thorax was opened, and the right heart was punctured with a 23-gauge needle attached to a PE-50 line and Heart Performance Analyzer (HPA-200 τ , Micro-Med. Inc., Louisville, KY). After a 30-min stabilization period, RVPSP was recorded over 30 min. Animals were euthanized by overdose of anesthetic (pentobarbital 200 mg/kg) and the heart and lungs were dissected and weighed. The right ventricle (RV) free wall was separated from the left ventricle (LV) and the septum (S), and the Fulton index (RV/LV+S ratio) was calculated. The lung was perfused with 10% buffered formalin under constant low pressure (25 mmHg), immersed in 10% buffered formalin and later embedded in paraffin.

Protocol 3: Effects of hemolysis on adenosine metabolism in vitro

Blood (3ml per rat) was collected from four donor rats and anticoagulated (25 μ l heparin/ml blood). Blood was frozen in liquid nitrogen and thawed three times. The hemolyzed blood was centrifuged (10,000 RPM for 10 min) to obtain clear, grossly hemolyzed plasma. Plasma samples were split in to identical 0.4ml aliquots. A 0.4ml portion of 3.5 mM adenosine solution was added to each aliquot (final adenosine solution 1.75mM). One aliquot from each sample was added to 20 μ l of erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) solution (final EHNA concentration 15 μ M). The other aliquot received 20 μ l of saline (vehicle). A 50 μ l portion was removed from each aliquot (time=0) and then all samples were incubated in a 37C water bath. A 50 μ l portion was removed from all samples at 10, 20, 40 and 60 minutes after the start of incubation. All 50 μ l portions were flash

frozen in liquid nitrogen immediately after removal and stored at -80C. The frozen samples were later placed into boiling water for 2 minutes, and the cooled heat-inactivated samples were treated with 150 μ l of ice-cold acetonitrile. Samples were centrifuged and supernatant was removed, taken to dryness, and reconstituted with 200 μ l water for adenosine and inosine analysis.

Protocol 4: Effects of L-Arginine on HAB-Induced Changes in Systemic, Pulmonary and Renal Circulation

Animals were anesthetized and instrumented for measurement of RV pressures and systemic blood pressures, as described in Protocol 2. Next, an abdominal midline incision was made and an ultrasonic flow probe (Model 1RB; Transonic Systems, Inc., Ithaca, NY, USA) was placed on the left renal artery for measurement of renal blood flow (RBF). Renal vascular resistance (RVR) was calculated. An intravenous infusion of saline (50 μ l/min; control group) or L-arginine (50 mg/kg + 1.66 mg/kg/50 μ l/min; L-Arginine group) into the right jugular vein was initiated. After 30 minutes, HAB infusion was initiated and changes in systemic blood pressure, RVPSP, and renal blood flow were monitored for 60 minutes.

Measurement of Adenosine, Inosine, ADA activity and Plasma Hemoglobin and Nitrite

Adenosine and inosine levels in vitro in hemolyzed rat blood adenosine metabolism experiments were measured by LC-MS/MS using the Thermo Electron TSQ Quantum-Ultra system as described previously [34]. Cell-free plasma hemoglobin and nitrite levels were measured as described previously [35, 36]. Plasma ADA activity was measured with a commercially available kit (Dyazyme Laboratories, Poway, CA).

Measurement of Arginase Activity

Rat plasma, hemolyzed rat plasma, and rat RBCs, as well as lung tissue collected from control animals and rats exposed to single dose (HAB 120 min) or 10 daily doses of HAB (HAB Day 10) were analyzed for arginase activity. Arginase activity in plasma and tissue homogenates was determined as the conversion of [^{14} C-guanidino]-L-arginine (American Radiolabeled Chemicals or Moravsek) to [^{14} C]urea (2.5-hr incubation), which was then converted to ^{14} CO $_2$ by urease and trapped as Na ^{214} CO $_3$ and counted on a scintilla-

tion counter as described previously [37]. Protein concentrations were determined by the BCA protein assay kit (Pierce) and used to calculate lung arginase specific activities. Plasma arginase activity was expressed as activity per volume plasma (nmol/ml/min).

Histopathological Analysis

Five-micrometer serial tissue sections from paraffin embedded lungs were deparaffinized and stained with hematoxylin and eosin (H&E), and Verhoeff's VanGieson (VVG) for histological and morphometric assessment. The Aperio ScanScope XT system (Aperio Inc., Vista, CA, USA) was used to scan entire glass slides at 20x or 40x magnification and the Aperio Image Scope software was used to analyze digital slides. Histopathological analysis of lung injury was performed on H&E slides by using a modified scoring system, as previously described [38, 39]. Five criteria were used to score lung injury: (i) infiltration or aggregation of inflammatory cells in alveolar spaces; (ii) hemorrhage (i.e., presence of RBCs or hemolyzed blood in alveolar spaces); (iii) alveolar wall thickening; and (iv) perivascular inflammation. Sections were scored as follows: 0, absent; 1, mild (<25% lung involvement); 2, moderate (25–50% lung involvement); 3, severe (50–75% lung involvement); 4, very severe (>75% lung involvement).

For assessment of medial remodeling, 6–8 vessels in the peripheral lung fields were located at approximately equal distances from the pleural lining. Only vessels with an approximately circular profile (cross-sectional cuts) were studied. For each blood vessel, luminal diameter and media thickness were measured along two perpendicular lines (diameter) through the center of the vessel (Figure 10-A). The media % index was calculated as: $2 \times \text{media}/\text{diameter} \times 100$; and is presented as the average of four medial measurements for a single vessel. DMSO induced a significant widening of the adventitia only in a small number of vessels which did not permit consistent measurement of adventitial surface area in all examined vessels and these data are not presented. The vessel size was calculated using the diameter that includes only media+lumen measurement. The diameters and respective surface areas were measured by the Java-based image processing program ImageJ.

Statistical Analysis

All data are presented as mean $\pm$ standard error of the mean. Statistical analysis was performed using the Number-Crunchers Statistical System software, and significance was defined as $p < 0.05$. Comparisons among multiple groups were performed by a 1-factor analysis of variance. If this analysis indicated a significant difference among the means, multiple comparisons were made with a post-hoc Fisher's least significant difference test. A Student t-test was used to compare specific pairs of groups determined a priori to be of particular importance.

RESULTS

Single 30-minute intravenous infusions of DMSO induced intravascular hemolysis as evidenced by dramatic increases in plasma free hemoglobin (Figure 2A). Plasma hemoglobin returned to baseline levels within 6 hours and the half-life of mono-exponential decrease of plasma hemoglobin was 53 min (Figure 2B). Administered doses of DMSO and HAB (1.5ml each) had similar "hemolytic potency" expressed as plasma concentration, half-life, and area under the curve (AUC) of free hemoglobin (Figure 2 A&B). DMSO induced acute pulmonary vasoconstriction (i.e., increased RVPSP; Figure 3A&3E) and had no effect on systemic blood pressure (Figure 3B&3F). HAB induced much greater increase in RVPSP (Figure 3C & 3E) and increased MABP (Figure 3D&3F). Pretreatment with the adenosine deaminase inhibitor EHNA nearly abolished DMSO-induced vasoconstriction in pulmonary circulation, as evidenced by minimal changes in RVPSP after DMSO administration (Figure 4A & 4B). Furthermore, pretreatment with EHNA attenuated the DMSO-induced drop in hematocrit (Figure 4C). Notably, CGS21680 (selective adenosine A2A receptor agonist) attenuated DMSO- and HAB-induced pulmonary vasoconstriction (Figure 4D & E). In hemolyzed rat blood there was rapid in vitro adenosine metabolism over 60-min incubation period (Figure 5), with 75% of adenosine being converted to inosine. Here again, pretreatment with EHNA completely prevented the time-related conversion of adenosine to inosine (Figure 5). This indicates that the observed rapid adenosine metabolism was due to RBC-derived ADA.

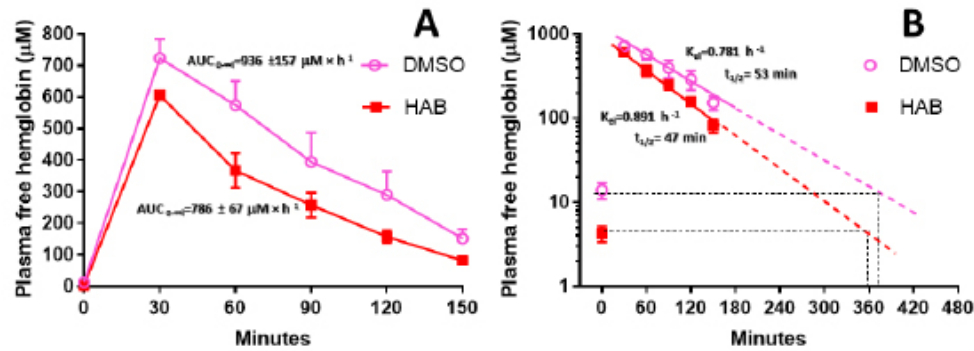


Figure 2. Relative in vivo hemolytic potency of dimethyl sulfoxide (DMSO; 1.5ml/30min i.v.; $n=6$) compared to hemolyzed autologous blood (HAB; 1.5ml/30 min i.v.; $n=6$) expressed as plasma free hemoglobin.

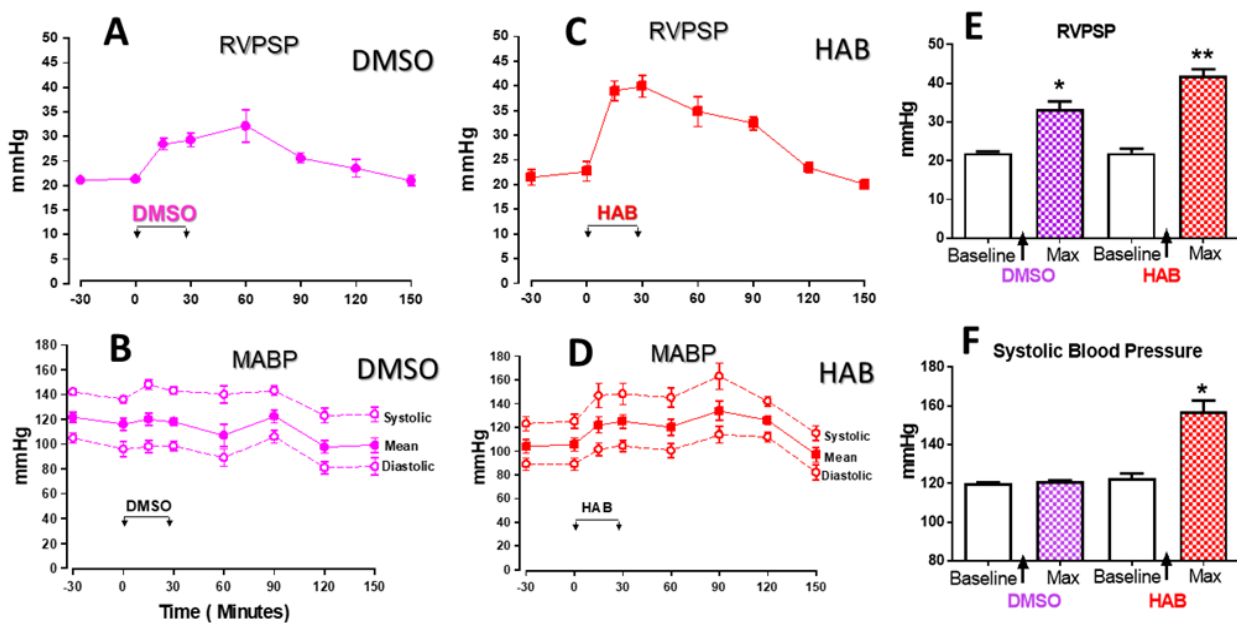


Figure 3. The effects of dimethyl sulfoxide (DMSO; $n=6$) and hemolyzed autologous blood (HAB) on right ventricular peak systolic pressure (RVPSP; $n=6$) and mean arterial blood pressure (MABP)

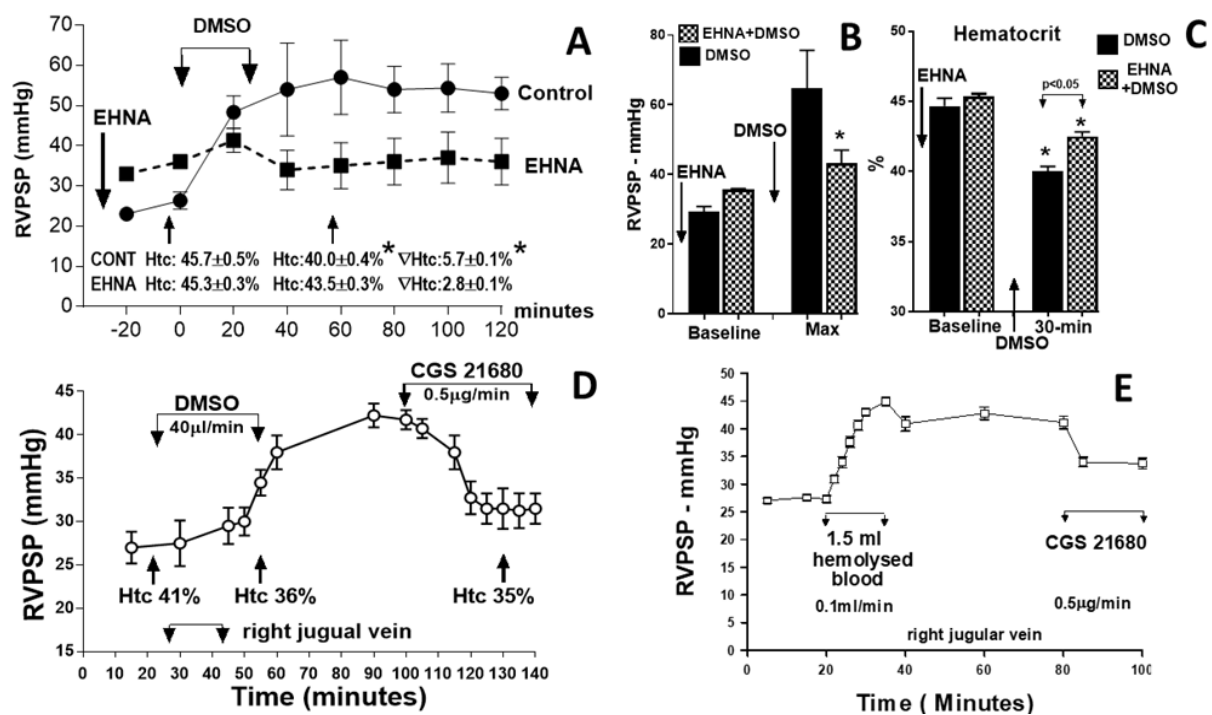


Figure 4. Pretreatment with adenosine deaminase inhibitor EHNA attenuates dimethyl sulfoxide [DMSO]-induced pulmonary vasoconstriction (A & B) and reduces DMSO-induced anemia (C); Treatment with selective adenosine A_{2A} receptor agonist CGS21680 attenuates pulmonary vasoconstriction, i.e., reduces the increase in right ventricular pressure induced by DMSO (D) or hemolyzed autologous blood [HAB] (E);

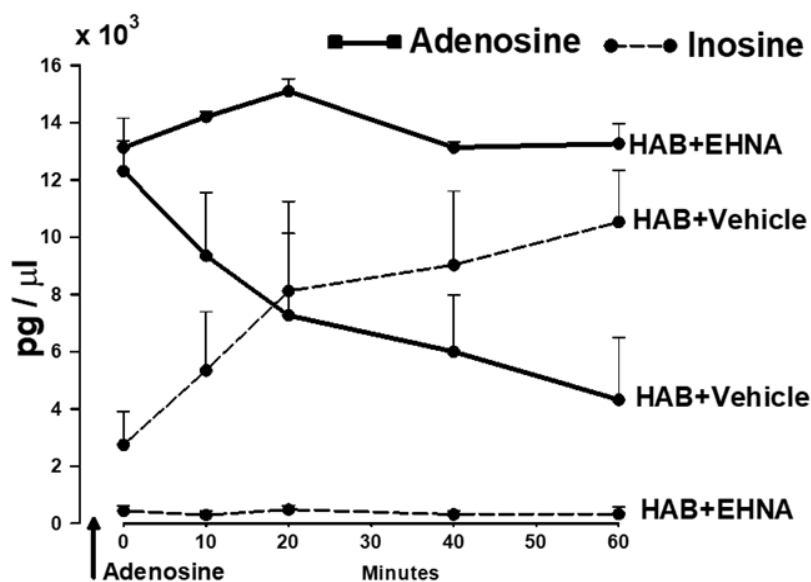


Figure 5. Erythrocytic adenosine deaminase (ADA) accelerates adenosine metabolism. - Plasma adenosine and inosine levels in hemolyzed blood in the presence [HAB+EHNA] and absence of adenosine deaminase inhibitor EHNA [HAB+Vehicle].

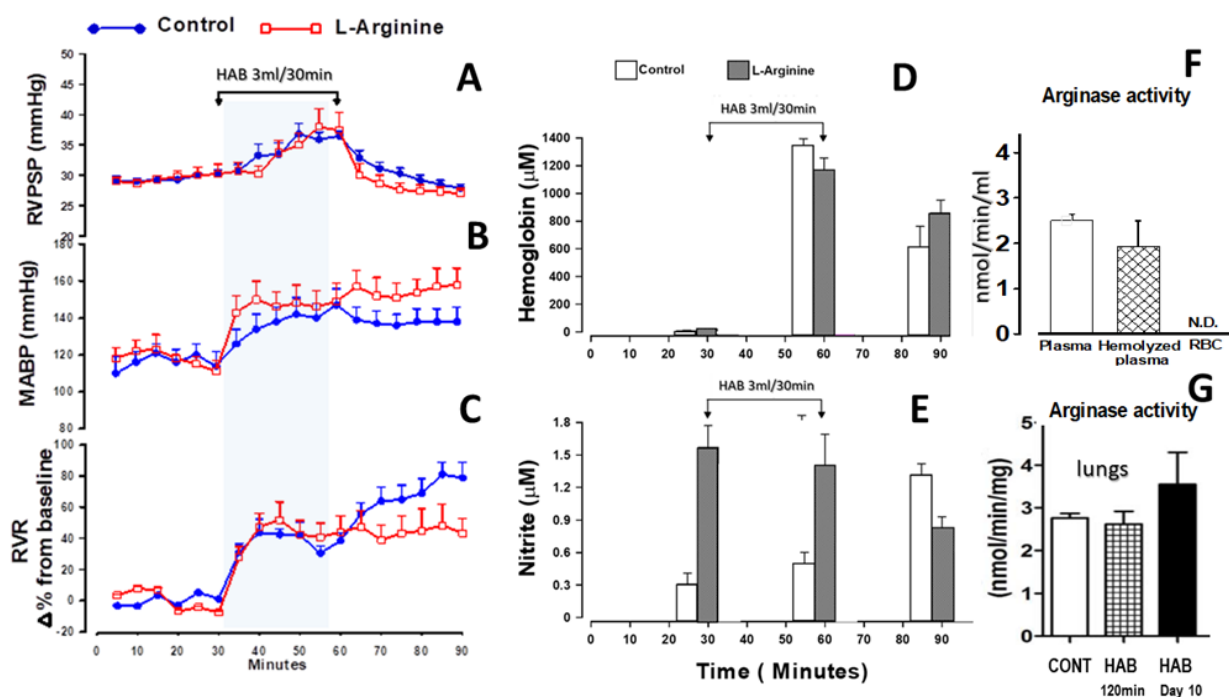


Figure 6. The effects of L-arginine on DMSO-induced changes in (A) right ventricular peak systolic pressure (RVSP), (B) mean arterial blood pressure (MABP) and (C) renal vascular resistance (RVR); (D) Plasma free hemoglobin and (E) plasma nitrite levels before and during hemolytic insult; (F) Arginase activity in red blood cells and control and hemolytic plasma; (G) Arginase activity in rat lung tissue after single dose (HAB 120min) or 10-day treatment with hemolyzed blood (HAB Day 10 group).

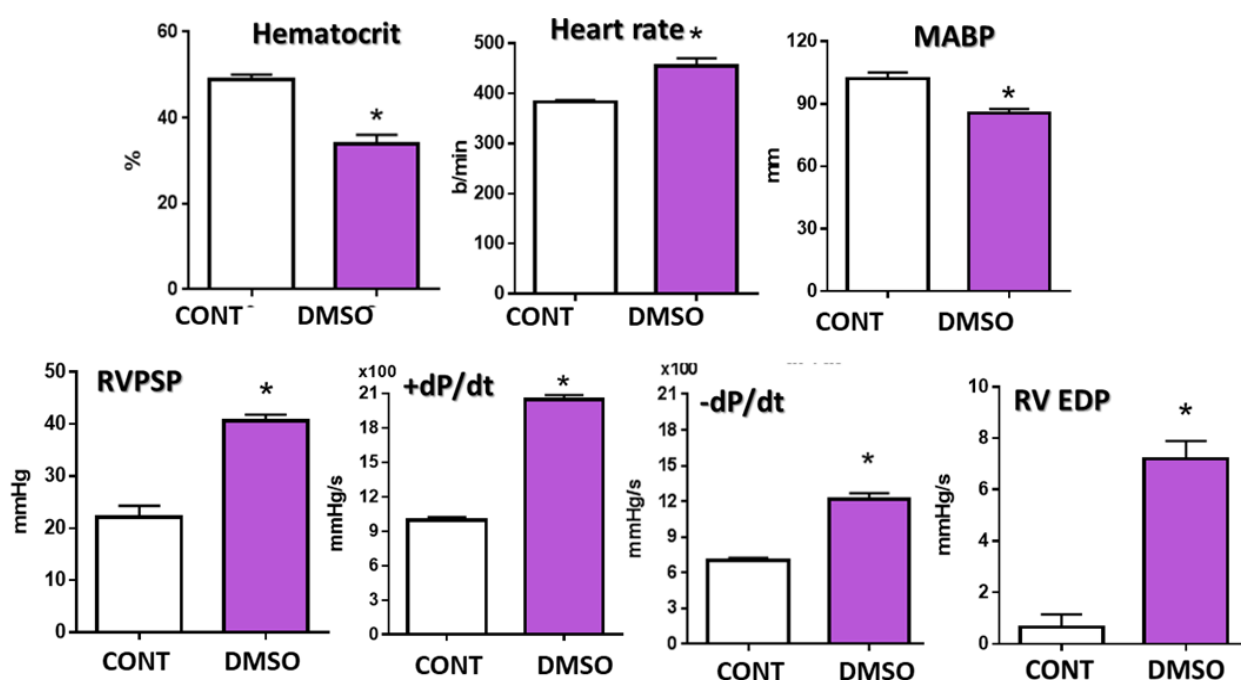


Figure 7. DMSO-induced repetitive hemolysis over 10-day period leads to development of anemia, pulmonary hypertension and ensuing right ventricular failure, i.e., reduces hematocrit and increases right ventricular peak systolic pressure (RVSP) and RV end diastolic pressure (RV EDP).

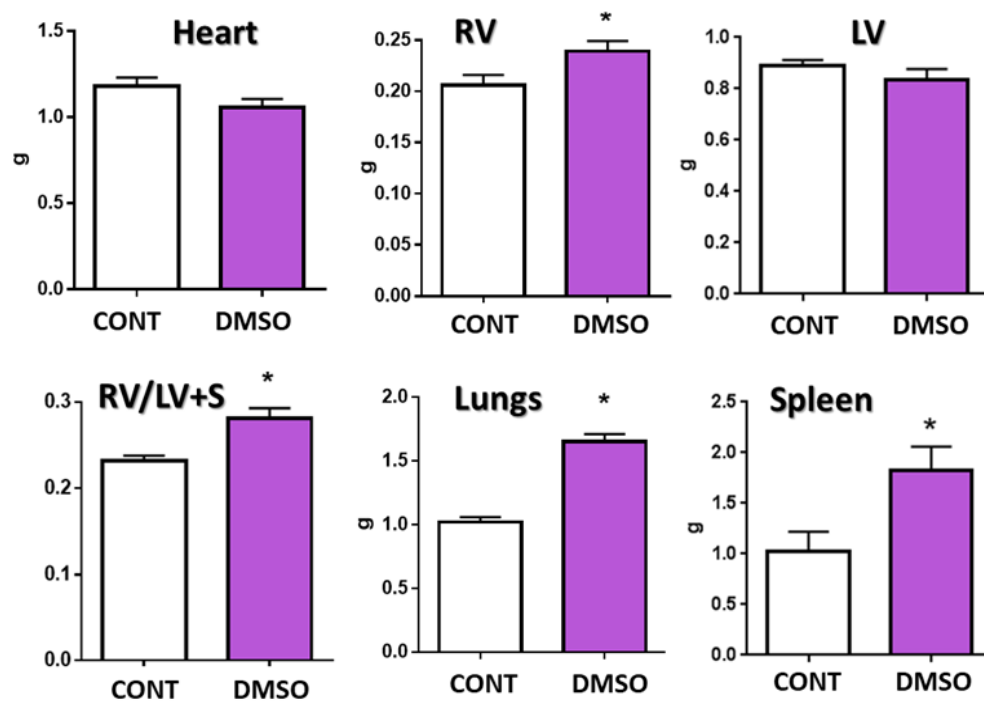


Figure 8. DMSO-induced repetitive hemolysis over 10-day period leads to development of splenomegaly and isolated right ventricular hypertrophy

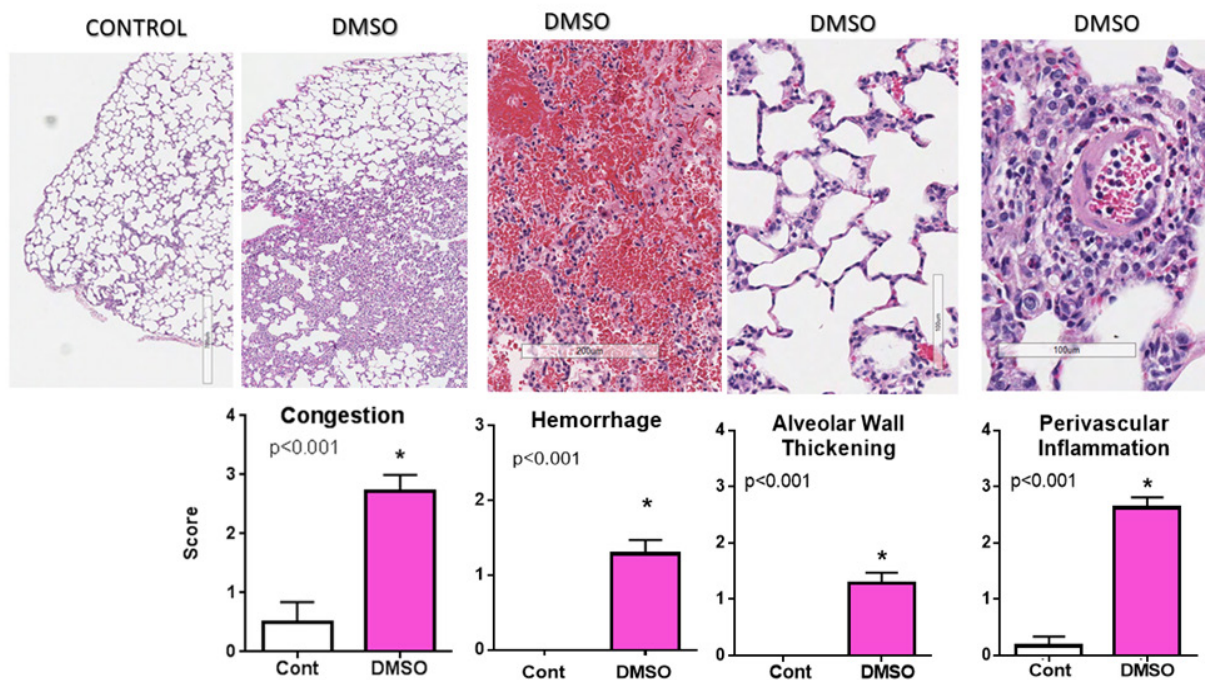


Figure 9. DMSO-induced repetitive hemolysis over 10-day period leads to development of moderate and unevenly distributed lung injury in the form of pulmonary congestion, hemorrhages, alveolar wall thickening and perivascular inflammatory infiltrates (H&E staining).

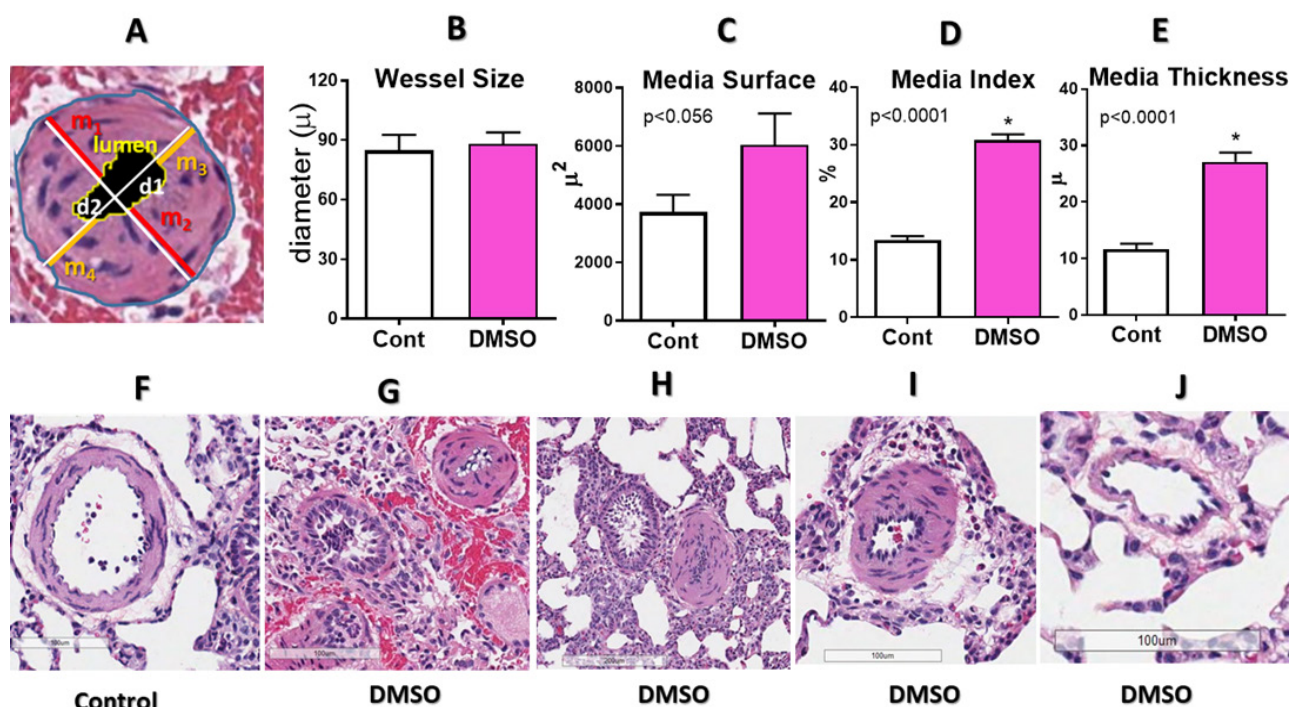


Figure 10. DMSO-induced repetitive hemolysis over 10-day period leads to vascular remodeling characterized by medial hypertrophy of medium and small size pulmonary arteries (C-E & G-I). Presence of hemorrhages (G) or inflammatory cells infiltrates (H & I) in proximity of remodeled vessels with occasional prominent endothelial cells (Figure 10J); H&E staining.

Pretreatment with L-arginine and its administration during the hemolytic episode, (i.e., HAB infusion) had no effect on HAB-induced pulmonary vasoconstriction nor the increase in systemic blood pressure and renal vascular resistance (Figure 6A-6C). Of note, L-arginine treatment resulted in a 2- to 5-fold increases in nitrite levels (Figure 6E). Control plasma and plasma from hemolyzed blood samples had similar arginase activity, and no arginase activity was detected in lysates of hemolyzed rat RBCs (Figure 6F). Regarding lung tissue, arginase activity in the control lungs was similar to that in lungs analyzed 120 minutes after administration of a single dose of HAB (Figure 6F) and lung arginase activity was comparable to enzyme activity in control and hemolytic plasma (Figure 6G vs. 6F). In rats exposed to a 10-day treatment with HAB, arginase activity tended to be higher compared to the control group and HAB-120 min groups (Figure 6G).

Daily administration of DMSO into the right atrium for 10 days induced anemia and hemoglobinuria, and compared to the control group, the DMSO group had significantly reduced blood pressure and increased heart rate

(Figure 7, top panels). DMSO treated animals developed moderate PH and had increased RV EDP (Figure 7, bottom panels), consistent with isolated RV hypertrophy and suggestive of impending RV failure. Morphometric analysis revealed splenomegaly, increased lung weight and, as predicted by hemodynamic parameters, isolated right ventricular hypertrophy (Figure 8). Histopathological semi-quantitative analysis revealed significant patchy lung injury in the form of moderate to severe congestion, perivascular inflammation, mild to moderate airspace hemorrhage, and alveolar wall thickening (Figure 9). In DMSO treated animals, vascular remodeling was characterized by medial hypertrophy of medium and small size pulmonary artery branches (Figure 10C-E & G-I), occasional prominent endothelial cells (Figure 10J) were detected. The changes did not affect all vessels in a given animal. Some remodeled vessels showed scattered perivascular inflammatory infiltrates (Figure 10H & I) or hemorrhages (Figure 10G), while others did not (Figure 10J). The inflammatory infiltrates were also seen in unaffected vessels of treated animals (not shown).

DISCUSSION

In the present study, we used DMSO to induce hemolytic vascular injury. Previously we have shown that hemolyzed autologous blood (HAB) acutely induces pulmonary vasoconstriction and that chronic treatment with HAB produced hemodynamic and histopathological PH [28]. Therefore, before conducting chronic experiments, we compared the “hemolytic potency” of DMSO to that of HAB. DMSO and HAB induced similar hemolytic insults, as measured by plasma concentrations, half-life ($t_{1/2}$) and area under the curve (AUC) of the released hemoglobin (Figure 2). Hemoglobin was cleared from the circulation within six hours of administration of DMSO or HAB. Although DMSO and HAB induced similar hemolytic insults, DMSO produced less severe pulmonary vasoconstriction and, in contrast to HAB, DMSO did not acutely increase systemic blood pressure. It is noteworthy that besides the precipitation of intravascular hemolysis, DMSO has a multitude of effects that may account for its overall vascular effects and are relevant to our findings; notably it is a potent reactive oxygen species scavenger and has anti-inflammatory and anti-fibrotic effects in experimental models and several diseases in humans [29, 40]. DMSO is the only FDA approved treatment for intravesical instillation in patients with classic interstitial cystitis [41, 42]. Furthermore, DMSO inhibits human platelet aggregation induced by ADP or collagen [29]. As intravascular hemolysis is associated with significant oxidative stress, inflammatory response, platelet aggregation and thrombosis, it is therefore plausible that through these effects DMSO may offset some of the adverse effects of repetitive hemolysis and result in only mild PH and pulmonary vascular injury.

The fact that CGS21680, a stable and selective A_{2A} receptor agonist attenuated DMSO- and HAB-induced increase in RVPSP, suggests that A_{2A} signaling opposes hemolytic pulmonary vasoconstriction and that accelerated adenosine metabolism and reduced A_{2A} signaling may have detrimental effects in hemolytic disease. Notably, pretreatment with the ADA inhibitor EHNA almost abolished hemolytic pulmonary vasoconstriction, indicating significant ADA release during hemolysis. This was consistent with *in vitro* experiments in which accelerated adenosine metabolism (i.e., conversion of adenosine

to inosine) in hemolyzed blood was completely prevented by pretreatment with EHNA.

Hemolysis is also associated with a significant release of arginase from erythrocytes. Therefore, by reducing the bioavailability of L-arginine, a substrate for nitric oxide, arginase may significantly contribute to initial hemolytic vascular injury and development of hemolytic vasculopathy. A growing body of evidence indicates depleted levels of L-arginine in SCD patients and in mouse models of SCD, while suggesting that L-arginine supplementation may have beneficial effects [43, 44]. The acute administration of HAB was not associated with increased arginase activity. Furthermore, although L-arginine supplementation markedly increased plasma nitrite levels, it had no effect on HAB-induced acute changes in systemic, pulmonary and renal circulation. Hemolytic and control plasma and lung tissue had similar arginase activity and no arginase activity was detected in RBCs. This was somewhat expected, due to the lack of arginase expression in rat RBCs [45], and indicates that, in contrast to humans, in rats there is no release of arginase during hemolysis and that arginase is not initially involved in development of hemolytic vasculopathy and PH in rats. Yet, in rat models of monocrotaline- and bleomycin-induced PH [46, 47] and in rats with hemolysis-induced angioproliferative PH [48], most likely due to increased presence of inflammatory cells, there is increased arginase expression and activity in the lungs; this suggests that although the putative “arginase pathway” is not initially involved in the development of disease, it may contribute to progression of hemolytic vascular injury and PH in our new rat models of hemolysis-associated PH [28, 48].

Repeated daily administration of DMSO for 10 days, with consequent intermittent 6-hour daily exposure to intravascular hemolysis effects, was sufficient to induce both hemodynamic and histopathological PH. DMSO-treated animals, in addition to elevated RVPSP, had increased RV EDP and isolated RV hypertrophy, suggesting impending RV failure. Similar to patients with hemolytic anemia, DMSO treated animals developed reduced systemic blood pressure and splenomegaly. Collectively, these data suggest a mechanistic link between hemolysis and PH.

Experiments with hemolyzed blood have demonstrated increased ADA release and accelerated adenosine metabolism during hemolytic conditions, suggesting that adenosine deficiency

may be involved in the underlying pathophysiology of hemolytic vasculopathy and PH. Adenosine is an ubiquitously occurring endogenous nucleoside that, by interacting with at least four cell surface receptor subtypes (A1, A2A, A2B and A3), induces a number of biological effects that, in aggregate, may reduce hemolysis-induced vasculopathy and risk for PH [24]. In this regard, adenosine is a selective pulmonary vasodilator that, via activation of high-affinity A2A receptors, induces both endothelium-dependent and endothelium-independent pulmonary vasodilation. A2A receptor-mediated pulmonary vasodilation is produced even under conditions of severe NO deficiency (i.e., conditions seen in patients with SCD and PH), and activation of A2A receptors attenuates ischemia-reperfusion injury in the lungs and reduces inflammation in several models of lung injury [23]. Furthermore, activation of A2B receptors due to increased adenosine levels during hypoxia or hypoxic preconditioning attenuates pulmonary inflammation by inhibition of the NF- κ B-mediated inflammatory signaling cascade [49]. Finally, genetic removal of ADO A2A receptors in mice not only enhances pulmonary inflammation but also, under conditions of hypoxia, induces PH and increases hypoxic pulmonary vascular remodeling [50]. Similarly, in mice with SCD, activation of the A2A receptor with the selective agonist ALT146e improves baseline pulmonary function, reduces pulmonary inflammation and prevents hypoxia-reoxygenation-induced exacerbation of pulmonary injury [51]. In SCD, increased release of ATP from damaged erythrocytes and tissue is very rapidly converted to adenosine by cell surface enzymes. The ecto-apyrase (CD39) converts ATP and ADP to AMP, and ecto-5'-nucleotidase (CD73) converts AMP to adenosine. Notably, mice genetically deficient in CD39 or CD73 show increased hypoxia-associated tissue damage and vascular leak syndrome during hypoxia [52, 53]. Adenosine levels are usually higher in the pulmonary circulation than in systemic circulation, yet lower adenosine levels have been reported in patients with primary PH compared to healthy subjects [54, 55], suggesting that a PH patient may benefit from administration of exogenous adenosine. Indeed, in newborns with persistent PH and in adults with refractory PH or post-surgical PH, administration of adenosine reduces pulmonary artery pressure [56-59]. Limited clinical data suggest adverse vascular effects of cell free ADA; similar to the present findings in patients with SCD, the active phase of Behcet's disease (characterized by endothelial dysfunction due to chronic relapsing inflammatory vasculitis)

is associated with increased serum ADA activity, oxidative injury, and reduced activity of antioxidant enzymes in erythrocytes [60, 61]. Furthermore, HIV positive patients (whose risk of developing PH is higher than in the general population) have increased activity of erythrocytic and plasma ADA [62, 63]. Overall, these data are indicative to the adverse vascular effects of extracellular ADA in hemolytic vasculopathy. Taken together, the above discussion and the presented data support the notion that the "ADA-Adenosine" pathway may play an important role in hemolytic vascular injury and development of PH [24].

CONCLUSION

In the present study, we provide evidence that DMSO-induced chronic hemolysis is mechanistically linked to PH development. We propose that in addition to free hemoglobin and arginase, adenosine deaminase plays a significant role in hemolysis-induced vascular injury and PH. Therefore, further studies are warranted to determine the relative contributions of free hemoglobin, plasma arginase, and the proposed ADA-adenosine pathway in the development and progression of disease in the new rat models of hemolysis-associated PH.

Conflict of Interest Disclosure: The authors have no conflict of interest to disclose.

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Резиме**ДИМЕТИЛ СУЛФОКСИДОТ ИНДУЦИРА ХЕМОЛИЗА И РАЗВОЈ НА БЕЛОДРОБНА ХИПЕРТЕНЗИЈА**

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Васкуларните и белодробните оштетувања се чести компликации поврзани со хемолитичките заболувања, кои заедно со белодробната хипертензија (БХ) поврзана со хемолиза се појави на најсериозна компликација на српестата анемија. Причинско-последичната врска помеѓу интраваскуларната хемолиза и развојот на БХ е сè уште предмет на истражување. Претходно ние покажавме дека повеќекратното администрирање аутологна хемолизирана крв кај стаорци доведува до развој на БХ. Диметил сулфоксидот (ДМСО), широко употребуван солвент и антиинфламаторен агенс, предизвикува хемолиза *in vivo*. Ние претпоставивме дека повеќекратното администрирање ДМСО кај стаорци ќе индуцира развој на БХ. Исто така, ние го испитавме ослободувањето на аденозин деаминаза (АДА) и аргиназа од еритроцитите, процес што може да го зголеми оштетувањето на крвните садови, поврзано со хемолизата. Акутната администрација на ДМСО (1,5мл/30мин во десниот атриум) предизвика хемолиза и белодробна вазоконстрикција. ДМСО го зголеми максималниот притисок во десната комора (МПДК), кој беше придружен со зголемено ослободување на АДА. Значајно е тоа што администрирањето на аденозин А2А рецептор агонист или претходен третман на животните со АДА инхибитор еритро-9-2-хидрокси-3-нонил аденин (ЕХНА) го намали акутното покачување на МПДК. Повеќекратното администрирање на ДМСО во текот на 10 дена доведе до анемија, хемоглобинурија, хемоглобинемија и спленомегалија. ДМСО-третираните животни развија белодробни оштетувања (конгестија, хеморагија, задебелување на алвеоларните септуми, периваскуларна инфламација), дисфункција и хипертрофија на десната комора (ДК) и белодробна хипертензија. Хистопатолошката анализа откри ремоделирање на малите (50–100 μ) белодробни крвни садови. Презентираните резултати опишуваат нов експериментален модел на хемолиза-индуцирана БХ, сугерираат дека е хемолизата механички поврзана со БХ и укажуваат на можната патогена улога што аденозин деаминазата и забрзаниот метаболизам на аденозинот можат да ја имаат во БХ поврзана со хемолиза.

Клучни зборови: хемолиза, белодробна хипертензија, аденозин, аденозин деаминаза