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Dimethyl Malonate Slows Succinate Accumulation and Preserves Cardiac Function in a Swine Model of Hemorrhagic Shock

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Abstract

Background: Succinate (SI) is a citric acid cycle metabolite that accumulates in tissues during hemorrhagic shock (HS) due to electron transport chain uncoupling. Dimethyl malonate (DMM) is a competitive inhibitor of succinate dehydrogenase, which has been shown to reduce SI accumulation and protect against reperfusion injury. Whether DMM can be therapeutic after severe HS is unknown. We hypothesized that DMM would prevent SI buildup during resuscitation

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in a swine model of hemorrhagic shock, leading to better physiological recovery after resuscitation (RES).

Methods: The carotid arteries of Yorkshire pigs were cannulated with a 5-French catheter. After placement of a Swan-Ganz catheter and femoral arterial line, the carotid catheters were opened and the animals were exsanguinated to a mean arterial pressure (MAP) of 45 mm. After 30 minutes in the shock state, the animals were resuscitated to a MAP of 60 mm using lactated ringers. A MAP above 60 mm was maintained throughout RES. One group received 10 mg/kg of DMM (n=6) while the control received sham injections (n=6). The primary end-point was SI levels. Secondary end-points included cardiac function and lactate.

Results: SI levels increased from baseline to the 20-minute RES point in control, while the DMM cohort remained unchanged. The DMM group required less IV fluid to maintain a MAP above 60 (450.0 vs. 229.0 mL, p=0.01). The DMM group had higher pulmonary capillary wedge pressure at the 20 and 40-minute RES points. The DMM group had better recovery of cardiac output and index during RES, while the control had no improvement. While lactate levels were similar, DMM may lead to increased ionized calcium levels.

Discussion: DMM slows SI accumulation during HS and helps preserve cardiac filling pressures and function during RES. In addition, DMM may protect against depletion of ionized calcium. DMM may have therapeutic potential during HS.

Study Type: Basic science paper

Keywords

hemorrhagic shock; dimethyl malonate; reactive oxygen species; endothelial glycocalyx

Background

Trauma remains the leading cause of death in people under the age of 45 (1) with hemorrhage being the most common cause of preventable death after traumatic injury (2). Succinate (SI) accumulation is a known sequalae of ischemia-reperfusion injury. Hemorrhagic shock leads to hypoxemia at the cellular level, causing increased anaerobic metabolism with accumulation of citric acid metabolites, such as succinate (3, 4). During fluid resuscitation after hemorrhagic shock, oxygen stores become available to the electron transport chain again, leading to increased release of succinate (5, 6). Succinate itself may be a driver of deleterious effects, leading to increased reactive oxygen species (ROS) after ischemia and reperfusion (5, 7). In addition, plasma succinate levels have been shown to be a biomarker of metabolic derangement after hemorrhagic shock in critically ill patients (8).

The presence of ROS has significant deleterious effects. ROS in the heart leads to mitochondrial dysfunction, cardiac cell death, and can lead to cardiomyopathy (9–11). Excessive generation of intracellular ROS and calcium play a key role in apoptosis and cell death after myocardial ischemia-reperfusion (12–15). ROS can also damage the endothelial cell layer by inducing shedding of the endothelial glycocalyx, a glycoprotein layer on the luminal side of endothelial cells that maintain transvascular fluid exchange

and the coagulation cascade (16–21). Our lab's previous work has shown that the lungs are preferentially damaged by oxidative stress after hemorrhagic shock and resuscitation (22).

Dimethyl Malonate (DMM) is a cell permeable, competitive inhibitor of succinate dehydrogenase and has been shown to reduce succinate levels (23). Succinate dehydrogenase is an enzyme complex that converts fumarate to succinate during anaerobic metabolism, where the citric acid cycle is reversed (7). DMM inhibits accumulation of succinate, which prevents rapid oxidization of succinate to form mitochondrial reactive oxygen species. DMM has been shown to protect several different tissue beds in the presence of ischemia-reperfusion, including the brain (24, 25), liver (26), and perhaps most studied, the heart (23, 27, 28). Whether DMM can be therapeutic after severe HS is unknown. We hypothesized that DMM would prevent SI buildup during resuscitation in a swine model of hemorrhagic shock, leading to better physiological recovery after resuscitation.

Methods

Animals

All animal care and experimentation were performed in accordance with the Tulane University Institutional Animal Care and Use Committee (IACUC)- approved protocols, and following guidelines from the Institute for Laboratory Animal Research. Study subjects were female Yorkshire swine (22–28 kg, Arkansas Tech). There was no difference in body weight between the control and treatment group (51.8 vs. 54.6 kilograms, p=0.99). After arrival at our institution, swine were acclimatized for a minimum of 48 hours before experimental procedures. Animals had free access to food and water up to initiation of anesthesia. The ARRIVE reporting guidelines were used for this study. A complete ARRIVE Checklist was uploaded as Supplemental Digital Content (SDC Table 1).

Surgical Setup

Anesthesia was induced using Tiletamine-Zolazepam/Xylazine (4–6/2–3 mg/kg) cocktail IM. Animals were then intubated and anesthesia was maintained using inhaled isoflurane in oxygen administered through the orotracheal tube. Animals were mechanically ventilated using a volume-controlled mode of 12–15 mL/kg with a respiratory rate of 10–15 breaths/min. Temperature management was performed using a warming blanket set to 38°C to help mitigate hypothermia.

The hemorrhagic shock model was performed as previously described (29, 30). Briefly, percutaneous arterial vascular access was obtained using ultrasound guidance to place a single 7.5 Fr catheter into a femoral artery. Venous access was also obtained using two 7.5 French catheters in the bilateral femoral veins. The neck was dissected to expose the right carotid artery and jugular vein. The carotid was cannulated using a 5 Fr catheter to be used for exsanguination.

An 8 Fr introducer sheath was placed in the right internal jugular vein and a flow directed Swan-Ganz catheter was introduced into the jugular vein sheath and advanced into the

pulmonary artery. Mean arterial pressure (MAP) was transduced through the femoral artery catheter using a digital pressure transducer.

Animals were hemorrhaged through the carotid line at 100 ml/min until a MAP of 45 mm Hg was reached. The MAP was maintained at this level by repeated blood draws from the carotid for 30 minutes. The shock time point refers to the end of this 30-minute shock state. Following this 30-minute period, animals were resuscitated with lactated Ringer's solution. Lactated Ringer's solution was perfused via the femoral vein lines at 150 mL/min until a MAP of ≥ 60mmHg was achieved. The resuscitation period was started once a MAP of 60 mmHg was reached. Lactated Ringer's solution was perfused as required to maintain a MAP of ≥60 mm Hg throughout the remainder of the surgical procedure. Animals were randomized into control (saline) (n=6) or dimethylmalonate (DMM, Millipore Sigma, 10 mg/kg IV) treatment (n=6) groups using computer allocation. All analyses were performed in a blinded fashion. Sample size was determined from unpublished data in a rat model of hemorrhagic shock. In this unpublished study, lung glycocalyx damage was decreased in DMM treated animals, with a mean glycocalyx intensity of 87 Arbitrary Units in untreated animals and 122 Arbitrary Units in treated animals, with a standard deviation of 22 Arbitrary Units. Using an alpha of 0.05 and power of 80%, this yielded a sample size of 6 animals per group (unpublished data). DMM was administered at the end of the 30-minute hemorrhage period, immediately prior to initiation of resuscitation. DMM was administered at a dose of 10 mg/kg based on unpublished data inducing hemorrhagic shock in small animal pilot studies using rats. DMM was administered as a bolus over five minutes (unpublished data).

Sampling

Venous and arterial blood samples were taken for analysis and preservation at baseline (before hemorrhage), after 30 minutes of hemorrhage, and after 20, 40, and 60 minutes of resuscitation. An iSTAT blood analyzer (Abbott) was used to assess blood chemistry at these time points (using CG4+, PT, and CHEM8+ cartridges). Blood was centrifuged at 800xg for 10 minutes at 4°C and plasma was collected for later analysis. After 60 minutes of resuscitation, animals were euthanized using IV injection of 150 mg/kg pentobarbital. Necropsy was then performed and heart left ventricle apex and tissue from the left lower lobe of the lung were snap frozen in Optimal Cutting Temperature compound (O.C.T, Sakura Finetek).

Dihydroethidium Staining

Dihydroethidium staining was performed as in prior studies (31). Left ventricle tissue snap frozen in O.C.T. as above was sectioned in a cryostat at −20°C at a section thickness of 10 μm; the sections were affixed to a positively charged glass slide coated with 0.1% gelatin in deionized (DI) water. Tissue sections were subsequently coated with 0.1% gelatin in DI water and incubated until dry. Tissue sections were then submerged in 50 mL of DI water for a minimum of 10 min to rehydrate the tissue as well as to remove excess OCT compound from the sections for improved staining. A 5 mM dihydroethidium (DHE) staining solution was prepared by diluting 25 mL of 10 mM DHE in dimethylsulfoxide into 50 mL of DI water. Tissue sections were incubated in 50 mL of the DHE staining solution for 20 min at room temperature and minimal light exposure. The sections were then washed three times

with 50 mL of DI water. Sections were then cover slipped with Fluoro Gel and immediately imaged using an Olympus BX51 fluorescence microscope (excitation wavelength 542 – 582 nm, emission wavelength 604–644 nm). Resulting images were analyzed by blinded reviewers and ROS was quantified using ImageJ software (ImageJ, RRID:SCR_003070).

Glycocalyx Imaging

Glycocalyx staining was performed to determine staining intensity of the endothelial glycocalyx as previously described. We performed these experiments because our prior work has shown that the endothelial glycocalyx in lung tissue is preferentially affected in hemorrhagic shock (22). Briefly, flash frozen tissue was sectioned in a cryostat at −20°C at a section thickness of 10 μm and the sections were affixed to room temperature glass slides. Fixation was performed by immersion in 4°C methanol for 10 min. Glycocalyx staining was performed with Fluorescein isothiocyanate-labeled wheat germ agglutinin (FITC-WGA, Millipore Sigma) in phosphate buffered saline (PBS) for 1 h at room temperature with 4’,6-diamidino-2-phenylindole (DAPI, Millipore Sigma). Sections were washed three times with PBS and coverslipped in Fluoro Gel (Electron Microscopy Sciences). Tissue was imaged on an Olympus BX51 fluorescence microscope. Glycocalyx intensity was analyzed using ImageJ software (ImageJ, RRID:SCR_003070) and intensity was measured using

Succinate Assay

Plasma succinate assay was performed using a colorimetric commercially available kit as per manufacturer instructions (LSBio) using venous plasma.

Statistical analysis

Measurements and data are expressed as mean ± SEM as data was normally distributed. A two-way repeated measures ANOVA was performed for difference between measurements for multiple time point experiments, with Tukey’s multiple comparisons post hoc analysis for differences between groups. Single time point studies used Student’s t test for comparison between groups. All t tests were two-tailed, and $P < 0.05$ was considered significant for all comparisons.

Results

Survival

Survival was not significantly different in the DMM and control group as five of six animals survived the shock and resuscitation phases (83.3% vs. 83.3%, p=0.99). Both animals that expired during the experiment did so during the resuscitation phase. The animal in the control group died 13 minutes into the resuscitation phase, while the animal that expired in the DMM group died 18 minutes into the resuscitation phase.

Succinate Levels

When compared to baseline, serum succinate levels (Figure 1) increased in the control group at the 20-minute resuscitation phase (264.7 vs. 407.4 mmol/L, p=0.01). Succinate levels did

not differ from baseline in the DMM group as shown in Figure 1 (260.6 vs. 395.6 mmol/L, p=0.06).

Hemodynamic Measurements

Cardiac output (2.4 vs. 3.0 L/min, p=0.049) and index (2.8 vs. 4.0 L/min/m^2, p=0.048) improved during the resuscitation phase within the DMM group from the 20-minute to 60-minute time point (Figure 2A and 2B). The control group did not demonstrate any change during the resuscitation phase. Pulmonary capillary wedge pressure increased during the resuscitation period within the DMM group and was significantly higher than the control group at 20- (5.2 vs. 9.0 mm Hg, p=0.04) and 40-minute (4.8 vs. 7.8 mm Hg, p=0.02) time periods (Figure 2C). Mean arterial pressure did not differ between the two groups (Figure 2D). Amount of resuscitation fluid required to maintain target MAP during the resuscitation phase was higher in the control group as compared to the DMM cohort (450.0 vs. 229.0 mL, p=0.01).

Ionized Calcium and Coagulation Parameters

Ionized calcium levels increased from the shock state to the 40- (1.2 vs. 1.4 mmol/L, p=0.04) and 60-minute (1.2 vs. 1.3 mmol/L, p=0.046) resuscitation phase (Figure 3A). In addition, ionized calcium in the DMM group increased from the 20- to 40- minute resuscitation time points (1.3 vs. 1.4 mmol/L, p=0.02). All comparisons of ionized calcium between the control and DMM groups at the various time points were non-significant. Hemoglobin levels in the DMM group were decreased at the 20- (7.5 vs. 7.0 grams/dL, p=0.003), 40- (7.5 vs. 6.7 grams/dL, p=0.002) and 60-minute (7.5 vs. 6.8 grams/dL, p=0.01) resuscitation time points when compared to the shock time point. In the control group, there was no difference in hemoglobin levels throughout the resuscitation phase, when compared to the shock time point (Figure 3B). All comparisons of hemoglobin between the control and DMM groups at the various time points were non-significant. Prothrombin time and INR did not vary between the DMM and control groups throughout the shock and resuscitation phase (Figure 3C and 3D).

Markers of End Organ Damage

Lactate and creatinine levels did not differ when comparing the two groups (Figures 4A and 4B). Arterial pH did not differ between the DMM and control group throughout the shock and resuscitation phases (Figure 4C).

Myocardial Reactive Oxygen Species

A comparison of reactive oxygen species as measured by DHE stain is shown in Figure 5. Representative images are shown in Figure 5A and 5B. Endothelial reactive oxygen species was higher in the control group as compared to the DMM group (113.5 vs. 87.1 Arbitrary Units, p=0.02) (Figure 5C). Myocardial reactive oxygen species was not different when comparing the control and DMM cohorts (49.3 vs. 48.4 Arbitrary Units, p=0.83) (Figure 5D).

Pulmonary Endothelial Glycocalyx

Representative images of pulmonary artery endothelial glycocalyx are shown in control and DMM groups in Figures 6A and 6B. Pulmonary artery glycocalyx staining intensity was higher in the DMM group (126.2 vs. 163.9 Arbitrary Units, $p<0.001$) (Figure 6C). Alveolar capillary glycocalyx staining intensity was higher in the DMM group (28.0 vs. 44.0 Arbitrary Units, $p<0.001$) (Figure 6D).

Discussion

Ischemia-reperfusion injury after hemorrhagic shock and resuscitation leads to accumulation of succinate. Succinate is one of several byproducts that drives formation of reactive oxygen species. The presence of ROS has many negative effects, leading to cardiac injury and endotheliopathy. DMM inhibits succinate dehydrogenase, inhibiting succinate formation. Whether DMM may be a novel therapeutic that reduces succinate levels and improves physiological recovery after hemorrhagic shock has yet to be examined. We found that DMM does decrease succinate accumulation and helps enhance recovery in a swine model of hemorrhagic shock and resuscitation.

We found that DMM may indeed blunt plasma succinate accumulation after hemorrhagic shock and resuscitation. While DMM is thought to have protective effects by reducing succinate levels after various ischemia-reperfusion insults by inhibiting succinate dehydrogenase (32), data showing that DMM actually reduces plasma or tissue succinate levels *in vivo* are lacking. A prior study has shown that DMM does not reduce succinate accumulation during renal ischemia alone in mice, however, it does reduce succinate accumulation in the kidneys after renal ischemia-reperfusion in this mouse model (33). Our study is the first to show that DMM may reduce plasma succinate levels in a large animal model of ischemia-reperfusion caused by hemorrhagic shock and fluid resuscitation.

The potential therapeutic benefit of DMM to mitigate cardiac injury has been shown in numerous studies (27). DMM can help reduce cardiac infarct size in a mouse model of myocardial infarction (34). Other studies have postulated that DMM is not protective in ischemia alone, however, it is beneficial when given at time of reperfusion (23, 27). The present study indicates that DMM can help short-term recovery of the heart after resuscitation from hemorrhagic shock. This enhanced short-term recovery seen in DMM treated animals appears to be, at least in part, due to enhanced filling pressures. The mechanism behind this finding needs further investigation. Decreased ROS in the heart with DMM treatment likely plays a role as shown in previous studies (35). Our findings confirmed that endothelial cells in the heart had decreased ROS with DMM treatment, which may explain the improved cardiac function seen in the DMM group during resuscitation. In addition, DMM may act as a vasopressor or scavenger of reactive oxygen species. Further studies are needed to examine the mechanism by which DMM improves hemodynamics during resuscitation.

Interestingly, we found that serum ionized calcium levels were higher in the DMM group. To our knowledge, this is the first study to show that dimethyl malonate increases plasma calcium levels. Succinate accumulation is known to increase intracellular calcium levels

(36–38), therefore it stands to reason that inhibition of succinate production by DMM may decrease intracellular calcium influx, leading to increased plasma calcium levels. However, the mechanism behind increase plasma calcium levels in DMM treated animals could not be elicited in this study and needs further investigation. The importance of preventing hypo- and hypercalcemia has been shown in prior studies (39). Hemorrhaging trauma patients are at risk of hypocalcemia, which in turn, can increase risk of mortality (40–42). Hypocalcemia can precipitate cardiac arrhythmias, heart failure, and hypotension (43–45). In our study, the improved cardiac function and preserved filling pressures in DMM treated animals may be due to increased calcium levels.

ROS causes degradation of the endothelial glycocalyx (16, 46). Our previous work has shown that the vasculature in the lung are disproportionately exposed to ROS after ischemia-reperfusion and are most vulnerable to glycocalyx injury (22). The present study confirmed that DMM protects the glycocalyx in the lung. This protective effect was seen in both large and small vessels, as DMM protected the glycocalyx in the pulmonary artery and the capillary vessels in the alveoli. Degradation of the glycocalyx contributes to acute lung injury and ARDS (47). Many factors, such as matrix metalloproteinases, toll-like receptors, and growth factors can play a role in glycocalyx shedding and preservation (47, 48). While the mechanism for the protective effect on the pulmonary glycocalyx could not be elucidated in our study, reduction of ROS likely played a role. The preserved endothelial glycocalyx seen in DMM treated animals may help keep fluid in the vasculature as the DMM group required less IV fluids in the resuscitation phase to maintain a similar MAP as the control group. The preserved endothelial glycocalyx may also explain why hemoglobin levels where lower in the DMM group. The glycocalyx maintains vascular permeability, which means that the DMM group has less capillary leak. Keeping fluid in the intravascular space may cause a hemodilution effect in the DMM treated animals, leading to a lower hemoglobin concentration.

Future pre-clinical studies on DMM should focus on the pharmokinetic profile of the compound and better delineating whether there are any toxic effects. Further pre-clinical studies are needed to better characterize its effect on calcium handling in hemorrhagic shock. This may pave the way for human trials to be performed to determine if it may be a novel therapy for trauma patients.

This study was not without limitations. First, animals were resuscitated with crystalloid fluid, which differs from the current practice of early and balanced transfusion of blood products in hemorrhagic shock. Secondly, we did not do survival studies to examine long-term effects of DMM. In addition, we used a controlled model of hemorrhagic shock, rather than an uncontrolled model of hemorrhage, which may more closely reflect traumatic injury. Finally, the power of this study was limited due to the small sample size in each group of animals, which may have limited our ability to detect a survival difference.

In conclusion, DMM may have therapeutic potential in reducing ischemia-reperfusion injury after hemorrhagic shock and reperfusion. DMM helps enhance cardiac recovery during resuscitation and preserves the endothelial glycocalyx. These protective effects are likely

due to preserving ionized calcium levels and decreasing ROS. Additional studies are needed to further examine these findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflicts of Interest and source of funding:

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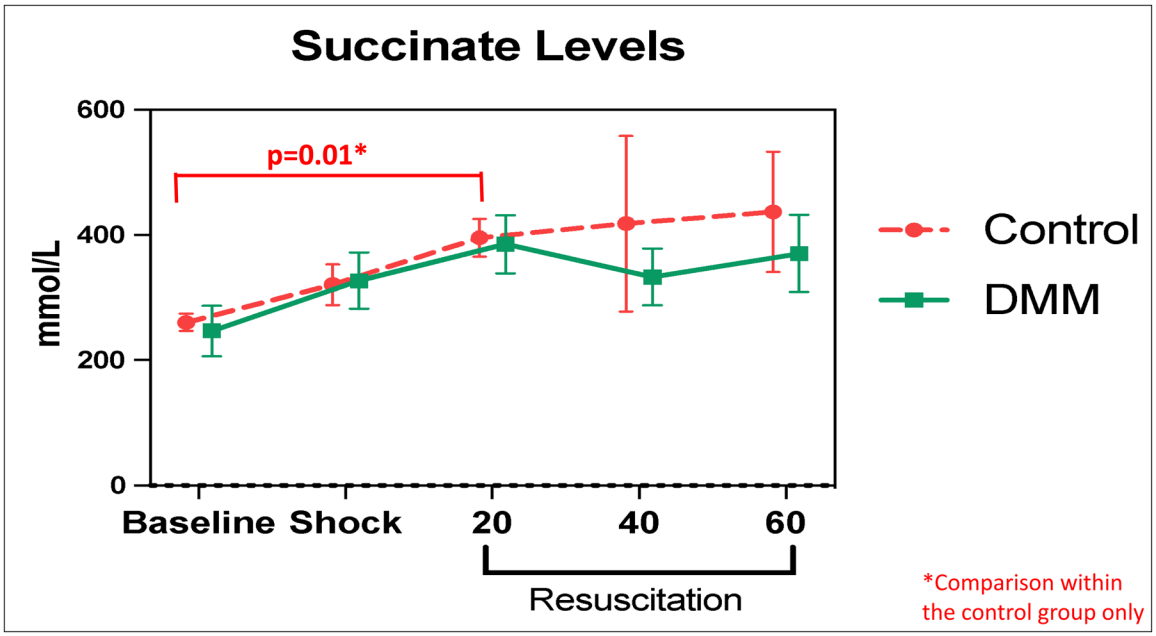

Figure 1 –.

A comparison of succinate levels showing that succinate accumulation does not have a statistically significant increase in the resuscitation phase for DMM treated animals.

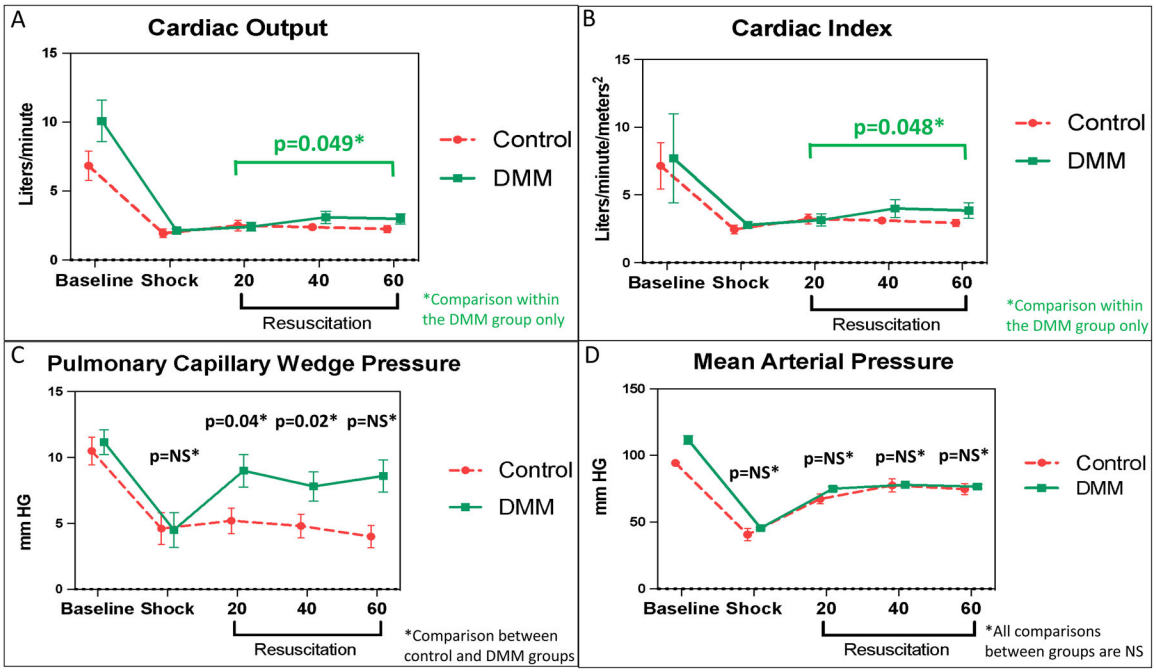

Figure 2 –.

A comparison of DMM treated and control animals showing improved A) cardiac output and B) cardiac index during resuscitation in DMM treated animals. Improved cardiac filling pressures during resuscitation as measured by C) pulmonary capillary wedge pressure while D) mean arterial pressure was similar.

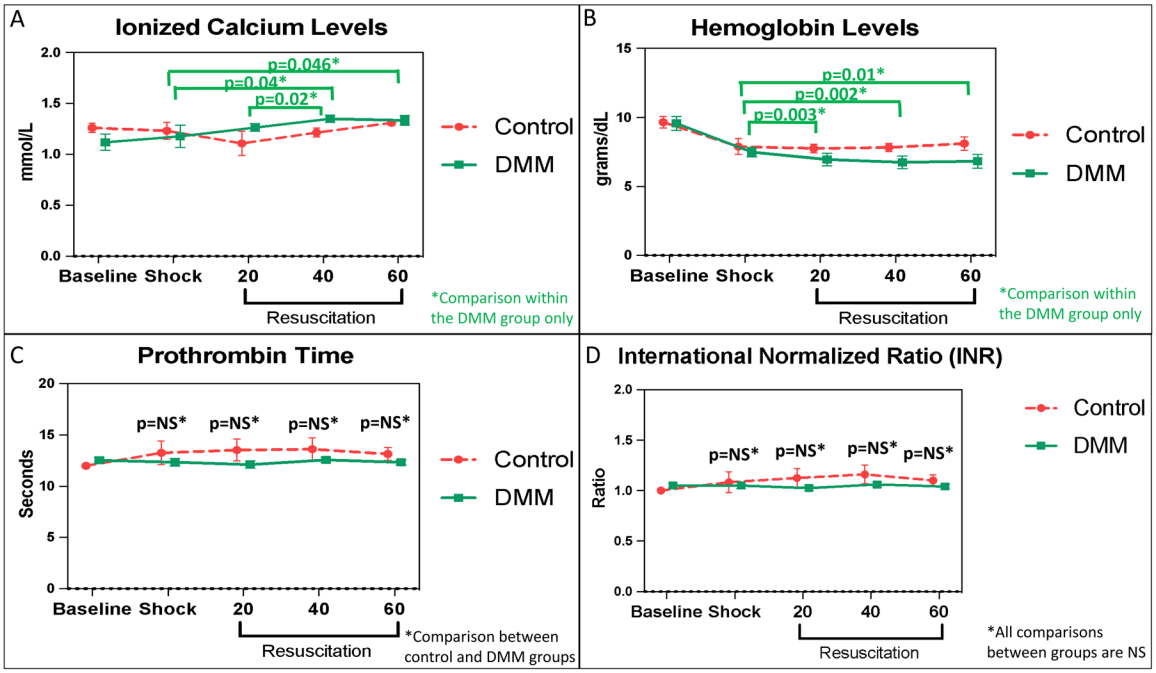

Figure 3 –.

A comparison of DMM treated animals and controls demonstrating A) preservation of ionized calcium levels in DMM treated, B) decreased hemoglobin levels in DMM treated, and similar C) prothrombin time and D) international normalized ratio (INR).

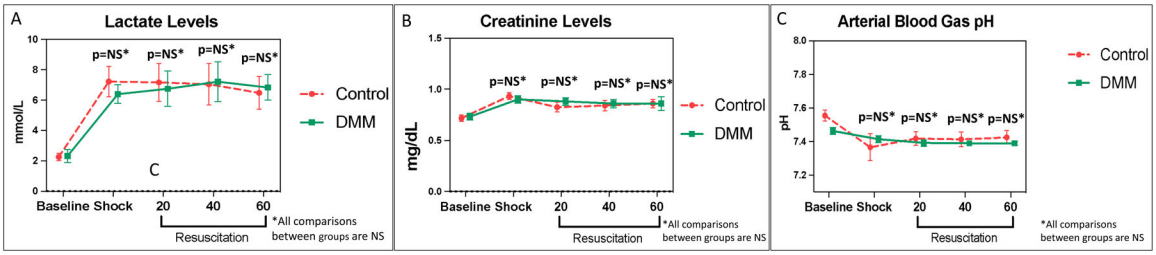

Figure 4 –.

A comparison of end organ damage in DMM and control animals shows no difference in A) lactate levels, B) creatinine levels, and C) arterial blood gas pH.

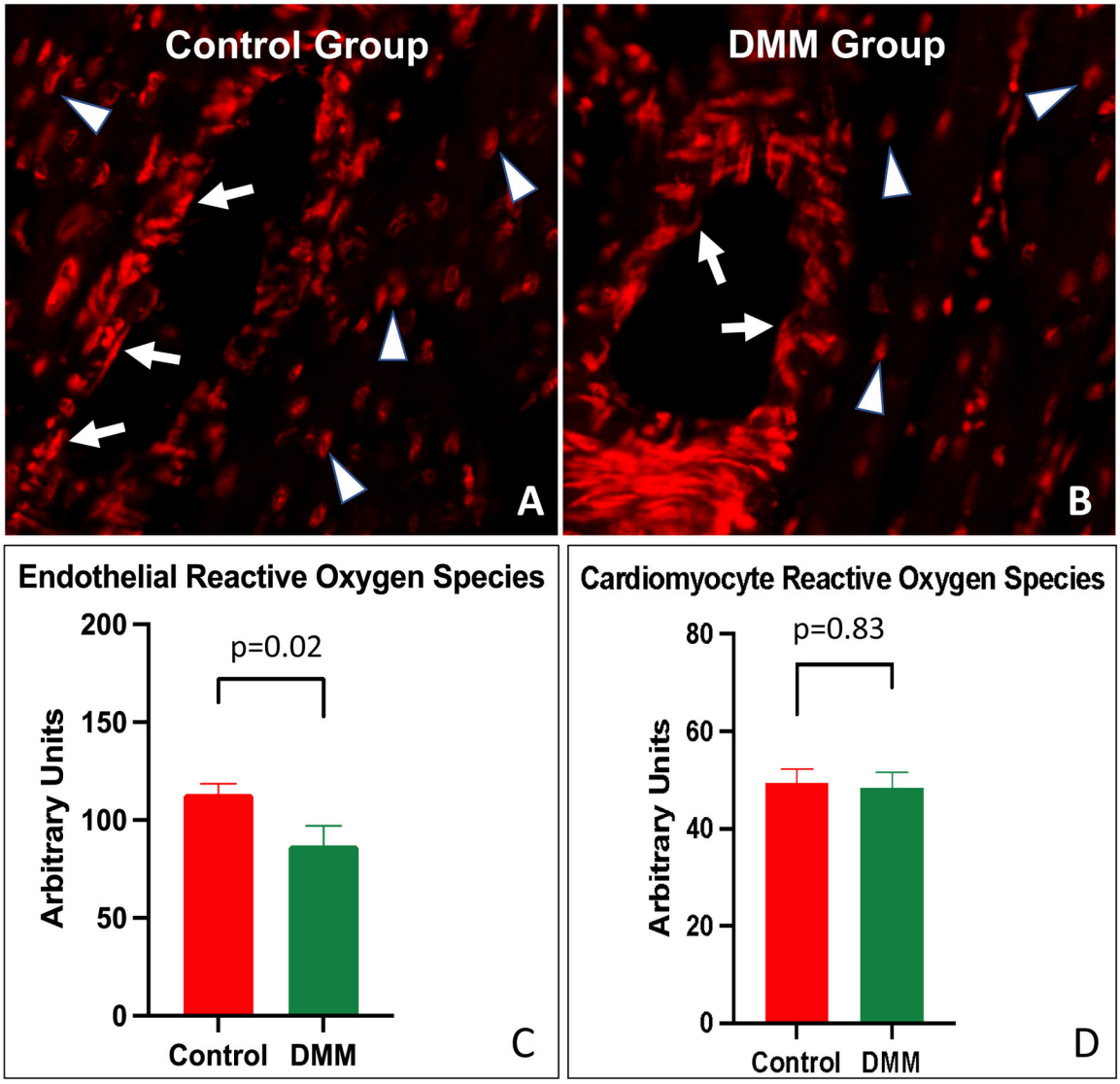

Figure 5 –.

A comparison of reactive oxygen species using dihydroethidium (DHE) staining in myocardium with representative images in A) and B) with arrow heads pointing to myocardium and arrows pointing to endothelium. C) DMM treated animals have less reactive oxygen species in myocardial endothelial cells. There is no difference in cardiomyocyte reactive oxygen species by DHE staining.

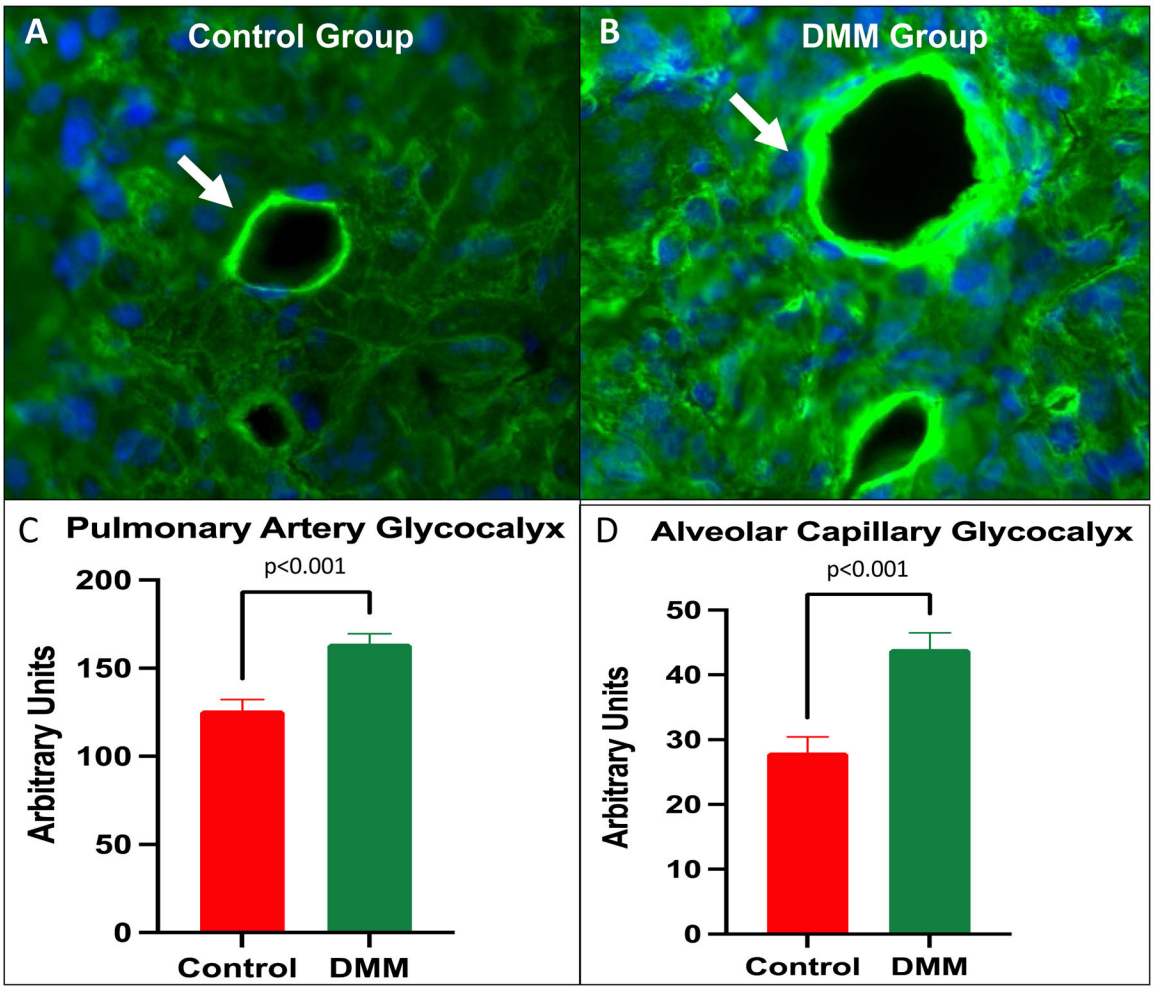

Figure 6 –.

A comparison of pulmonary endothelial glycocalyx with representative images from the pulmonary artery shown in A) control and B) DMM treated groups as measured by fluorescein isothiocyanate-labeled wheat germ agglutinin staining. DMM protected the endothelial glycocalyx as there is increased glycocalyx staining intensity in DMM treated in both the pulmonary artery glycocalyx and alveolar capillary glycocalyx.