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PLASMA AND MYOCARDIAL VISFATIN EXPRESSION CHANGES ARE ASSOCIATED WITH THERAPEUTIC HYPOTHERMIA PROTECTION DURING MURINE HEMORRHAGIC SHOCK/ RESUSCITATION

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Abstract

Aim—Cytokine production during hemorrhagic shock (HS) could affect cardiac function during the hours after resuscitation. Visfatin is a recently described protein that functions both as a proinflammatory plasma cytokine and an intracellular enzyme within the nicotinamide adenine dinucleotide (NAD⁺) salvage pathway. We developed a mouse model of HS to study the effect of therapeutic hypothermia (TH) on hemodynamic outcomes and associated plasma and tissue visfatin content.

Methods—Mice were bled and maintained at a mean arterial pressure (MAP) of 35 mmHg. After 30 min, animals (n=52) were randomized to normothermia (NT, 37±0.5°C) or TH (33±0.5°C) followed by rewarming at 60 min following resuscitation. After 90 min of HS (S90), mice were resuscitated and monitored for 180 min (R180). Visfatin, interleukin 6 (IL-6), keratinocyte-derived chemokine (KC), tumor necrosis factor- α (TNF- α), and myoglobin were measured by ELISA.

Results—Compared to NT, TH animals exhibited improved R180 survival (23/26 [88.5%] vs. 13/26 [50%]; p = 0.001). Plasma visfatin, IL-6, KC, and TNF- α increased by S90 in both groups (p<0.05). TH attenuated S90 plasma visfatin and, after rewarming, decreased R180 plasma IL-6, KC, and myoglobin (p<0.05) relative to NT. Heart and gut KC increased at S90 while IL-6 increases were

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Conflict of Interest Statement

The authors have no relevant conflicts of interest to disclose.

delayed until R180 ($p < 0.05$). NT produced sustained elevations of myocardial KC but decreased visfatin by R180, effects abrogated by TH. ($p < 0.05$).

Conclusions—In a mouse model of HS, TH improves hemodynamics and alters plasma and tissue proinflammatory cytokines including the novel cytokine visfatin. TH modulation of cytokines may attenuate cardiac dysfunction following HS.

Keywords

inflammation; temperature; resuscitation; cytokine; visfatin; pre-B-cell colonyenhancing factor; Nampt; interleukin-6; Tumor Necrosis Factor-alpha; keratinocyte-derived chemokine; CXCL1; hemorrhagic shock

1. INTRODUCTION

Hemorrhagic shock (HS) following trauma can often lead to circulatory collapse despite aggressive resuscitation.^{1, 2} Although exsanguination due to uncontrolled blood loss remains an important cause of death, pump failure due to depressed cardiac function also contributes to such early cardiovascular collapse.²⁻⁴ Cardiac dysfunction may be induced, in part, by the expression of NF κ B transcribed proinflammatory cytokines such as tumor necrosis factor-alpha (TNF- α) and interleukin 6 (IL-6) which are rapidly elevated in the plasma of patients following trauma or HS.²⁻⁵

Visfatin (i.e. pre-B-cell colony-enhancing factor, PBEF), a highly conserved cytokine, potentiates inflammation within the lung, heart, gut, and circulating plasma⁶ by driving the transcription of NF κ B pathway proinflammatory genes⁷ such as IL-6 and keratinocyte-derived chemokine (KC). In addition to existing as an extracellular cytokine, visfatin exists as an essential intracellular enzyme within the NAD⁺ salvage pathway.⁸ Given these intriguing dual roles in inflammation and tissue bioenergetics, visfatin could play a key role in modulating cardiac function in the setting of HS. Preclinical literature suggests that the induction of mild to moderate therapeutic hypothermia (TH) may forestall hemodynamic collapse and improve survival outcomes following HS.⁹⁻¹⁴ In the setting of cardiac arrest, a related disease of whole-body ischemia/reperfusion (I/R) injury¹⁵, the use of mild hypothermia following successful resuscitation has been established as the standard of care for treating comatose victims of out-of-hospital ventricular fibrillation arrest.¹⁶ Furthermore, researchers from our lab and others have demonstrated that the use of intra-arrest TH attenuates cardiac dysfunction following cardiac arrest though the effects of TH on cytokine expression are not well understood.¹⁷⁻¹⁹ In this study, we developed a mouse model of pressure-controlled HS to study the effect of TH on hemodynamics and the expression of visfatin, IL-6, KC, and TNF- α in plasma, heart, and gut.

2. MATERIALS AND METHODS

2. 1. Animal Preparation

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Chicago. Adult female C57BL/6 mice (Taconic Farms, Germantown, NY) were anesthetized with 80 μ g/g of ketamine (Phoenix Scientific, Inc. St. Joseph, MO) and 12 μ g/g xylazine (Ben Venue Laboratories, Bedford, Ohio) via intraperitoneal injection. Following resuscitation, animals received periodic re-dosing of 20 – 30% of the initial ketamine dose as required to maintain surgical anesthesia. Rectal temperature was monitored with a thermocouple (Omega Engineering Inc, Stamford, CT) and maintained at 37 ± 0.5 °C with a warming lamp. Using aseptic surgical technique, a 1cm sagittal incision was made over the ventral neck to expose the trachea which was orally-intubated with a 20 gauge

angiocath (Becton Dickinson, Franklin Lakes, NJ). The left femoral and right carotid arteries were then isolated and cannulated with 0.016" O.D. micro-cannula catheters (BioTime, Berkeley, CA) flushed with heparinized saline (100 IU/L) prior to insertion. Carotid arterial blood pressure was measured via a disposable blood pressure transducer (Hospira, Lake Forest, IL). Mechanical ventilation (Scireq Scientific Respiratory Equipment Inc, Montreal, CA) was maintained with a tidal volume of 12.5 μ L/g, a respiratory rate of 110 breaths per min, a FiO_2 of 1.0, and a positive end-expiratory pressure of 2 cm H_2O . In-line end-tidal CO_2 (ETCO_2) was measured by continuous microcapnography (Columbus Instruments, Columbus, OH). Needle probes provided 3-lead ECG measurements. All continuous physiologic signals were acquired using PowerLab Chart (ADInstruments, Colorado Springs, CO).

2.2. HS Protocol and Experimental Groups

A highly-lethal model of pressure-controlled HS, based on that of Wiggers and others, was developed with a target short-term mortality of 50%.²⁰ Following a 30 min baseline (BL) equilibration period, mice meeting the inclusion criteria of a mean arterial pressure (MAP) ≥ 70 mmHg underwent a prescribed bleed (2.25mL/100g over 10 min) via the femoral artery cannula into a heparinized syringe pump (Harvard Apparatus, Holliston, MA). MAP was then maintained at 35 ± 5 mmHg for 90 min through further withdrawal or autotransfusion of shed arterial blood via the syringe pump. After 90 min of shock (S90), animals received all shed blood remaining within the syringe pump followed by intravenous infusion with L-lactated Ringer's solution (Baxter, Deerfield, IL) at a volume of three times the initial shed blood volume. After 30 min of shock, animals ($n = 52$) were randomly assigned to therapeutic hypothermia (TH, 33 ± 0.5 °C) or normothermia (NT, 37 ± 0.5 °C). TH animals were cooled over 5 min through the application of 70% isopropyl alcohol to their ventral surface followed by hand fanning. At 60 min following the start of resuscitation (R60), TH animals were rewarmed over 20 min and maintained at 37 ± 0.5 °C. Animals were monitored for up to 180 min (R180) following start of resuscitation. Non-survivors were identified as animals with values of MAP ≤ 20 mmHg persisting for greater than 1 min.

2.4. ELISA Methods

Whole hearts, ileum, and plasma were harvested at the end of the protocol from surviving animals in the NT ($n = 5$) and TH ($n = 5$) study groups. A separate group of animals was randomized to NT or TH and sacrificed at BL ($n = 5$), S90 ($n = 10$, or 5 per HT or NT group), and R60 ($n = 10$, or 5 per group) for tissue and plasma sampling. Samples were snap frozen in liquid nitrogen and stored at -80 °C.

Tissues were pulverized to a fine powder under liquid nitrogen and then added to a lysis buffer consisting of 1X PBS, Protease Inhibitor Cocktail tablet (Roche, Pleasanton, CA), and 0.1% Triton X100 (Sigma-Aldrich, St. Louis, MO). After 15 min of ice incubation, samples were centrifuged at 13,000 rpm for 10 min. The resulting supernatant was transferred to a fresh tube and stored at -80 °C until assayed. Blood samples were collected and centrifuged for 10 min at 13,000 rpm. Plasma was then transferred to microcentrifuge tubes and stored at -80 °C.

Measurement of plasma and tissue for visfatin, IL-6, TNF- α , and KC were made using enzyme-linked immunosorbent assay (ELISA) antibodies (Biosource International, Camarillo, CA; MBL International, Woburn, MA; R&D Systems, Minneapolis, MN) according to manufacturer's guidelines. Plasma myoglobin was measured using an ELISA kit (Life Diagnostics Inc., Westchester, PA).

2.5. Statistical Methods

Statistical computations were performed using GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA) with significance levels of $p < 0.05$ except where noted. Continuous data were

described by means and standard error measures. Treatment group differences were identified by Mann-Whitney U testing. Within group differences between selected time points were identified by Kruskal-Wallis ANOVA by ranks with post-hoc Dunn's testing for multiple comparisons. Kaplan-Meier survival comparisons were made using log-rank (Mantel-Cox) test.

3. RESULTS

3.1. Survival and Hemodynamic Outcomes

A total of 52 animals weighing an average of 29.5 ± 2.3 g were instrumented and randomized to NT or TH. Baseline hemodynamics and temperatures prior to randomization were indistinguishable between groups (Table 1). At S90, TH animals displayed lower heart rate and ETCO_2 than NT animals ($p < 0.05$). In addition, TH animals required less autotransfusion of shed blood by S90 ($p < 0.001$). At R60, following resuscitation and re-warming, TH animals displayed higher MAP (Figure 1) with lower heart rates and ETCO_2 ($p < 0.05$). Group differences in MAP and heart rate persisted throughout the post-resuscitation period. Figure 2 depicts the pattern of mortality within the NT group with non-survivors surviving a median of 118 min (IQR, 104.5 – 156.5) following the start of resuscitation. All animals survived to S90. However, survival to the R180 time point was significantly higher in the TH group compared to NT [23/26 (88.5%) vs. 13/26 (50%), $p = 0.001$].

3.2. Plasma Cytokines

In both groups, circulating plasma visfatin, IL-6, KC, and TNF- α concentrations increased relative to BL by S90 (Figure 3) and remained elevated at R60 ($p < 0.05$). The TH group exhibited lower plasma visfatin concentrations at S90 relative to the NT comparison group ($p < 0.05$). Concurrently, plasma TNF- α concentrations trended higher ($p = 0.09$) in TH compared to NT while IL-6 concentrations trended lower ($p = 0.15$). By R180, plasma visfatin and IL-6 remained elevated in both groups while TNF- α returned to baseline. In the TH group, plasma IL-6 and KC at R180 were significantly lower than the NT comparison group ($p < 0.05$).

3.3. Cardiac Tissue Cytokines

Cardiac tissue visfatin (Figure 4) was not significantly modulated by S90 or R60. However by R180, heart visfatin decreased in the NT group relative to baseline ($p < 0.05$) but was maintained near baseline in the TH group. Heart IL-6 expression increased over baseline by R180 ($p < 0.05$) and was not significantly modulated by TH. Heart KC increased by S90 in the TH group with a similar trend in NT. Heart KC remained elevated in the NT group until R180. By contrast, heart KC decreased to baseline in the TH group. Heart TNF- α expression was not significantly modulated across the shock and resuscitation protocol.

3.4. Ileal Tissue Cytokines

Visfatin content in the ileum (Figure 5) was attenuated relative to baseline by R180 in both groups ($p < 0.05$) and not altered by temperature. Ileal IL-6 was elevated by R180 ($p < 0.05$) with a trend towards lower expression values in TH treated animals. Ileal KC was elevated at S90 in TH animals relative to baseline ($p < 0.05$). By R60, ileal KC was increased over baseline in both groups and remained elevated at R180 ($p < 0.05$). Though elevated over baseline, ileal KC values were significantly lower in TH, as compared to NT, at R180 ($p < 0.05$). TNF- α expression in the ileum exhibited a trend towards increased expression during shock and resuscitation which did not reach significance.

3.5. Plasma Myoglobin

Plasma myoglobin concentrations in both groups were elevated over BL at R180 ($p < 0.05$), consistent with cardiac injury. At R180, TH animals displayed significantly lower plasma myoglobin than NT animals (2043 ± 227 ng/ml vs. 5104 ± 200 ng, $p < 0.05$).

4. DISCUSSION

In this study, we demonstrate the hemodynamic benefits of TH in a highly-lethal mouse model of pressure-controlled HS. We also provide the first report that plasma visfatin is modulated by HS and resuscitation. Furthermore, our results suggest that TH attenuates plasma visfatin during the early phases of HS, prior to resuscitation, while maintaining heart tissue visfatin content at later time points following resuscitation. In addition, we demonstrate that TH modulates the cytokine profiles of plasma IL-6 and KC, and ileal tissue KC at later time points following resuscitation. Finally, we show that TH reduces plasma myoglobin release and thus may have direct cardioprotective effects.

4.1. Effect of TH on Hemodynamics

Our results demonstrate that the induction of TH, prior to resuscitation, improves hemodynamics during the shock period. Enhanced shock tolerance has been noted in previous models of pressure-controlled HS in larger species^{9, 10} and in a variety of studies in models of unresuscitated HS^{12, 21} and thus suggest a role for TH as a temporizing therapy during the pre-resuscitation period. TH also appears to provide hemodynamic benefit for at least 2 hours following rewarming, which is consistent with previous reports in a limited number of studies performed in larger species.^{10, 13} While not modeled by our study, TH has also been shown to improve long-term survival rates in a variety of shock models with and without associated trauma.^{11, 13, 14}

The timing of TH induction during the shock period, prior to resuscitation, may be an important aspect of this therapy. In one pressure-controlled model of HS, TH instituted just 5 min following the start of resuscitation resulted in lower post-resuscitation heart rates, but did not improve mean arterial pressure or 72h survival rates.²² The impact of TH duration is less clear as in one study of pressure-controlled shock, animals treated with 75 min of TH (34 °C) followed by a prolonged 12h period of mild hypothermia (35 °C) showed a trend towards improved survival at 72h relative to animals receiving just 75 min of TH.¹⁰ A separate model of trauma-hemorrhage where animals were allowed to develop moderate hypothermia (30 °C) spontaneously during the hemorrhage period had contrasting results.^{23, 24} In that study, animals rewarmed to 37 °C during resuscitation exhibited improved cardiac output and left-ventricular contractility at 4 and 24 hours as compared to animals maintained at 32 °C for 240 min following resuscitation.^{23, 24} Differences in outcome between this model and ours may, in part, reflect different physiological responses to mild versus moderate and induced versus spontaneous hypothermia.

While the induction of TH prior to full resuscitation presents challenges to the clinician, we considered the translational relevance of such a protocol prior to embarking on this study. Specifically, we considered a significant literature regarding resuscitation regimens utilizing permissive hypotension or low-volume partial resuscitation strategies.²⁵⁻²⁸ Permissive hypotension strategies are somewhat analogous to, though arguably less severe than, the pressure-controlled shock period utilized in the present study and thus could be to include the induction of TH prior to full resuscitation.

4.2. Effect of TH on Plasma Cytokines

The release of plasma cytokines is an early feature of the innate immune response to HS injury.²⁹ Clinical studies suggest that persistent elevations in specific cytokines, such as IL-6, may correlate with injury severity, development of organ failure, and survival outcomes in the setting of trauma.³⁰ The rapid and sustained release of plasma IL-6 during the shock and resuscitation periods in our study is consistent with results from an earlier study in a rat model of combined trauma-hemorrhage.³¹ Work by others has also suggested trends towards attenuation of plasma IL-6 at 1h following resuscitation in TH treated animals.^{11, 13} In one animal study of volume-controlled HS without trauma, hypothermia maintained throughout the resuscitation period was associated with attenuation of IL-6 at 120 min.³² Our results extend this literature by demonstrating that TH-attenuation of IL-6 is sustained for 2h following rewarming. In addition to its well-described role in mediating the transition from innate to adaptive immunity,³³ IL-6 can also directly produce negative inotropic or chronotropic effects in the setting of HS.³⁴ Thus treatment-related differences in the expression of IL-6 cytokines could contribute to the observed differences in hemodynamics noted at R180.

In agreement with our results, the chemokine KC has previously been shown to be elevated in the plasma following resuscitation in a mouse model of trauma-hemorrhage.³⁵ Our work adds to this literature by demonstrating that plasma KC concentrations increase during shock prior to resuscitation. In addition, this is the first study to report attenuation of plasma KC by TH in the setting of HS. Functionally, such attenuation could limit the diapedesis of polymorphonuclear (PMN) cells into vital organs.³⁵

The rapid rise and fall of plasma TNF- α in our model is consistent with its reported hyper-acute kinetics.³¹ The observed trend towards higher plasma TNF- α at 1h following resuscitation in the TH group is consistent with reports in rat models exploring the benefits of TH in the setting of HS.^{11, 13} Furthermore, our findings are consistent with data from cultured monocytes showing that hypothermia (32 °C) potentiates TNF- α promoter activity and mRNA accumulation.³⁶ Given that exogenous TNF- α has been shown to pharmacologically-mimic cardioprotective preconditioning in I/R models,³⁷ the treatment-related trend towards elevated TNF- α during the shock period could be beneficial.

4.3. Effect of TH on tissue cytokine expression

The observed effect of shock and resuscitation on heart and gut cytokines is consistent with previous lab reports.^{3, 38} IL-6, which is minimally expressed in the healthy heart, is upregulated by a variety of overload or injury conditions including HS.^{3, 38} The functional impact of elevated cardiac tissue cytokines on cardiovascular outcomes is poorly understood. However, recent work in cardiomyocytes, freshly isolated from hearts following trauma-hemorrhage, suggests an inverse relationship between cardiomyocyte IL-6 levels and cardiac function.³

This is one of the first reports to explore heart or gut KC following HS and the first to note differential attenuation of ileal KC expression in TH treated animals. While the role of tissue KC is not well-understood, such attenuation could impact inflammation and barrier function of the gut by limiting subsequent PMN infiltration. This finding is particularly relevant to the small bowel, which has a preponderance of tissue-resident macrophages, as it has often been posited as a major source of circulating cytokines following HS or gut I/R injury.³⁹ The lack of myocardial TNF- α expression in our model is surprising as investigators in a murine model of trauma-hemorrhage have demonstrated that left-ventricular dysfunction at 1h following resuscitation, which was associated with elevated expression of myocardial TNF- α , could be reduced through the administration of anti-TNF antibodies.³⁸

4.4. Modulation of Visfatin in HS

Visfatin is a recently described cytokine which exhibits both extracellular proinflammatory cytokine effects and also functions as an intracellular enzyme involved in the NAD⁺ salvage pathway.⁴⁰ Elevated plasma visfatin is associated with a variety of disease states including rheumatic disease, obesity, diabetes mellitus, coronary artery disease, acute coronary syndrome, and acute lung injury.⁴¹⁻⁴³ We have also previously demonstrated that visfatin produces vascular dysfunction⁴⁴ and is also involved in inflammatory lung injury.^{45, 46} We extend this literature as the first report of plasma visfatin in HS. As an extracellular cytokine, visfatin stimulates expression of a variety of inflammatory cytokines^{45, 46} and may sustain inflammation through inhibition of neutrophil apoptosis⁴⁷ as well as directly serving as a neutrophil chemotactic agent.⁴⁶ Thus, attenuation of plasma visfatin by TH during the shock period, which occurs at a much earlier time point than that of IL-6 or KC, could serve to limit the later innate immune response thus improving outcomes.

Visfatin has also been identified as the intracellular enzyme, nicotinamide phosphoribosyl transferase (Nampt), which plays a vital role in the coenzyme nicotinamide adenine dinucleotide (NAD) salvage pathway.^{47, 48} The reduction in cardiac visfatin noted in our normothermic group following resuscitation is consistent with reports of similar decreases in models of focal ischemia and simulated ischemia⁸ and could impair cellular bioenergetics and thus contribute to cardiovascular collapse during the early phases of resuscitation. Conversely, the preservation of visfatin protein expression in the heart by TH could lead to the maintenance of intracellular NAD⁺ and thus could represent a possible mechanism of hypothermic protection.

4.4. Other putative mechanisms of TH protection

In addition to group cytokine differences, several other putative mechanisms may contribute to the observed treatment benefits of TH. For example, the observed improvements in hemodynamic indices could simply reflect the direct impact of temperature on metabolism which has been shown to decrease total body and myocardial O₂ consumption along with myocardial blood flow during HS.⁹ In addition, improved hemodynamics could reflect unmeasured changes in stroke volume, ventricular preload, and systemic vascular resistance that are mediated, in part, by the sustained effects of temperature on central autonomic and intrinsic vascular reflexes.⁹

4.5 Limitations

While TH improves hemodynamics and alters cytokine profiles in our model, additional studies are needed to establish a mechanistic connection between early cytokine modulation and improved hemodynamic outcomes. Furthermore, while visfatin exhibits a prominent response in our model, it is unclear whether it represents a vital link in the innate immune response to HS or merely an epiphenomenal biomarker. Additional studies will be necessary to isolate the role of visfatin in HS and TH protection.

5. CONCLUSIONS

HS triggers a robust cytokine response prior to resuscitation which can be modified at both the humoral and tissue levels by the induction of TH prior to resuscitation. TH attenuates the early release of circulating visfatin while preserving its expression in the heart following resuscitation. Such modulation could serve to attenuate the innate immune response to HS and potentially improve myocardial energetics and function. Visfatin may represent a therapeutic target for improving outcomes following shock and resuscitation.

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Figure 1.

Average ($\pm$ S.E.M.) mean arterial pressure (MAP) for NT and TH groups. Following baseline period, animals underwent a controlled-hemorrhage to a MAP of 35 mmHg which was maintained for 90 min (S90). TH was induced after 30 min of shock (S30) and maintained until 60 min after resuscitation (R60). Note that group size decreases due to mortality following resuscitation as depicted in Figure 2.

Figure 2.

Kaplan-Meier survival curves of TH and NT groups. TH improved survival (*) by log-rank survival analysis ($p = 0.001$).

Figure 3.

Plasma visfatin, IL-6, KC, and TNF- α expression (n = 5 each time point) displayed as mean $\pm$ standard error. Significant differences relative to baseline (*) and between NT and TH (#) are noted (p<0.05).

Figure 4.

Cardiac tissue visfatin, IL-6, KC, and TNF- α expression (n = 5 each point) displayed as mean $\pm$ standard error. Significant differences relative to baseline (*) are noted (p < 0.05).

Figure 5.

Ileum tissue visfatin, IL-6, KC, and TNF- α expression (n = 5 each point) displayed as mean $\pm$ standard error. Significant differences relative to baseline (*) and between NT and TH (#) are noted (p<0.05).

Table 1

Characteristics of normothermia (NT) and therapeutic hypothermia (TH) groups

		NT (n=26)	TH (n=26)
S0	Temperature, °C	36.9 ± 0.2	36.9 ± 0.3
	MAP, mmHg	81.8 ± 7.3	80.1 ± 7.5
	HR, bpm	259 ± 46	264 ± 40
	ETCO ₂ , mmHg	40.1 ± 4.7	40.7 ± 4.0
	Shed Blood Volume, mL/kg	19.2 ± 2.0	19.5 ± 2.1
S90	Temperature, °C	37.0 ± 0.2	33.0 ± 0.1 [†]
	MAP, mmHg	34.7 ± 1.2	35.0 ± 1.1
	HR, bpm	508 ± 69	331 ± 71 [†]
	ETCO ₂ , mmHg	40.2 ± 6.2	29.7 ± 3.2 [†]
	Autotransfusion Volume, mL/kg	8.0 ± 4.9	1.7 ± 4.3 [†]
R60	Temperature, °C	37.0 ± 0.2	33.0 ± 0.3 [†]
	MAP, mmHg	43.1 ± 12.6	57.1 ± 8.6 [†]
	HR, bpm	533 ± 88	342 ± 65 [†]
	ETCO ₂ , mmHg	37.8 ± 4.8	32.4 ± 4.2 [†]
R180	Temperature, °C	37.1 ± 0.3	37.0 ± 0.3
	MAP, mmHg	30.5 ± 12.8	43.6 ± 13.2 [†]
	HR, bpm	585 ± 66	538 ± 81 [†]
	ETCO ₂ , mmHg	31.9 ± 6.7	36.2 ± 6.6
	Survival, n (%)	13 (50.0)	23 (88.5) [†]

[†]P < 0.05 between groups. Values displayed as mean ± standard deviation except where noted.