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EXOGENOUS NITRIC OXIDE INDUCES PROTECTION DURING HEMORRHAGIC SHOCK

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Abstract

This study analyzed the systemic and microvascular hemodynamic changes related to increased nitric oxide (NO) availability during the early phase of hemorrhagic shock. Hemodynamic responses to hemorrhagic shock were studied in the hamster window chamber. Exogenous NO was administered in the form of nitrosothiols (nitrosylated glutathione, GSNO) and was given prior the onset of hemorrhage. Moderate hemorrhage was induced by arterial controlled bleeding of 50% of the blood volume, and the hypovolemic shock was followed over 90 min. Animals pre-treated with GSNO maintained systemic and microvascular conditions during hypovolemic hemorrhagic shock, when compared to animal treated with glutathione (GSH) or the sham group. Low concentrations of NO released during the early phase of hypovolemic shock from GSNO mitigated arteriolar vasoconstriction, increased capillary perfusion and venous return, and improved cardiac function (recovered of blood pressure and stabilized heart rate). GSNO's effect on resistance vessels influenced intravascular pressure redistribution and blood flow, preventing tissue ischemia. In conclusion, increases in NO availability during the early phase of hypovolemic shock, could preserve cardiac function and microvascular perfusion, sustaining organ function. Direct translation into a clinical scenario may be limited, although the pathophysiological importance of NO in the early phase of hypovolemia is clearly highlighted here.

Keywords

Microcirculation; hemorrhage; glutathione; nitrosoglutathione; functional capillary density

INTRODUCTION

Cardiovascular adaptation to hemorrhagic shock is dynamically controlled by the sympathetic and parasympathetic systems.¹ Different circulating endocrine and local paracrine factors such as nitric oxide (NO) have been postulated to modulate the cardiovascular responses to hypovolemia.² Excessive formation of NO was reported to be associated with the vascular hyporeactivity induced during hypovolemia, whereas myocardial autonomic dysfunction has a crucial role during the decompensation stage.^{3,4} There is increasing evidence that NO produced both during and after ischemia, may be an important factor in the pathogenesis of ischemic injury.⁵

NO production keeps the vasculature relaxed, regulating blood pressure and tissue perfusion. In addition, NO modulates synaptic signaling, cellular defense and mitochondria oxygen

utilization.⁶ It also regulates biological processes by modifying amino acid residues in peptides and proteins, specially sulfhydryl groups (thiols) of cysteine residues.^{7,8} The NO short half-life limits its biological activity, leading to the proposal, that binding NO to carrier molecules is more stable and active, than free NO gas, because NO can be released at its site of action.^{9,10} Low MW thiols such as glutathione (GSH) react with NO or oxides of nitrogen to form nitrosothiols (RS-NOs) and are likely intracellular NO-carriers.^{11,12} High MW thiols, such as albumin, act as intravascular NO storages.^{9,13,14}

The objectives of the current study were: i) to determine any protective effect of exogenous NO, stabilized in the form of low MW RSNO during hypovolemic shock; ii) to define the basic mechanisms to potential therapeutic applications of exogenous NO donors as an alternative—to restore microvascular perfusion, in the absence of volume resuscitation. To examine these objectives, we pretreated our experimental hamster model with nitrosylated GSH (GSNO) or GSH prior to being subjected to a severe hemorrhagic shock (50% of blood volume). Systemic and microvascular parameters were studied up to 90 min after hemorrhage.

METHODS

Materials

Nitrosylated GSH (GSNO) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MI).

Animal Preparation

Investigations were performed in 55 – 65 g male Golden Syrian Hamsters (Charles River Laboratories, Boston, MA) fitted with a dorsal skinfold window chamber. Animal handling and care followed the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the local animal care committee. The hamster window chamber model is widely used for microvascular studies in the unanesthetized state, and the complete surgical technique is described in detail elsewhere.^{15,16} The experimental animal was allowed at least 2 days for recovery before the preparation was assessed under the microscope for any signs of edema, bleeding, or unusual neovascularization. Animals were anesthetized again, and arterial and venous catheters filled with a heparinized saline solution (30 IU/ml) were implanted. Catheters were tunneled under the skin, exteriorized at the dorsal side of the neck, and securely attached to the window frame. The microvasculature was examined 3 to 4 days after the initial surgery and only animals with window chambers whose tissue did not present regions of low perfusion, inflammation, and edema were entered into the study.¹⁷

Inclusion Criteria

Animals were suitable for the experiments if: 1) systemic parameters were within normal range, namely, heart rate (HR) > 340 beat/min, mean arterial blood pressure (MAP) > 80 mmHg, systemic Hct > 45%, and arterial oxygen partial pressure (PaO₂) > 50 mmHg; and 2) microscopic examination of the tissue in the chamber observed under a ×650 magnification did not reveal signs of edema or bleeding. Hamsters are a fossorial species with a lower arterial PO₂ than other rodents due to their adaptation to the subterranean environment. However, microvascular PO₂ distribution in the chamber window model is the same as in other rodents.¹⁷

Acute severe hemorrhage protocol

Acute hemorrhage was induced by withdrawing 50% of estimated total blood volume (BV) via the carotid artery catheter within 5 min. Total BV was estimated as 7% of body weight. Animals were followed over 90 min after hemorrhage induction.

Experimental groups

Animals were randomly divided into three experimental groups after baseline assessment, according to a sorting scheme based on a list of random numbers.¹⁸ Animals received group treatment 30 minutes before hemorrhage in 100 μ l normal saline: 1) **GSNO**, 1 mg/kg nitrosoglutathione (GSNO, Sigma, St Louis, Mo, USA); 2) **GSH**, 1 mg/kg glutathione (GSH, Sigma, St Louis, Mo, USA); 3) **Sham**, volume control only the vehicle (normal saline). 30 minutes before hemorrhage in 100 μ l of normal saline.

Eighteen animals were entered into the study, they were randomly assigned to the following experimental groups: GSNO (n = 6); GSH (n = 6); and Sham (n = 6). Systemic data for baseline and shock were obtained by combining all experimental groups.

Systemic Parameters

MAP and heart rate (HR) were recorded continuously (MP 150, Biopac kSystem; Santa Barbara, CA). Hct was measured from centrifuged arterial blood samples taken in heparinized capillary tubes (25 μ l, ~50% of the heparinized glass capillary tube is filled). Hb content was determined spectrophotometrically from a single drop of blood (B-Hemoglobin, Hemocue, Stockholm, Sweden).

Blood Chemistry and Biophysical Properties

Arterial blood was collected in heparinized glass capillaries (0.05 ml) and immediately analyzed for PaO₂, PaCO₂, base excess (BE) and pH (Blood Chemistry Analyzer 248, Bayer, Norwood, MA). Hamsters are a fossorial species with a lower arterial PO₂ than other rodents, due to their adaptation to the subterranean environment.¹⁹ However, microvascular PO₂ distribution in the chamber window model is the same as in other rodents, such as mice.¹⁷

Microvascular Experimental Setup

The unanesthetized animal was placed in a restraining tube with a longitudinal slit from which the window chamber protruded, then fixed to the microscopic stage of a transillumination intravital microscope (BX51WI, Olympus, New Hyde Park, NY). The animals were given 20 min to adjust to the change in the tube environment before measurements. The tissue image was projected onto a charge-coupled device camera (COHU 4815) connected to a videocassette recorder and viewed on a monitor. Measurements were carried out using a 40X (LUMPFL-WIR, numerical aperture 0.8, Olympus) water immersion objective. The same sites of study were followed throughout the experiment so comparisons could be made directly to baseline levels.

Functional Capillary Density (FCD)

Functional capillaries, defined as those capillary segments that have RBC transit of at least one RBC in a 45s period in 10 successive microscopic fields were assessed, totaling a region of 0.46 mm². Each field had between two and five capillary segments with RBC flow. FCD (cm⁻¹), i.e., total length of RBC perfused capillaries divided by the area of the microscopic field of view, was evaluated by measuring and adding the length of capillaries that had RBC transit in the field of view. The relative change in FCD from baseline levels after each intervention is indicative of the extent of capillary perfusion.¹⁷

Microhemodynamics

Arteriolar and venular blood flow velocities were measured online by using the photodiode cross-correlation method²⁰ (Photo Diode/Velocity Tracker Model 102B, Vista Electronics, San Diego, CA). The measured centerline velocity (V) was corrected according to vessel size to obtain the mean RBC velocity.²¹ A video image-shearing method was used to measure vessel diameter (D).²² Blood flow (Q) was calculated from the measured values as $Q = \pi \times V (D/2)^2$. Changes in arteriolar and venular diameter from baseline were used as indicators of a change in vascular tone. This calculation assumes a parabolic velocity profile and has been found to be applicable to tubes of 15 – 80 μm internal diameters and for Hcts in the range of 6 – 60%.²¹

Data analysis

Results are presented as mean $\pm$ standard deviation. Data within each group were analyzed using analysis of variance for repeated measurements (ANOVA, Kruskal-Wallis test). When appropriate, post hoc analyses were performed with the Dunns multiple comparison test. Microhemodynamic measurements were compared to baseline levels obtained before the experimental procedure. The box-whisker plot separates the data into quartiles, with the top of the box defining the 75th percentile, the line within the box giving the median, and the bottom of the box showing the 25th percentile. The upper “whisker” defines the 95th percentile; the lower whisker, the 5th percentile. Microhemodynamic data are presented as absolute values and ratios relative to baseline values. A ratio of 1.0 signifies no change from baseline while lower and higher ratios are indicative of changes proportionally lower and higher than baseline (i.e., 1.5 would mean a 50% increase from the baseline level). The same vessels and capillary fields were followed so that direct comparisons to their baseline levels could be performed, allowing for more robust statistics for small sample populations. Kurtosis test was used to characterize the relative peakedness or flatness of microvascular measurements compared to the normal distribution. Positive Kurtosis indicates a relatively peaked distribution; negative Kurtosis indicates a relatively flat distribution. Skewness test was used to characterize the degree of asymmetry of microvascular measurements around its mean. Positive skewness indicates a distribution with an asymmetric tail extending towards more positive values; negative skewness indicates a distribution with an asymmetric tail extending towards more negative values. Statistics were calculated using GraphPad Prism 4.01 (GraphPad Software, Inc., San Diego, CA). Changes were considered statistically significant if $P < 0.05$.

RESULTS

Prehemorrhage

Blood gas parameters are presented in detail on Table 1. Changes in MAP and HR after infusion of GSNO and GSH are presented in Figure 1. No statistically significant changes in MAP or HR were observed after infusion. GSNO and GSH did not affect arterial blood gas parameters or acid base balance.

Microvascular diameter and blood flow after GSNO and GSH are presented in Figure 2. Arteriolar and venular diameters changes were not significantly affected by the infusion of GSNO and GSH when compared to Sham or baseline. Arteriolar and venular flows were not statistically significantly affected by the infusion of GSNO and GSH. Location and variability (Kurtosis and Skewness, K/S) of microhemodynamics were slightly affected after infusion: GSNO, arteriolar: diameter (0.04/–0.28) and flow (–0.02/–0.11) and venular diameter (–0.34/–0.29) and flow (–0.01/–1.22); GSH: arteriolar: diameter (0.02/–1.20) and flow (–0.29/–1.12) and venular diameter (–0.37/–0.90) and flow (–0.12/–0.80); compared to Sham: arteriolar: diameter (–0.28/–0.64) and flow (0.29/0.86) and venular diameter (–0.27/–0.78) and flow (0.04/1.21). Analysis of this result indicates a non-consistent response in diameters with a

tendency to increase blood flow post-infusion of GSNO and GSH. FCD after infusion of GSNO and GSH are not significantly different from baseline (*GSNO*: 0.98 ± 0.06 ; *GSH*: 0.97 ± 0.07 ; *Sham*: 0.98 ± 0.5 of baseline).

Systemic and microvascular response to acute severe hemorrhage

Systemic hemodynamic and blood parameters are presented in detail on **Table 2**. All animals tolerated the entire hemorrhage shock protocol without visible signs of discomfort.

Hemorrhage statistically reduced Hct and Hb from baseline in all the experimental groups. The hamster hemorrhage model has low variability as noted by the consistency in the results observed during shock.

Changes in MAP and HR during the hemorrhagic shock are presented in Figure 3. Hemorrhage and shock statistically decreased MAP from baseline in all groups. MAP for animals pretreated with GSH and GSNO presented the same recovery trend over time. 90 min after hemorrhage MAP was higher for GSH and GSNO than Sham ($P < 0.05$). During the early phase after hemorrhage (30 min and 60 min), HR was increased from baseline for GSH and Sham and remained unchanged for GSNO. 90 min after hemorrhage HR was significantly reduced for Sham when compared to GSH and GSNO. Animals pretreated with GSNO maintained stable HR not different from baseline.

Gas laboratory parameters and calculated acid-base balance are presented in Table 1. Hemorrhage decreased Hct and Hb in all groups. Different pretreatments did not affect Hct and Hb after hemorrhage. Hemorrhagic shock significantly decreased arterial pH and pCO_2 .

Microvascular diameter and blood flow are presented in Figure 4. Arteriolar and venular diameters were significantly constricted from baseline during hemorrhagic shock for all groups. Microvascular flows were significantly decreased from baseline for all groups, at all time points after hemorrhage. Arteriolar flows decreased after the hemorrhage for GSH and Sham groups. Animals pretreated with GSNO maintained higher arteriolar flows at 60 and 90 min after hemorrhage, than the GSH and Sham group.

Changes in capillary perfusion during the protocol are presented in Figure 5. FCD was significantly reduced after hemorrhage for all groups, at all time points. FCD decreased as a function of time in the Sham group. Animals pretreated with GSNO had higher FCD than Sham animal at 60 and 90 min, and higher than GSH animals at 90 min, respectively.

DISCUSSION

The principal finding of the study is that hemorrhagic shock pretreatment with GSNO maintains superior systemic and microvascular hemodynamic conditions than GSH or the vehicle (Sham group). The importance of exogenous stable NO, during anemic and hypovolemic conditions is mostly evidenced by the resulting cardiac performance and microvascular perfusion. Hemorrhage is responsible for many deaths within the first few hours, possibly within the first few minutes, thus our findings relate to scenarios where an increase in NO bioavailability could make a difference in survival until volume resuscitation is available. Our findings support the hypothesis that severe hemorrhage induces vascular decompensation associated with low availability of NO in the early phase of hemorrhage, and that NO supplementation during this phase reduces the risks of profound hemorrhage and may prevent circulatory arrest. Several limitations of the current research include those inherent to hemorrhage pre-treatment. Additionally, direct translation of this hypothesis into the clinical scenario, was not included in the objectives of the current study. However, future studies focusing in this arena would be very promising.

Exogenous NO in the form of GSNO maintained systemic and microvascular conditions during hypovolemic hemorrhagic shock, via the superior maintenance of systemic and microhemodynamic function. Perfusion maintenance during hypovolemia in animals pretreated with GSNO could be initially explained by the reduction of precapillary resistance when compared to animals treated with only GSH or the vehicle. This finding suggests that NO is released from GSNO during the early phase of the hemorrhage, which then prevented arteriolar constriction, increasing capillary perfusion and venous return. Additional to the microcirculatory effects, the NO released from GSNO prevented cardiac function disturbance and HR remained unchanged during hypovolemia. This result alone presents the importance of NO availability in the early phase of hemorrhage, to regulate heart contractility and contraction rate. NO's effect on resistance vessels directly influences pressure redistribution in the circulation, sustaining blood flow and preventing tissue ischemia. Furthermore, our results show that the principal factor in ensuring hemodynamic restoration by GSNO is not related to a volume effect since hematocrits were not different between groups.

The outcome of hemorrhagic shock is related to the degree of hypovolemia, the magnitude of acquired oxygen debt, and the delay in treatment. Monitoring the microcirculation is crucial in determining the effect of changes in intravascular volume in tissue hypoperfusion. Application of various techniques, including intravital microscopy, has shown the presence of major microcirculatory alterations during hemorrhage^{23–25}, the persistence of these microcirculatory alterations has been associated with multiorgan failure and death.²⁶ Severe hemorrhage induces vascular decompensation associated with low availability of NO in the early phase of hemorrhage, and NO supplementation partially decreases microcirculatory alterations. Based on previous studies during hypovolemic shock, we also believe that microvascular dysfunctions observed in the hamster window model, reflects the conditions of major organs and their compartments, due to anatomical location and analyzed circulatory regulation.¹⁷ Notably, after the discovery of NO as an important regulator of vascular tone, the role of NO during shock was assumed to be negative and NOS inhibitors were evaluated to prevent, revert, or at least minimize hypotension induced during shock, with negative outcome.^{27,28}

Results show a strong correlation between cardiac stability and microvascular flow. Central cardiac effects could reflect increases in cardiac output mediated by lower arteriolar afterload and increased preload (venous return), due to NO vasodilatory properties and cardiac chronotropic effects.²⁹ During hypovolemia compensatory mechanisms attempt to restore blood pressure by increasing systemic vascular resistance through arteriolar constriction, as observed in the Sham group. Contrarily, animals treated with GSNO increased perfusion through dilatation, vascular recruitment and collateral arterio-venous shunts, which facilitates pressure transmission from the arterial system to the venous circulation, and creates a favorable pressure gradient to increase venous return. NO chronotropic effects may be due to decreased venous constriction, favoring cardiac filling.²⁹ A number of recently published studies provide evidence that exogenous NO can contribute to ischemic preconditioning.³⁰

The results presented in Figure 3 showed that exogenous NO maintained central hemodynamic function by preventing rhythm disturbances, which has been observed before, implying a protective role of NO on cardiac over-drive and pacing.³¹ Exogenous NO attenuated microvascular complications during hypovolemia, which targets a pivotal protective function by maintaining tissue perfusion, assuring wash out of metabolic residues preventing future damage during reperfusion. Short term fluctuations of HR reflect the dynamic responses of the cardiovascular control system to naturally occurring perturbations.

In conclusion, this study shows that severe hemorrhage induces vascular decompensation due to low availability of NO during the early phase of hemorrhage, and that exogenous NO during

this stage can prevent circulatory arrest and further multiorgan failure. Mechanistically, partial preservation of microvascular perfusion during hypovolemic shock can be obtained by increasing NO bioavailability, which acts through vascular tone regulation and pressure redistribution, maintaining capillary pressure and metabolite exchange. NO has also a fundamental signaling role in cellular function with implications in cardiac chronotropic function that appear to be as important as its well known vasodilator effects. Thus, suppression of NO bioavailability during hemorrhagic shock treatment may aggravate conditions, while the administration of exogenous NO donors may improve outcome.

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Figure 1.

Relative changes in mean arterial pressure (MAP) and heart rate (HR) after pretreatment for all groups prior hemorrhage. Broken line represents baseline level. MAP (mmHg, mean $\pm$ SD) for each animal group were as follows: Baseline: **SHAM**, 112 ± 7 , $n = 6$; **GSH**, 118 ± 6 , $n = 6$; **GSNO**, 114 ± 8 , $n = 6$. $n =$ number of animals. HR (bpm, mean $\pm$ SD) for each animal group were as follows: Baseline: **SHAM**, 432 ± 34 ; **GSH**, 428 ± 36 ; **GSNO**, 434 ± 32 .

Figure 2.

Relative changes in arteriolar and venular diameter and blood flow after pretreatment for all groups, prior hemorrhage. Broken line represents baseline level. Diameters (μm , mean $\pm$ SD) for each animal group were as follows: Baseline: **SHAM** (arterioles (A): 59.1 ± 9.4 , $n = 42$; venules (V): 59.9 ± 8.0 , $n = 44$); **GSH** (A: 58.1 ± 9.4 , $n = 44$; V: 56.8 ± 9.6 , $n = 47$); **GSNO** (A: 57.9 ± 8.6 , $n = 43$, V: 58.6 ± 8.9 , $n = 44$). n = number of vessels studied. RBC velocities (mm/s, mean $\pm$ SD for each animal group were as follows: Baseline: **SHAM** (A: 4.4 ± 1.1 , V: 2.4 ± 0.8); **GSH** (A: 4.4 ± 0.9 ; V: 2.4 ± 0.8); **GSNO** (A: 4.5 ± 0.8 ; V: 2.6 ± 0.6). Calculated flows (nl/s, mean $\pm$ SD) in **Figures 2E** (arteriolar) and **2F** (venular) for each animal group were as follows: Baseline: **SHAM** (A: 11.5 ± 3.6 ; V: 6.7 ± 2.3); **GSH** (A: 11.5 ± 3.1 ; V: 6.6 ± 2.0); **GSNO** (A: 11.0 ± 2.5 ; V: 6.5 ± 2.1).

Figure 3.

Relative changes in mean arterial pressure (MAP) and heart rate (HR) after hemorrhage for all groups. Broken line represents baseline level. †, $P < 0.05$ compared to *baseline*; ‡, $P < 0.05$ compared to *Sham*; §, $P < 0.05$. Baseline MAP and HR are presented in the legend for Figure 1.

Figure 4.

Relative changes in arteriolar and venular diameter and blood flow after pretreatment for all groups, prior hemorrhage. Broken line represents baseline level. †, P<0.05 compared to **baseline**; ‡, P<0.05 compared to **Sham**; ¥, P<0.05 compared to **GSH**; §, P<0.05. Baseline diameter, RBC velocity and blood flow are presented in the legend for Figure 2.

Figure 5.

Changes in Functional capillary density (FCD) during hypovolemic shock post hemorrhage for all groups. †, $P < 0.05$ compared to *baseline*; ‡, $P < 0.05$ compared to *Sham*; ¥, $P < 0.05$ compared to *GSH*; §, $P < 0.05$.

Table 1

Laboratory Parameters

	Baseline	Post Treatment			Shock 60 min		
		Sham	GSH	GSNO	Sham	GSH	GSNO
Hct, %	49 ± 1	49 ± 2	48 ± 2	49 ± 2	31 ± 2 [†]	30 ± 2 [†]	29 ± 2 [†]
Hb, g/dl	14.8 ± 0.4	14.6 ± 0.6	14.7 ± 0.8	14.7 ± 0.6	9.3 ± 0.7 [†]	9.1 ± 0.6 [†]	8.9 ± 0.7 [†]
PaO ₂ , mmHg	55.7 ± 5.9	54.6 ± 6.1	57.3 ± 5.6	55.8 ± 5.3	82.3 ± 6.1 [†]	78.3 ± 5.8 [†]	72.7 ± 6.5 ^{†§}
PaCO ₂ , mmHg	53.1 ± 4.2	52.9 ± 4.1	52.4 ± 4.8	51.8 ± 5.2	38.7 ± 6.2 [†]	40.8 ± 5.7 [†]	43.6 ± 5.8 [†]
Arterial pH	7.345 ± 0.014	7.342 ± 0.021	7.336 ± 0.018	7.352 ± 0.022	7.321 ± 0.023 [†]	7.326 ± 0.017 [†]	7.331 ± 0.018 [†]
BE, mmol/l	2.8 ± 1.2	2.1 ± 1.5	2.4 ± 1.3	2.9 ± 1.8	-3.6 ± 1.7 [†]	-1.8 ± 1.5 [†]	-0.8 ± 1.1 [†]

Sham, infused with saline; *GSH*, infused with GSH; *GSNO*, infused with GSNO. Values are means ± SD. Baseline included all the animals in the study. Hct, systemic hematocrit; Hb, hemoglobin content of blood; MAP, mean arterial blood pressure; PaO₂, arterial partial O₂ pressure; PaCO₂, arterial partial pressure of CO₂; BE, base excess. Shear rate of 160 s⁻¹ at 37°C; COP, colloid osmotic pressure at 27°C. Values are means ± SD.

[†] P<0.05 compared to *Baseline*

[§] P<0.05 compared to *Sham*.