Repletion of *S*-Nitrosohemoglobin Improves Organ Function and Physiologic Status in Swine Following Brain Death

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Structured Abstract

Objective—To determine if deficiency of nitric oxide bioactivity contributes to the physiologic instability that occurs following brain death and, if so, to also determine in this setting whether administration of a renitrosylating agent could improve systemic physiologic status.

Summary Background Data—Organ function following brain death is negatively impacted by reduced perfusion and increased inflammation; the magnitude of these responses can impact post-graft function. Perfusion and inflammation are normally regulated by protein *S*-nitrosylation but systemic assessments of nitric oxide bioactivity following brain death have not been performed.

Methods—Brain death was induced in instrumented swine by inflation of a balloon catheter placed under the cranium. The subjects were then serially assigned to receive either standard supportive care or care augmented by 20 ppm of the nitrosylating agent ethyl nitrite blended into the ventilation circuit.

Results—Circulating nitric oxide bioactivity (in the form of *S*-nitrosohemoglobin) was markedly diminished 10 h after induction of brain death—a decline that was obviated by administration of ethyl nitrite. Maintenance of *S*-nitrosohemoglobin was associated with improvements in tissue

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Conflicts of Interest

JDR has financial interests in Miach Medical Innovations and N30. JSS has financial interests in LifeHealth, N30, and Vindica Therapeutics. Miach is an early-stage medical device company, the others are all early stage biotech companies developing nitric oxide related technologies. These companies had no involvement in this study. There are no conflicts to report for the other co-authors.

List of Supplemental Digital Content

The supplemental digital content consists of a single file titled Yurcisin *et al* Supplemental Digital Content Ann Surg. The document contains additional physiologic and serum chemistry data.

blood flow and oxygenation, reductions in markers of immune activation and cellular injury, and with preservation of organ function.

Conclusions—In humans the parameters monitored in this study are predictive of post-graft function. As such, maintenance of endocrine nitric oxide bioactivity after brain death may provide a novel means to improve the quality of organs available for donation.

Introduction

Transplantation is an accepted intervention to correct innate organ failure but a successful outcome is highly dependent upon the physiologic status of the donor. On average, more than 75% of transplanted organs are recovered from deceased individuals, the majority of which are classified as brain dead heart beating donors (Organ Procurement and Transplantation Network; http://optn.transplant.hrsa.gov/). Five year survival rates for recipients of these organs range from 80% for kidneys to just over 50% for lungs; where comparisons can be made (kidney, liver) survival rates are significantly higher for those patients who received their new organ from a living donor[1, 2]. Stressors placed upon organs, particularly brain death, are believed to contribute to these survival rate differences. Stressors are also significant contributors to the poor procurement rate of suitable organs from _consented_ donors (<50%) due primarily to *in situ* organ failure[3].

The period during and following brain death is characterized by physiologic instability, the result first of intense sympathetic stimulation and significant release of catecholamines (the Cushing Response) with transient vasoconstriction, hypertension, and tachycardia, followed by the complete loss of sympathetic activity leading to hypotension and a profound reduction in systemic vascular resistance[4]. Hemodynamic homeostasis is regulated in part by nitric oxide (NO)[5]. A disruption of endocrine NO bioactivity may contribute to the tissue ischemia and organ damage that has previously been ascribed to loss of autonomic function. Post-brain death organ function can be further impaired by an inflammatory state[6], and this too may reflect a deficiency of NO[7].

Once brain death is confirmed, care of the patient switches from restorative to supportive as organ donation status is assessed[8]. Supportive care aims to maintain organ perfusion and, to some extent, the acidosis and systemic effects of brain death can be controlled by altering ventilation rates and/or administration of vasoactive agents. However, such interventions may have minimal ability to preserve end-organ oxygen delivery, which is primarily a function of local tissue perfusion. Local blood flow is "auto-regulated" at least in part through NO bioactivity derived from red blood cells[9, 10]. Hemoglobin (Hb) plays a central role in this auto-regulation by coupling the binding and release of oxygen with that of NO[11]. Hypoxic vasodilation by red blood cells is linked to Hb desaturation, which effects release of NO from thiols of Hb (*S*-nitrosoHb; SNO-Hb), thereby providing a regulated mechanism for matching blood flow (oxygen delivery) with local metabolic demand[5].

Decreased levels and/or impaired processing of SNO-Hb have been observed in diseases characterized by tissue hypoxemia[12–18]; where examined, red blood cells from these disparate patient populations exhibited impaired vasodilatory capacity. Such data suggest that red blood cell-derived NO bioactivity may play an important role in the respiratory cycle and that impairment of this activity might contribute to the pathophysiology of ischemic conditions. Based on these findings, we reasoned that a similar disruption of this endocrine function of red blood cells might occur following cessation of central nervous system activity, thus contributing to systemic hemodynamic instability. We further reasoned that in this setting an intervention directed towards increasing NO bioactivity could be beneficial.

Methods

This study was approved by the Duke University IACUC and all procedures complied with The Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act. Experiments were conducted on young adult swine with the experimental treatment cohort receiving 20 ppm of the *S*-nitrosylating agent ethyl nitrite (ENO; blended in nitrogen by Custom Gas Solutions of Durham, NC).

Surgical Instrumentation

Animals were sedated with acepromazine (0.8 mg/kg) and ketamine (20 mg/kg; both im) then anesthesia was induced with thiopental (10 mg/kg, iv). After endotracheal intubation, anesthesia was maintained with isoflurane (1.5 to 2.0%). Ventilatory settings were adjusted to maintain end-tidal CO_2 below 35 mm Hg. Isoflurane and end-tidal CO_2 concentrations were continually measured by an airway gas monitor (Datex Instrumentation Corporation, Helsinki Finland). Body temperature was maintained at 38 ($\pm$0.5) °C with a Bair-Hugger warming system (Arizant Healthcare Incorporated, Eden Prairie, MN). EKG leads were placed, vascular access was obtained at various sites (right internal jugular for the central line, femoral artery and vein for the peripheral lines), and bladder access was secured through a peritoneal incision. Hemodynamic parameters (cardiac output, arterial pressures, etc) were monitored using a Vigilance system from Edwards Lifesciences (Irvine, CA). After revealing the scalp, a small hole was bored for placement of an intra-cranial pressure (ICP) probe (Aesculap Inc. USA, Center Valley, PA) and a forehead sensor was placed to monitor brain electrical activity (Aspect Medical Systems, Inc. Norwood, MA). A second trepanation was conducted for insertion of a balloon catheter.

Brain Death

Brain death was induced by inflation of the balloon catheter over a period of 10–15 min until ICP exceeded systolic blood pressure. This state of elevated ICP was maintained throughout the study to keep cerebral perfusion pressure at 0 mm Hg. Loss of brain electrical activity was confirmed and then the vaporizer was turned off. When expired isoflurane concentration reached 0, a brain death assessment was conducted (pupils fixed and non-reactive to light, no lash or corneal touch response, absent oculo-cephalic reflex, absent vestibulo-ocular reflex following auricular washing with cold saline, no gag reflex, and no cough with tracheal suction). A ventilatory drive test was not conducted because we determined during model development that this procedure induced cardiac instability. ENO treatment was begun after confirmation of brain death and continued for 10 h. Cardiac instability was treated with fluids and pharmacologic therapy[19]; hormone replacement was not initiated. Ventricular fibrillation refractory to electro-cardioversion was cause for early study termination.

Physiologic Analyses

Tissue oxygenation and tissue Hb levels (the latter serving as a surrogate for tissue blood flow) measured in the buccal mucosa using a spectrophotometric monitoring system (T-Stat; Spectros Corporation, Portola Valley CA)[20]. Arterial and venous blood gas parameters were measured at 60 min intervals during the study using a Gem Premier 3000 (Instrumentation Laboratory, Lexington, MA) Additional venous blood samples were collected before and then again 10 h after the determination of brain death for clinical chemistry analyses (Antech Diagnostic; Cary, NC) and for determination of red blood cell SNO-Hb levels, the latter using mercury-coupled photolysis-chemiluminescence[21, 22].

Glomerular filtration rate (GFR) was estimated from the serum chemistry data using the following formula: $170 \times [\text{creatinine}]^{-0.999} \times [\text{blood urea nitrogen}]^{-0.170} \times [\text{albumin}]^{+0.318}$,

modified from Levey *et al*[23]. Inulin clearance was another methods used to measure kidney function: 60 mg/kg of inulin (Alfa Aesar, Ward Hill, MA) in 250 ml of normal saline was rapidly infused (iv), urine was collected for 1 h, and at the end of this collection period a venous blood sample was obtained; inulin clearance assessments were also conducted before and then started 9 h after brain death to account for the 60 min interval between inulin infusion and blood/urine collection. Blood and urine inulin levels were then quantitated using an established chromatographic method[24, 25].

Statistical Analyses

Data are presented as means ± standard deviations (SD) except for changes in oxygen content, which are presented as medians ± first and third quartile deviations. For finite parameters, testing involved paired t-tests to determine differences before and after brain death; where significant changes were identified analysis of variance (ANOVA) was used to test if the magnitude of the changes were different between groups.

To determine change in oxygen utilization, arterial and venous blood oxygen contents were calculated using the standard clinical formula ([blood oxygen saturation $\times$ Hb $\times$ 1.34] + [0.003 $\times$ blood oxygen partial pressure]) from which the difference was determined by subtracting the venous value from the arterial value. Subsequent changes in the A/V difference were determined by subtraction: $A/V_{experiment} - A/V_{baseline}$. Positive values indicate an decrease in venous blood oxygen content and thus an increase in oxygen utilization since arterial oxygen content was essentially constant; negative values reflect an increase in venous blood oxygen content and thus an reduction in oxygen utilization.

For continual parameters, median values were calculated at 60-min intervals and averaged to obtain group data for presentation. Area under the curve values for percent change from baseline of tissue oxygenation and tissue-Hb, and of the A/V differences were calculated and then tested for treatment effects using ANOVA. Tukey's test was used to identify group differences when the F statistic was significant. Linear regression, where noted, was conducted to test for temporal relationships. Comparisons were made using the Prism Graphpad software package (Graphpad, La Jolla, CA); p values of < 0.05 were considered significant.

Results

General Physiologic Status

A total of 39 adult swine were utilized for this study: 16 were employed for model development and initial ENO dose titration assessments; 3 developed intractable ventricular fibrillations and cessation of cardiac activity after induction of brain death but before the end of the study (2 in the control group and 1 in the ENO group); and 20 completed the study (n=10 per group). Data on the cardiovascular status for these latter swine are presented in Figure 1. Parameters for all animals that completed the study met the United Network for Organ Sharing guidelines, specifically cardiac output was > 3.8 l/min and mean arterial pressure was > 60 mm Hg. Ventilation was actively regulated to keep end-tidal CO_2 below 35 mm Hg and the PaO_2/FiO_2 ratio significantly higher than 300 mm Hg (data depicted in the Supplemental Digital Content Figure 1).

SNO-Hb, Brain Death, and Tissue Oxygenation

SNO-Hb levels were measured in venous blood samples obtained after securing vascular access and then again 10 h after the determination of brain death (Table 1). In the control cohort, SNO-Hb concentrations declined from 0.52 ± 0.68 to 0.21 ± 0.22 moles of SNO per moles of Hb tetramer $\times 10^{-3}$ (p=0.04 paired t-test; n=8 with two samples lost during

processing) whereas there was minimal decline in SNO-Hb in the 20 ppm cohort (0.38 ± 0.64 to 0.35 ± 0.15 SNO per Hb tetramer $\times 10^{-3}$; p>0.05, n=9 with one sample lost during processing). The decline in SNO-Hb in the control group was associated with alterations in oxygen utilization and local blood flow (Figure 2) as demonstrated by the changes from baseline in A/V oxygen content differences and buccal skeletal muscle bed tissue oxygenation and tissue-Hb values. In the control group, the changes in A/V difference following brain death were negative, indicative of a rise in venous oxygen content. This was accompanied by and correlated with (p<0.001) declines in both tissue oxygenation and tissue blood flow (i.e. a reduction in tissue Hb). ENO treatment reversed these effects: the A/V oxygen content difference was greater after brain death (i.e. venous blood oxygen content declined) and tissue oxygenation and tissue blood flow increased. Similar to the control group, the changes in A/V oxygen content correlated with tissue oxygenation and tissue blood flow (p<0.001). Area under the curve comparisons determined that the three parameters were all significantly different between the control and 20 ppm ENO groups (p=0.028, p=0.0037, and p=0.021 for A/V content, tissue oxygenation, and tissue Hb, respectively).

Metabolic Parameters

Values for various serum enzymes and metabolic markers before and 10 h after confirmation of brain death are presented in Table 1. (Additional serum chemistry results can be found in the Supplemental Digital Content Table 1.) Changes were most notable in the control group. Serum glucose levels increased, presumably reflecting an increase in glycogenolysis[26]. Total protein decreased, which along with the changes in serum electrolytes and increase in urine volume is indicative of induction of diabetes insipidus[27]. Lipase (but not amylase or alkaline phosphatase) increased in both groups. Markers of liver (alanine aminotranferease, ALT; aspartate aminotransferase, AST), kidney (creatinine), and tissue (creatine phosphokinase; CPK) injury were all significantly elevated in the control animals. While AST and CPK also increased in the ENO group, the magnitudes of these increases were significantly smaller. Modest elevations in hematocrit, and hemoglobin occurred in both groups. White blood cell (WBC) count only rose in the control group, which represented statistically significant increases in circulating neutrophils and monocytes.

Kidney Function

GFR and inulin clearance were assessed before and after brain death with clearance calculated as the plasma to urine inulin concentration ratio. These parameters along with mean serum creatinine levels are presented in Figure 3. In the control group, the mean inulin/plasma ratio increased significantly 10 h after brain death (from 0.15 to 1.89; p=0.011 paired t-test). This was accompanied by a significant decline in GFR (from 102 ± 13 to 77 ± 18 arbitrary units; p=0.0009) and a significant increase in serum creatinine (p=0.02). In contrast, neither the before/after inulin ratios (0.21 versus 0.17; p=0.49) nor GFR (91 ± 15 versus 84 ± 22; p=0.10) were different in the 20 ppm ENO group nor was there a change in the treated subjects' serum creatinine (p=0.90).

Discussion

Altered levels of SNOs and impaired vasodilatory activity of red blood cells have been observed in several chronic disease states characterized by aberrant tissue oxygenation. More recently, it has become apparent that various medical interventions, including blood transfusion[28] and peritoneal insufflation with carbon dioxide[29] can adversely affect red blood cell NO bioactivity with resultant reductions in organ blood flow. Based on the current results, brain death may constitute an acute stress characterized by relatively rapid reductions in SNO-Hb (i.e. within 10 h), leading to decreases in oxygen utilization (Figure

2). Our data thus provide a new perspective on the peripheral response to cessation of central nervous system activity in which the sequelae represent a system-wide failure of microvascular oxygen delivery rather than a disparate collection of failing organs.

The mechanism to account for the depletion of SNO-Hb following brain death is probably mutli-fold. Increasing acidosis disfavors the binding of NO to the beta cysteine residue of Hb[21]. The post-brain death catecholamine surge with resultant vasoconstriction and progressive lung injury may also contribute to this decline through effects on Hb oxygenation[5].

The inability of SNO-Hb-depleted red blood cells to facilitate hypoxic vasodilation has been well-documented[16, 28, 30] and thus provides a potential therapeutic target. Indeed, nitrosylating therapies directed towards increasing SNO-Hb have recently been developed and have shown therapeutic potential. In particular, ENO has been shown to correct impairments in tissue perfusion in a number of clinical and preclinical conditions that have in common a deficiency in SNO-Hb[16, 29, 31–34]. In the current setting, inhalation of ENO following brain death restored SNO-Hb concentration, enhanced tissue oxygenation and blood flow, and reduced markers of injury and inflammation. Collectively, these measures of organ viability are predictive of post-graft function[35].

Particularly notable were the effects of ENO on kidney function where 10 h after brain death no significant changes were seen in inulin clearance, GFR, or serum creatinine concentration (Figure 3). In the control group, the > 0.3 mg/dl increase in creatinine (Table 1) meets the criteria for diagnosing acute kidney injury (AKI)[36]. Serum creatinine can increase during donor support[37] with some authors reporting > 20% elevations (a level observed in our study) in more than 40% of human donors prior to organ procurement[38]. Elevated donor creatinine and reduced GFR are significant risk factors for delayed graft function and graft failure in kidney transplant recipients[39]. The major non-drug based cause of AKI is renal hypo-perfusion[40], which implies there were differences in kidney blood flow between the two experimental groups. ENO increased buccal blood flow (Figure 2), a finding that is consistent with our previous work demonstrating this agent can maintain flow to multiple organs[29]. As such, our data support the notion that brain death induced perturbations in blood flow to the kidneys (and to other organs) are reversed by inhalation of ENO.

Nitrosylation therapy with ENO may confer additional benefits. Hemoglobin is the major target of inhaled ENO but SNO-Hb can exchange NO with other proteins via transnitrosylation reactions to distribute NO bioactivity *in vivo*[42]. Under normal conditions, the activities of many inflammatory and pro-apoptotic proteins including toll-like receptors, interleukins, tumor necrosis factors, caspases, etc are tightly-controlled by S-nitrosylation[47–49]; their subsequent over-expression following loss of CNS function[43–46] may well reflect a loss of this regulatory control and are thus potential targets for ENO. Indeed, administration of a nitrosylating agent (or an exogenous SNO) can produce beneficial results in a number of acute and chronic inflammatory conditions where SNO levels are reduced[50–55]. Since neutrophil infiltration appears to negatively impact post-graft function[56], a more in-depth elucidation of ENO's actions on the inflammatory cascade following brain death would be worthwhile. In addition, nitrosylating therapy may also provide the means to enhance the activities of protective genes such as HIF-1[57] and HO-1[58] (a more selective approach than current therapies such as using dopamine to induce HO-1 activity[59]).

With respect to recording blood flow and tissue oxygenation, we selected the buccal mucosa as a monitoring site because this location allowed for easy sensor placement and it provided for continued access in case of displacement. Previous studies with the T-Stat have

demonstrated measurements of the buccal mucosa correlate well with recordings from more invasive locations and that changes in buccal mucosa oxygen levels and blood flow are sensitive indicators of global oxygenation status[20]. In addition, it has long been recognized that the external carotid arteries can remain well-perfused following brain death, even as blood flow within the internal carotids is significantly impaired[41]. While additional testing is needed, the current findings suggest that this site may be a convenient location to monitor for perfusion changes during donor support.

Our study has limitations. The method of brain death induction was rapid whereas in humans there can be a more delayed increase in ICP and this can subsequently alter the pattern of systemic organ disruption[60]. We were not in a position to conduct histologic assessments to determine if the functional benefits of ENO also prevented structural damage. The period of monitoring was short compared to the interval between declaration of death and organ procurement for some brain dead human donors. Nonetheless, it is doubtful that the observed depletion of SNO-Hb in the standard therapy control group would have resolved if the experimental duration increased. We deliberately chose to not initiate any hormone therapy so as to focus on documenting the effects of ENO inhalation. As administration of vasopressin, thyroxine, insulin, etc to human donors may confer benefits with respect to post-graft function[61], it is tempting to speculate that such benefits could be augmented when NO bioactivity levels are maintained following brain death.

In summary, inhalation of the *S*-nitrosylating agent ENO is an effective means to attenuate brain death-induced reductions in SNO-Hb concentrations, maintain tissue oxygenation, and reduce tissue injury, inflammation, and organ damage. As a result, clinical assessments of the utility and benefits of this intervention may be warranted. More generally, the present findings further support the importance of endocrine NO bioactivity in the regulation of organ function and they provide a novel example in which aberrant *S*-nitrosylation can be reversed with beneficial effects. Our results suggest that ENO has potential to improve physiologic status and organ function following brain death and to possibly expand the pool of organs available for donation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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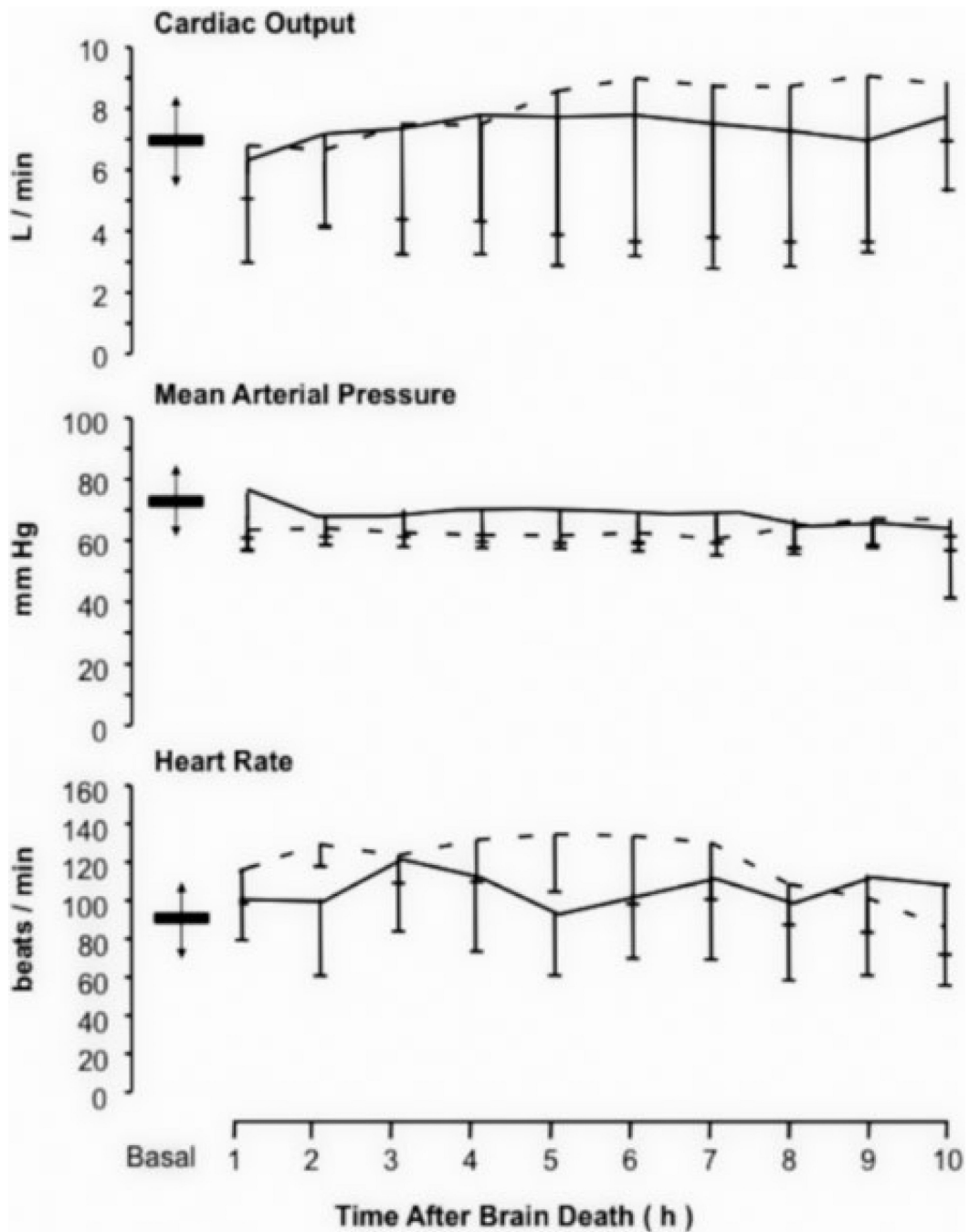
Figure 1.
Cardiovascular status. Mean (± SD) time courses for cardiac output, mean arterial pressure, and heart rate after the determination of brain death for the untreated (solid line) and ethyl nitrite (ENO) exposed (small dashes) cohorts. Within each graph, baseline mean (± SD) values are depicted by the bar and arrows. These parameters met the United Network Organ Sharing guidelines for donor maintenance with cardiac output > 3.8 l/min and mean arterial pressure > 60 mm Hg. Assessing for cardiovascular treatment differences was not a component of this study.
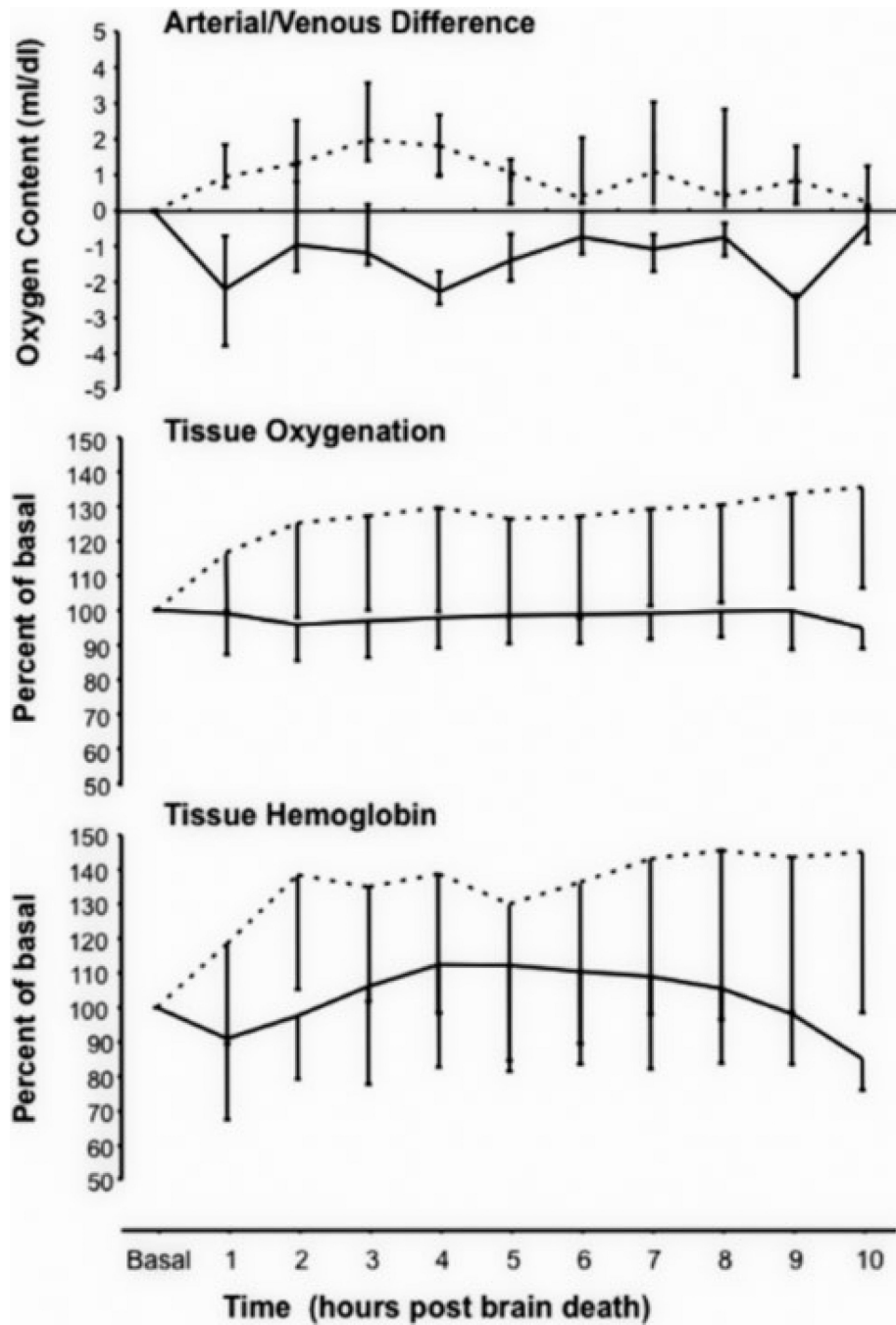
Figure 2.
Oxygen utilization and tissue oxygenation. Median (± QD) changes in arterial/venous (A/V) oxygen content differences along with mean (± SD) tissue oxygenation and tissue hemoglobin (a measure of blood flow) percent changes from baseline after the determination of brain death for the untreated (solid line) and ethyl nitrite (ENO) exposed (small dashes) cohorts. A/V values were determined by the formula $A/V_{experimental} - A/V_{baseline}$. Negative values, as seen in the control group, reflect an increase in venous blood oxygen content following brain death and thus a reduction in oxygen utilization since arterial oxygen content was constant. The positive values, as seen with the ENO treatment, indicate a decrease in venous blood oxygen content and thus an increase in oxygen utilization. Tissue

oxygenation and tissue hemoglobin were both significantly increased by 20 ppm ENO compared to the control values.
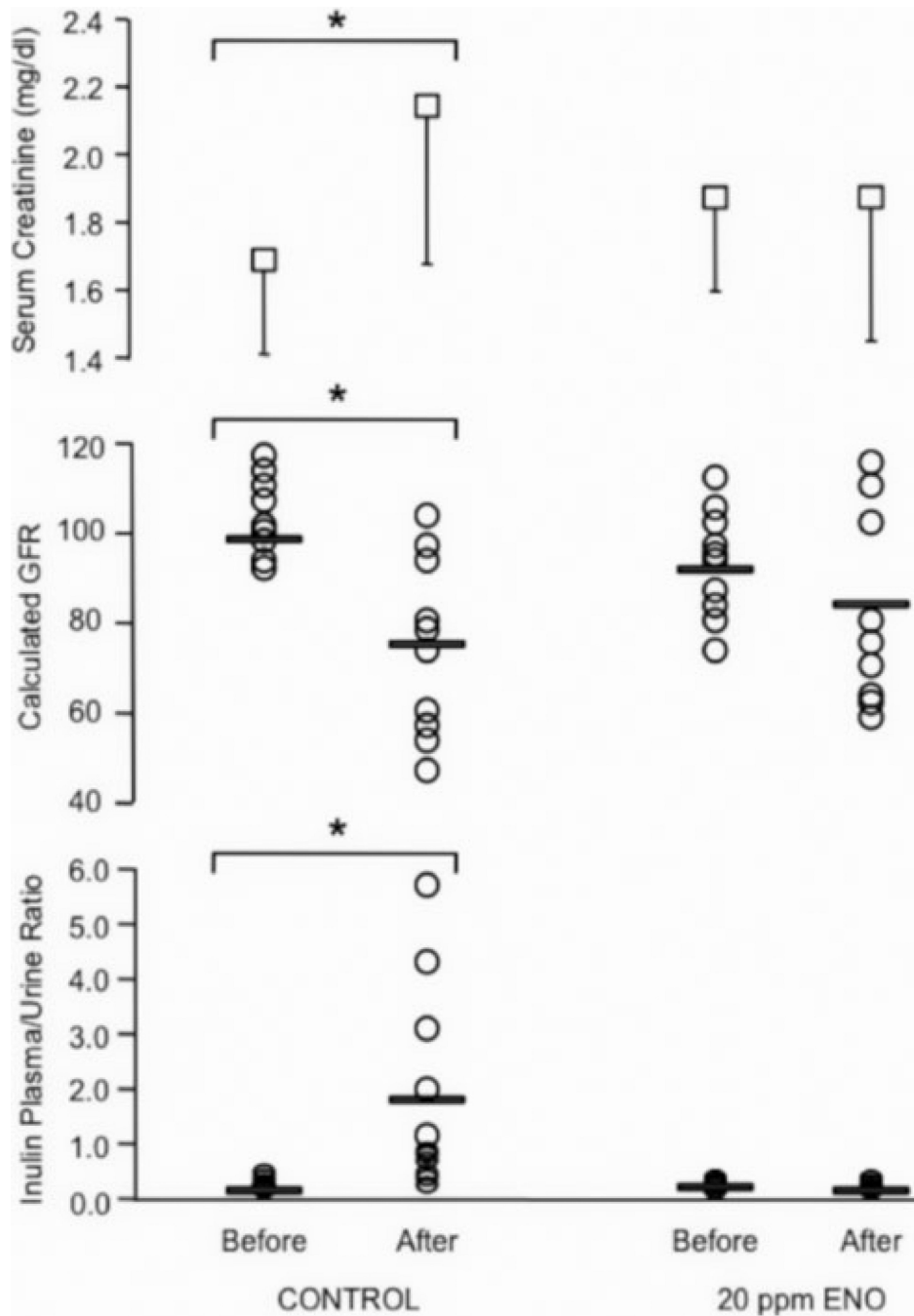
Figure 3.
Kidney function and serum creatinine. Mean (± SD) serum creatinine (top panel), calculated individual glomerular filtration rates (GFR; middle panel), and individual inulin plasma/urine ratios (bottom panel) before and 10 h after determination of brain death. The group means for GFR and inulin ratios are demarcated with bars. Serum creatinine and the plasma/urine inulin ratios significantly increased and GFR significantly decreased in the untreated control cohort after brain death (p < 0.05; brackets with asterisks). In the 20 ppm ethyl nitrite (ENO) treatment group, the before and after brain death values for all three parameters were not statistically different.

Table 1

Metabolic Parameters

Data on S-nitroso hemoglobin (moles of SNO per moles of Hb tetramere $\times 10^{-3}$) along with various serum enzymes and metabolic markers before and 10 h after determination of brain death for the two experimental groups.

Parameter (units)	Control		20 ppm ENO	
	before	after	before	after
SNO-Hb (Hbx10^{-3})	0.52 ± 0.68	0.21 ± 0.22[*]	0.28 ± 0.64	0.35 ± 0.15
Glucose (mg/dl)	95 ± 32	120 ± 35[*]	91 ± 24	113± 32
Total Protein (g/dl)	5.9 ± 0.6	5.1 ± 0.6[*]	5.5 ± 0.4	4.6 ± 0.4[*]
Lipase (U/l)	15 ± 28	45 ± 30[*]	3.8 ± 3.6	41 ± 48[*]
Amylase (U/l)	2755 ± 1531	2842 ± 1234	2084 ± 687	2110 ± 984
Alk Phosphatase (U/l)	128 ± 29	127 ± 29	137 ± 35	149 ± 48
Creatinine (mg/dl)	1.7 ± 0.3	2.1 ± 0.5[*]	1.9 ± 0.3	1.9 ± 0.4
ALT (U/l)	27 ± 6	48 ± 33[*]	30 ± 8	34 ± 8
AST (U/l)	37 ± 12	248 ± 175[*†]	37 ± 11	147 ± 64[*]
CPK (U/l)	2963 ± 1368	32418 ± 18770[*†]	3444 ± 1377	16422 ± 6730[*]
Hemoglobin (g/dl)	10.6 ± 1.5	11.4 ± 1.8[*]	10.5 ± 0.9	11.2 ± 1.4
Hematocrit	0.33 ± 0.05	0.34 ± 0.05	0.32 ± 0.03	0.34 ± 0.04
WBC (per mm^3)	12.5 ± 3.4	15.7 ± 3.4[*]	13.4 ± 2.7	12.6 ± 3.9
Neutrophils (cell count)	5940 ± 2621	8304 ± 2215[*]	6558 ± 1404	5453 ± 2684
Monocytes (cell count)	245 ± 152	939 ± 620[*]	285 ± 151	208 ± 231

Values in the "after" columns that are followed by an asterisk (*) indicate a significant difference from the pre brain death values (p<0.05).

Values in the "after" column of the control group also followed by a cross (†) indicate that the magnitude of the increase was significantly larger than the change observed in the ENO treatment group (again, p<0.05).

Abbreviations: ENO, ethyl nitrite; SNO-Hb,; Alk, alkaline; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CPK, creatine phosphokinase.