Background: Devastating extremity injuries are prevalent but often survivable on the modern battlefield. These complex injuries require advanced methods of reconstruction, involving prolonged ischemic periods and reperfusion injury. Using our group’s validated porcine model of gracilis myocutaneous flap transplantation, this study demonstrates that an interim perfusion of hydrogen sulfide (H2S) mitigates the effects of reperfusion injury in the setting of delayed restoration of blood flow.

Methods: A gracilis myocutaneous flap (200–400 g; surface area, 250 cm²) was procured from the hind limb of a Yorkshire swine (70–90 kg, n = 16). The right external carotid artery and the internal jugular vein are the recipient axis. Group 1 (control, n = 6) underwent delayed anastomosis with a 3-hour ischemic period. Group 2 (n = 10) underwent a similar delayed anastomosis with an interim perfusion of H2S during the ischemic period. The animals survived for 14 days. Systemic biomarker assays for skeletal muscle tissue injury (creatinine kinase, lactate dehydrogenase, and aspartate transaminase) and proinflammatory markers (tumor necrosis factor alpha and interleukin 6) provide assessment of reperfusion injury at the cellular level.

Results: The control animals (3 hours of ischemia with an interim perfusion of hemeperinated saline) demonstrated increased levels of injury biomarkers and proinflammatory cytokines compared with the animals receiving H2S infusion and identical ischemic interval. The control flaps had a mean creatine kinase level of 280 ± 10³ U/L (80 ± 10³), compared with the H2S group, which had a mean of 99 ± 10³ U/L (14 ± 10³; P = 0.0007 at postoperative day 2). Lactate dehydrogenase levels (mean) were 26 ± 10³ U/L (18 ± 10³) versus 9 ± 10³ U/L (±3 ± 10³; P = 0.0004) and aspartate transaminase levels (mean) were 1651 U/L (±324) versus 873 U/L (±279; P = 0.0013) for the control and treatment groups, respectively. Similarly, an intergroup difference in IL-6 was found, although not statistically significant. Tumor necrosis factor alpha levels (mean) were 93 pg/mL (±14) versus 39 pg/mL (±4; P = 0.0013) for the control and treatment groups, respectively.

Conclusions: This study demonstrated the mitigating properties of H2S on reperfusion injury. Interim perfusion with H2S resulted in diminution of ischemia-dependent biomarkers after 3 hours of ischemia. Follow-up studies will translate these findings as an evolving method for reconstructing previously unreconstructable injuries.

Key Words: hydrogen sulfide, gracilis myocutaneous flap, vascularized composite tissue autotransplantation, vascularized composite allotransplantation, porcine, ischemia and reperfusion injury, microvascular, microsurgery

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METHODS

The animals were handled and cared for under institutional guidelines in compliance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, DC: National Academy...
Hydrogen sulfide mitigates reperfusion injury in a porcine model of vascularized composite autotransplantation

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Press, 1996) and the American Association for the Accreditation of Laboratory Animal Care. Experimental protocols were approved by the Wilford Hall Ambulatory Surgical Center Clinical Research Division Institutional Animal Care and Use Committee and were performed in accordance with the recommendations of Good Laboratory Practices. The animals were entered into the study after a 5-day period of acclimation in the housing facilities of the Wilford Hall Ambulatory Surgical Center Clinical Research Division; Yorkshire swine (70–90 kg) were used for all experiments. All animals were inspected before the study by a veterinarian and monitored daily by a technician.

Thirty to 45 minutes before induction of anesthesia, the animals were sedated with ketamine (15–20 mg/kg) and atropine (0.04–0.4 mg/kg) administered intramuscularly by veterinary technicians. The animals were preoxygenated for 2 to 3 minutes by mask with 100% ambient oxygen. Anesthesia was initiated with 3% to 4.5% isoflurane in an air-oxygen mixture of 40% to 60%. The animals were orally intubated with an appropriate size (6.0–7.5 mm) endotracheal tube. Anesthesia was maintained with 2% to 3% isoflurane in an air-oxygen mixture of 40% to 60% by a nurse anesthetist or a qualified surgical technician. The animal’s heart rate, respiratory rate, oxygen saturation, and carbon dioxide levels were continuously monitored. The animals were sedated with ketamine (15–20 mg/kg) and atropine (0.04–0.4 mg/kg) administered intramuscularly by veterinary technicians. The animals were preoxygenated for 2 to 3 minutes by mask with 100% ambient oxygen. Anesthesia was initiated with 3% to 4.5% isoflurane in an air-oxygen mixture of 40% to 60%. The animals were orally intubated with an appropriate size (6.0–7.5 mm) endotracheal tube. Anesthesia was maintained with 2% to 3% isoflurane in an air-oxygen mixture of 40% to 60% by a nurse anesthetist or a qualified surgical technician. The animal’s heart rate, respiratory rate, oxygen saturation, and carbon dioxide levels were continuously monitored.

TABLE 1. Flaps by Group

<table>
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<tr>
<th>Group</th>
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<th>Flaps That Did Not Survive 14 d</th>
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<tr>
<td>3-h Ischemia (control)</td>
<td>6</td>
<td>2</td>
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<tr>
<td>3-h Ischemia (H2S)</td>
<td>10</td>
<td>3</td>
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The 10 animals that underwent treatment with H2S after initiation of the 3-hour ischemic period were compared with the control animals that underwent a similar ischemic interval with interim perfusion of heparinized saline solution. Systemic levels of CK were maximal for both the control and treatment groups at 24 hours (Fig. 1). The control flaps subjected to 3 hours of ischemia had a mean CK level of 280 × 103 U/L (±80 × 103), compared with the H2S group, which had a mean of 99 × 103 U/L (±14 × 103). The difference was statistically significant on POD 2 ($P = 0.0007$). After POD 1, the CK levels for both groups asymptotically returned toward baseline through POD 14.

Statistical Analysis

The experimental design of this study was a mixed-effects, randomized complete block design with repeated measures. Subject was a random effect because the subjects were a sample randomly selected and randomly assigned to a group. Fixed effects were ischemic period, treatment group, and time of repeated measures because these effects cannot be generalized to other treatments and times.

Sample means and SEs were calculated for continuous dependent variables and were stratified by group. Frequency distributions, by group, were determined for categorical variables. Main effects (group, time, and group by time) for continuous data were tested (at $\alpha = 0.05$) using a mixed-effects repeated-measures multivariate analysis of variance. The Bonferroni method was used to correct the level of significance for multiple comparisons while investigating significant main effects. For categorical variables, frequency distributions by group were compared using the Fisher exact test ($\alpha = 0.05$). Analyses were performed using the SASR 9.2, SAS Institute, Inc, Cary, NC.

RESULTS

The biomarker and cytokine levels on PODs 1, 2, 7, and 14 demonstrated the effects of IRI at the cellular level. Creatine kinase, LDH, and AST were compared because these enzymes are prevalent in the skeletal muscle and routinely measured as markers for reperfusion injury. Similarly, TNF-$\alpha$ and IL-6 levels were assessed because these cytokines are crucial in initiating, sustaining, and regulating the inflammatory processes.

The venous anastomosis was performed first with the use of a 2.5-mm venous coupler (Synovis GEM FLOW COUPLER, St Paul, Minn). Once the venous anastomosis is completed, the arterial anastomosis is performed with 2 running hemicircumferential 8.0 nylon sutures. The vascular clamps are then removed, and perfusion is reestablished. Once reperfusion is confirmed, blood is drawn systemically. The venous coupler wire remains attached to the external monitor while the neck incision is closed to ensure that there is no positional obstruction of venous flow. The neck incision is closed in layers.

Anesthesia was weaned, and the animal is extubated. During the postoperative survival period, the animals were housed in individual runs with freedom of movement and positioning. On postoperative days (PODs) 1, 2, 7, and 14, the animals were sedated with ketamine (15–20 mg/kg) and placed supine on the operating table for reassessment. Blood samples were collected and assayed for systemic biomarkers (complete blood count, chemistry, arterial blood gases, lactic acid, creatine kinase [CK], lactate dehydrogenase [LDH], and aspartate transaminase [AST]) and cytokines (tumor necrosis factor $\alpha$ [TNF-$\alpha$] and interleukin 6 [IL-6]). The flap appearance and incisions are examined. Patency of the venous anastomosis is assessed via external attachment of the venous FLOW COUPLER. Sixteen animals were used for this study.

**FIGURE 1.** creatine kinase levels during the experimental course; $*P = 0.0007$. 

**TABLE 1.** Flaps by Group
The LDH levels in both groups demonstrated peak levels within 24 hours of operation. Similar to CK, there was a statistically significant difference between the mean LDH levels between the 2 groups at POD 2. The mean LDH for the 3-hour ischemic control group was 26 x 10^3 U/L (±8 x 10^2) versus 9 x 10^3 U/L (±3 x 10^2; P = 0.0004) in the group that received an interim perfusion of H2S (Fig. 2).

Aspartate transaminase also demonstrates a statistically significant difference between the 3-hour control versus the H2S group (1651 U/L [±324] vs 873 U/L [±279], respectively; P = 0.0013; Fig. 3).

There were also differences between the systemic levels of proinflammatory cytokines between the experimental groups. There is a difference when comparing the IL-6 levels between the control and treatment animals; however, these differences were not noted to be of statistical significance (Fig. 4). The difference in TNF-α levels between the groups, however, was found to be statistically significant at POD 1 and 7. On POD 1, the control group had a mean level of 68 pg/mL (±7), whereas the treatment group had a mean level of 30 pg/mL (±5; P = 0.0010). On POD 7, the TNF-α levels were 93 pg/mL (±14) and 39 pg/mL (±4; P = 0.0013) for the control and treatment groups, respectively (Fig. 5).

Eleven of 16 flaps survived for the duration of the protocol. Five flaps failed early, likely for technical reasons, and these animals were euthanized on POD 7; biochemical results from the flaps that did not last the full 14 days were included in the statistical analysis (Table 1). Postmortem analysis of the failed flaps showed that failure was due to technical failures of either the arterial or the venous anastomosis. Flap failure rates were not significantly different between the control and experimental groups.

The results of complete blood counts and blood chemistry showed no significant differences between the 2 groups.

By POD 14, the flaps from both control and H2S groups showed good integration with the surrounding tissues.

**DISCUSSION**

Long-term (2-week) composite tissue viability was demonstrated in the setting of delayed reperfusion, with the effects of delayed restoration of blood flow ameliorated by H2S. All flaps subjected to ischemia exhibited a significant rise in biomarkers when compared with baseline. The flaps treated with an interim perfusion of H2S demonstrated a reduction in cellular lysis, reflected in a significantly blunted elevation of markers for skeletal muscle damage (CK, AST, and LDH). The effect of H2S is such that a flap subjected to 3 hours of ischemia but treated with H2S causes a smaller rise in the biomarkers when compared with the control flaps of both 1 and 3 hours of ischemic interval. A similar trend was also noted with the proinflammatory cytokines. The finding that the markers of muscle damage were relatively more affected than were the markers of inflammation suggests that the principal mode of action of H2S may be cytoprotective, rather than reducing inflammation itself.
The effects of H₂S on the flaps in the longer term are not defined by this 14-day survival model, and no previous studies have used H₂S this way. However, in this study, the experiment flaps were well integrated clinically at the 14-day point, and the biochemical markers of cellular injury and inflammation had returned to normal levels. On the basis of the excretion of the compound, it is reasonable to expect that no further effects would be exerted on the transplanted tissue composite.

The safety of using H₂S is a potential concern particularly because it is clearly a toxic substance in higher doses, with the cytoprotective effects being exerted only at lower levels. Hydrogen sulfide is metabolized peripherally and renally excreted as sulphate compounds. The toxic effects of H₂S are exerted primarily on the nervous and the respiratory system. The Occupational Health and Safety Administration has reviewed the safety of H₂S and found that levels in excess of 10 ppm for 10 minutes are considered potentially harmful, based on effects on humans and animals after industrial or agricultural exposure.18 Similar studies have found subtle effects on cellular immunity functions, which may have implications in the field of allotransplantation, including increases in levels of leukocytes, neutrophils, lymphocytes, CD8+ lymphocytes, and complement C3.19

It is beyond the scope of this experiment to establish mechanistic information of H₂S, and indeed, this is being actively investigated by the Defense Advanced Research Projects Agency. Imminent protocols transitioning H₂S into an allotransplant model will show what macro effects on immune reaction H₂S may have.

This study adds to a growing body of evidence relating to the role of H₂S in tissue preservation and stabilization. Blackstone et al12 demonstrated the ability of H₂S to induce a state of suspendedanimation in mice by decreasing metabolic rate and oxygen requirements. Other groups have followed to investigate the cytoprotective effects of H₂S on skeletal muscle and solid organs subjected to the insults of ischemia and reperfusion injury. Henderson et al9 demonstrated that pretreatment with H₂S conferred protection to muscle subjected to ischemia followed by a period of reperfusion. Simon et al11 later examined the effects of H₂S on organ dysfunction in a porcine model of aortic occlusion–induced IRI. The study revealed that sulfide pretreatment attenuated renal damage, which corresponded with a less severe systemic inflammatory response. Data from our study corroborate previous findings that H₂S plays a role in attenuation of ischemia-reperfusion–induced injury. However, unlike previous studies, this is the first validation of its effect in a large animal using composite tissue blocks.

The authors believe that this is a robust model of composite tissue autotransplantation; however, limitations inherent in research using animal species must be taken into account. It is not possible in this experiment to demonstrate whether the differences in the biomarkers of reperfusion injury, demonstrated above, have clinical significance with respect to flap function or viability because there was no significant difference in composite tissue survival between the control and treatment groups or, indeed, to look at potential effects on the animal or the flap beyond 14 days. The flap failure rates within this model of 5 (31%) of 16 in all groups largely reflect the demands the flaps must withstand in the postoperative period. The animals were left untreated with full freedom of mobility in the postoperative period. Although the design of this model is to maximize flap viability and reliability as well as to limit morbidity, flap failure is predictably higher than that found in the clinical arena, wherein the free tissue transfer is protected and under strict surveillance.

The overt objective of this line of research is translation of the model to allotransplantation, in which the flap is transplanted into a second pig using the same technique. The principles designed to quell reperfusion injury by suppressing excessive antigen release secondary to oxidative and inflammatory injury will be important adjuncts to an allotransplantation model. Subsequent studies by this group will investigate whether the demonstrated effects of H₂S in reducing circulating biomarkers of reperfusion injury will translate to attenuation of acute rejection in VCA and reduce requirements for systemic immunosuppression.

Demands for extensive complex vascularized composite tissue reconstruction in the military and civilian settings call for improvements in the current techniques. Despite significant advances in microsurgical techniques in recent decades, prolonged ischemia and the resultant reperfusion injury remain a challenge in these complex reconstructions. Reperfusion injury results in a multifactorial inflammatory condition with both immediate and long-term effects.20,21 Hydrogen sulfide was implemented as a measure to block the IRI process before its onset. This preemptive inhibition of inflammatory mediators may prevent the deleterious overstimulation of the inflammatory process and therefore ameliorate oxidative injury. Manipulation of the physiologic milieu before injury provides additional insight into reperfusion injury mechanisms and countermechanisms.

It is hypothesized that optimization of the posts ischemic physiologic milieu, by ameliorating the effects of reperfusion injury during vascularized composite tissue transfer, will offer advantages to the field of reconstructive allotransplantation. It may also enable free tissue transfer procedures to be performed in more critically ill patients, enabling earlier reconstruction with concomitant benefits in functional outcomes.

CONCLUSIONS
In the setting of delayed restoration of blood flow, H₂S demonstrated mitigating effects on reperfusion injury as evidenced by reduction of the systemic biomarkers of skeletal muscle tissue injury (CK, LDH, and AST) and proinflammatory cytokines (IL-6 and TNF-α). Follow-up studies will transition these findings to potentiate VCA.

REFERENCES


