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14. ABSTRACT
The goal of this project was to evaluate adjunctive use of purified poloxamer 188 (P188) in hypotensive shock resuscitation. The efficacy threshold specified by the RFA was to sustain hemorrhaging casualty through 4 hours of hypotensive resuscitation using specified protocols. Our studies using the proscribed model demonstrated a mean survival time was 9.6 hours with a range of 5-20 hours. The mean animals in this study was more than 3 hours longer that the controls treated with Hextend in a protocol designed to simulate current protocols in use by the military. P188 also improved auto resuscitation and reduced fluid requirements. Additional studies assessed tissue damage after shock and hypotensive resuscitation with Hextend followed by full resuscitation with crystalloid. In these studies, P188 blunted the no reflow phenomenon and largely prevented myocardial injury, pulmonary inflammation, small bowel damage, renal tubular necrosis, hepatic central lobular necrosis and apoptosis of splenic germinal centers that occurred during full resuscitation compared with the formulation currently used by Special Forces Medics in a rat model designed to simulate current battlefield practices. Finally, P188 increased survival from 0% to 75% in 50% volume controlled hemorrhage and did not increase bleeding in uncontrolled hemorrhage..

15. SUBJECT TERMS
Hemorrhagic shock, resuscitation, Poloxamer, drug, survival, inflammation

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Table of Contents

Introduction:

This project was a result of Funding Opportunity Number: W81XWY-BAA-AFRRF issued as a supplement to the U.S. Army Medical Research Materiel Command Broad Agency Announcement (BAA) 04-1: Candidate technologies for advanced first-responder resuscitation fluid (AFRRF). The purpose of this program was to evaluate candidate technologies/products for fluid resuscitation that can be used by first responders and combat medics to resuscitate wounded military personnel on the battlefield. The RFA specified the protocols to be used and the threshold results desired. This project accomplished all of these objectives. In addition, it produced data that P188 reduced ischemia-reperfusion injury of multiple organs and improved survival.

Body

Current state of battlefield resuscitation: Despite tremendous efforts, death from bleeding remains a leading cause of death in both civilian and military trauma. Some people die immediately from devastating wounds. Others survive with minimal therapy. A subset of injured people, however, survive the initial injury only to develop decompensated shock and die usually within 6 hours. Even if first responders reach these patients within minutes, definitive hemorrhage control is often delayed because of difficulty with extrication, transport, diagnosing the source of bleeding and/or controlling bleeding. Advanced Trauma Life Support (ATLS) of such patients called for resuscitation with large volumes of fluid. However, such therapy may lead to increased hemorrhage and mortality.

Hypotensive resuscitation was developed to maintain perfusion of vital organs without increased bleeding until hemorrhage can be controlled. It is based on the observations that a blood pressure of 60 mmHg is sufficient to maintain perfusion of vital organs without producing further bleeding. Accumulating evidence demonstrates that hypotensive resuscitation does improve survival compared to traditional normotensive resuscitation. The success of hypotensive resuscitation varies with the type of fluid used. In one study, Hextend was identified as the best available fluid for hypotensive resuscitation (1). It has been adopted by the military such that the current battlefield standard of care for Special Operation medics is permissive hypotensive resuscitation with a small volume of colloid (i.e. Hextend 500 ml x 2) combined with site specific hemorrhage control (i.e. direct pressure and limb tourniquets) (2). Hypotensive resuscitation is also becoming the standard of care for victims of penetrating trauma in urban trauma systems with rapid response time and probably has role in the massively injured victims of blunt trauma (3).

Hypotensive resuscitation still has limitations. Blood pressure low enough to discourage bleeding from severed vessels is too low for adequate perfusion of all organs. Consequently, while perfusion is sufficient to sustain life for a period of time, it may not be able to prevent ischemia of the gut and other vulnerable organs that results in the no reflow phenomenon and/or reperfusion injury with full resuscitation. This sets the stage for acute lung injury (ALI), abdominal compartment syndrome (ACS) and subsequent multiple organ failure (MOF). Further progress in increasing the duration and decreasing complications of hypotensive resuscitation will require novel interventions to protect cells from ischemia and prevent the no reflow phenomenon and reperfusion injury.

Purified poloxamer 188 (P188) is a synthetic, nonionic, block copolymer of ethylene oxide and propylene oxide that has been purified to reduce low molecular polymeric impurities. The copolymer with average Mw of 8500 $\pm$ 1000 Daltons is composed of a single chain (block) of hydrophobic

polyoxypropylene, flanked by two chains (blocks) of hydrophilic polyoxyethylene, and has the following structural formula:

$$HO(CH_2CH_2O)_a(\underset{\substack{| \\ CH_3}}{CH}CH_2O)_b(CH_2CH_2O)_aH$$

Current clinical supplies of P188 are formulated as a clear, colorless, sterile, non-pyrogenic solution intended for intravenous (IV) administration with or without dilution Each 100 mL vial contains 15 g of purified poloxamer 188 (150 mg/ml), 308 mg sodium chloride USP, 238 mg sodium citrate USP, 36.6 mg citric acid USP and Water for Injection USP Qs to 100 ml. The pH of the solution is approximately 6.0 and has an osmolarity of 312 mOsm/L. It contains no bacteriostatic agents or preservatives.

PRODUCT CHARACTERIZATION. SynthRx has an active IND No. 31,246 for purified P188. The formulated product is exceptionally stable if kept free of oxygen. The FDA has approved use of 5 year old clinical supplies for Phase III trials if they continue to meet specifications.

Pharmacokinetics: P188 is not metabolized and is excreted intact by the kidney. Pharmacokinetic parameters have been calculated from plasma concentration values determined following intravenous administration in sickle cell patients and healthy volunteers. The clearance rate ranged from 1.0 to 1.5 ml/min/kg with a half life approximating 4 hours.

ADMINISTRATION: A therapeutic dose formulated as above can be delivered i.v. in as little as 100 ml. No incompatibilities with other drugs have been identified. P188 is not irritating in tissue.

SAFETY DATA: Poloxamer 188 has been infused into nearly 4000 acutely ill patients with acute myocardial infarction, acute crisis of sickle cell disease, bypass surgery and other conditions. A Phase III myocardial infarction trial revealed that high concentrations of P188 administered to elderly patients with poor kidney functions cause an elevation of creatinine. This renal toxicity proved to be reversible and due to low molecular weight impurities. These impurities have been removed from the current formulation and renal effects were eliminated in preclinical studies and did not recur in clinical studies. The FDA has stated that the preclinical safety package is complete and that the agent may be used in Phase III studies of patients with acute crisis of sickle cell disease. 15 clinical studies have been completed.

Pertinent Prior Results:

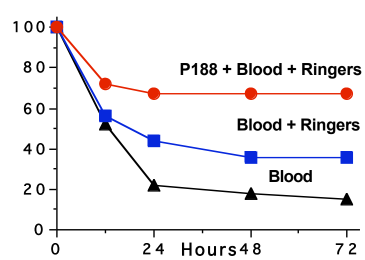

Figure1 . Survival of dogs following hemorrhagic shock. Dog were bled into shock for two hours as described. All of the withdrawn blood was then returned. This study tests the effects of P188 to restore perfusion of globally ischemic tissues (4).

Hemorrhagic Shock in Dogs: A dog study subjected 76 male beagles to nearly lethal hemorrhagic shock (4). Each animal was anesthetized, intubated, heparinized and placed on a respirator. Blood was withdrawn from the femoral artery until the blood pressure reached 75 mm Hg and was maintained at this level for 90 minutes. Additional blood was then removed to a blood pressure of 30 mm Hg for 30 minutes. After this period of time, the animals were randomized to three groups. The first group was

treated with their shed blood only. The second group received shed blood plus two times the shed blood volume of Ringer's lactate solution. The third group was treated identically to the second group except that 400 mg/100 ml of P188 was dissolved in the Ringer's lactate (LR). P188 improved survival from 15% for blood alone to 40% for blood plus LR to 78% of dogs treated with blood plus LR plus P188 ($p<0,01$), **Figure1.** P188 also produced multiple clinical signs of improved microvascular perfusion.

Brain Protection in Shock: Several investigators have reported that P188 can protect the brain from ischemic, traumatic and chemical injury. A single dose of P188 protects against early neuronal loss after hemorrhag (5). P188 demonstrated highly significant brain protection from prolonged ischemia during deep hypothermic circulatory arrest (6). Dogs were cooled to 10^{o} C, the heart was arrested for 150 minutes before they were rewarmed and weaned from bypass. Seven dogs were treated with P188 throughout. Six control dogs were treated with saline. All of the P188-treated dogs survived while half of the controls died ($p > 0.01$), **Figure 2**. All of the surviving control dogs had severe neurologic damage in that they could only hold their heads up. All but one of the P188 treated dogs had minor or no detectable neurologic deficit. The one P188 treated dog with significant neurologic deficit still had less neurologic damage than the best of the control group.

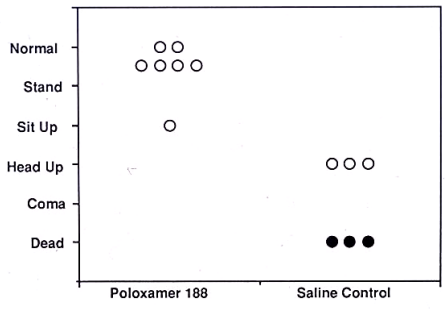

Figure 2. Neurologic Function Following DHCA. Beagle dogs were subjected to 2.5 hours of DHCA with or without P188 and were then monitored for 7 days. Each symbol represents the neurologic status of one dog at the end of the study. P188 protected brain function in a highly significant fashion in that the worst dog treated with P188 was better than the best control (6).

This study also demonstrated clinically significant protection of other organs as well as the brain. Histologic sections of the liver of the three saline treated animals that survived showed moderate to evidence of severe ischemic damage (centrilobular necrosis) while sections of 4 of 7 P188 treated animals showed no evidence of damage and 3 showed only mild changes. Finally, the P188 treated dogs produced nearly a liter of urine each during the 8 hours after DHCA, while the surviving control animals produced only about 300 ml each. This study shows that P188 has a significant impact in improving brain, kidney and liver function during and after exceptionally long periods of shock.

Use of P188 with hypotensive resuscitation (7): The studies supported by USAMRMC Contract #W81XWH-06-1-0570 (UNCLASSIFIED) were undertaken to determine if P188 could prolong the duration of hypotensive resuscitation and reduce its complications facilitating better survival and recovery with full resuscitation (7). The first experiment was designed to evaluate the effects of P188 in combination with Hextend on survival during hypotensive resuscitation. Hextend was chosen because it is being used clinically for hypotensive resuscitation. All of the animals had the same degree of hypotension documented by continuous computer monitoring. Nevertheless, 70% of P188 plus Hextend treated animals survived hypotension for 3 hrs longer than the Hextend treated controls and the remaining 30% survived 7-14 hours after the last control had died ($p=0.002$). It is known that the blood pressure of animals undergoing hypotensive resuscitation spontaneously increases after cessation of fluid administration at MAP of 60 mmHg. This is known as spontaneous resuscitation. The combination of Hextend plus P188 facilitated greater and longer lasting spontaneous resuscitation than Hextend alone documented by continuous computer monitoring, **Table 1.** This required much less volume of fluid that the controls ($p=0.0005$) (7).

Additional studies investigated the effects of P188 on the complications of hypotensive resuscitation in protocols designed to simulate clinical conditions of hemorrhagic shock. Most control animals survived hypotensive resuscitation but died after initiation of full resuscitation. Tissue damage progressed in spite of full resuscitation in control animals. Several studies suggested that this was due to the no reflow phenomenon and reperfusion injury. The volume of resuscitation fluid required to maintain blood pressure was markedly reduced by P188. This was confirmed by measurements of Evans Blue and tissue water. In both assays, P188 reduced leakage of fluid into tissue of each organ studied. The reduction in fluid loss was especially significant in the lung and GI tract that suffered the largest extravasation of fluid as a result of prolonged hypotension. These results suggest that P188 protected the integrity of the endothelial cells of the microvasculature. These results are particularly interesting because Hextend had previously been shown to reduce the volume of fluid required for hypotensive resuscitation more than any other agent tested (1).

Group	Hextend	Hextend + P188	p (t-test)
Number of animals	10	10	
Animal Weight (g)	266±4.	270±4	0.46
Shed Blood to BP 30 mmHg (% Total volume)	64±3%	61±2%	0.29
Time of decompensated (min)	13.1±2.2	10.2±2.4	0.37
Fluids to maintain BP at 30 mmHg	5.9±1.2	3.9±0.9	0.18
Initial Resuscitation volume to BP 60 mmHg (ml/kg)	8.9±0.6	7.0±1.0	0.11
Volume (ml/kg/h) to maintain BP at 60 mmHg for 6hrs	7.7±0.7	4.7±0.4	0.0002
Total Resuscitation volume till death (ml/kg/h)	11.2±1.4	4.7±0.8	0.0005
Survival time from onset of hemorrhage (min)	289±37	589.±99	0.002

Table 1. Effects of P188 on hypotensive resuscitation with Hextend. Rats were bled to a MAP of 30 mmHg for 30 min followed by hypotensive resuscitation at 60mmHg till death. There were no significant differences in any of the parameters measured prior to beginning of hypotensive resuscitation. During hypotensive resuscitation, there were highly significant differences in the volume of fluid required and length of survival.

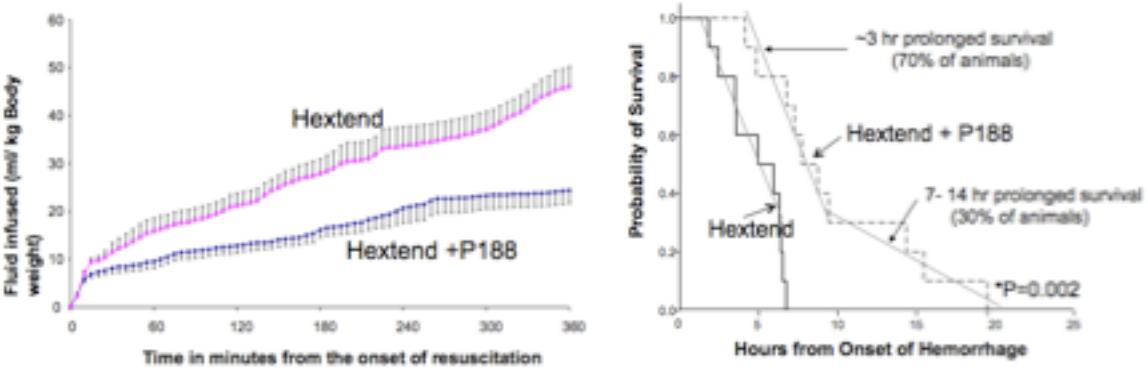

Figure 3: Fluid infusion requirements during hypotensive resuscitation. The mean ± SD fluid required to maintain MAP at 60 mm/Hg is shown. P188 decreased the amount of fluid required.

Figure 4. Effects of P188 on the survival during prolonged hypotensive resuscitation after lethal hemorrhage. Rats in the study of Fig. 1 were bled to a MAP of 30 mmHg for 30 min followed by hypotensive resuscitation at 60mmHg with either Hextend or Hextend + P188 till death.

The no reflow phenomenon occurs when blood fails to flow adequately when blood pressure is restored after a period of ischemia. It has been studied extensively in ischemic heart disease and is probably related to microvascular collapse that has been extensively studies in hemorrhagic shock. It is due to a combination of many factors including increased neutrophil adhesion, aggregation of red blood cells and damage to cells of the microvasculature. The finding of aggregated red blood cells in the microvasculature of the heart 1 hour into full resuscitation is evidence that no reflow occurred in our model. Similar findings were observed in the liver, kidney and small intestine. We are not aware of previous reports of morphologic evidence of the no reflow phenomenon. Congestion of vessels by uniformly packed red blood cells is common, especially in autopsy specimens. However, the presence of dense aggregates of red blood cells in an organ largely free of congestion is novel and consistent with observations of sludged blood flow in post ischemic states. By 5 hours, RBCs had disappeared entirely from the large areas of myocardium. In contrast, RBCs remained present in the microvasculature of P188 treated animals in a nearly normal distribution throughout the study. Similar changes were observed in each of the organs studied.

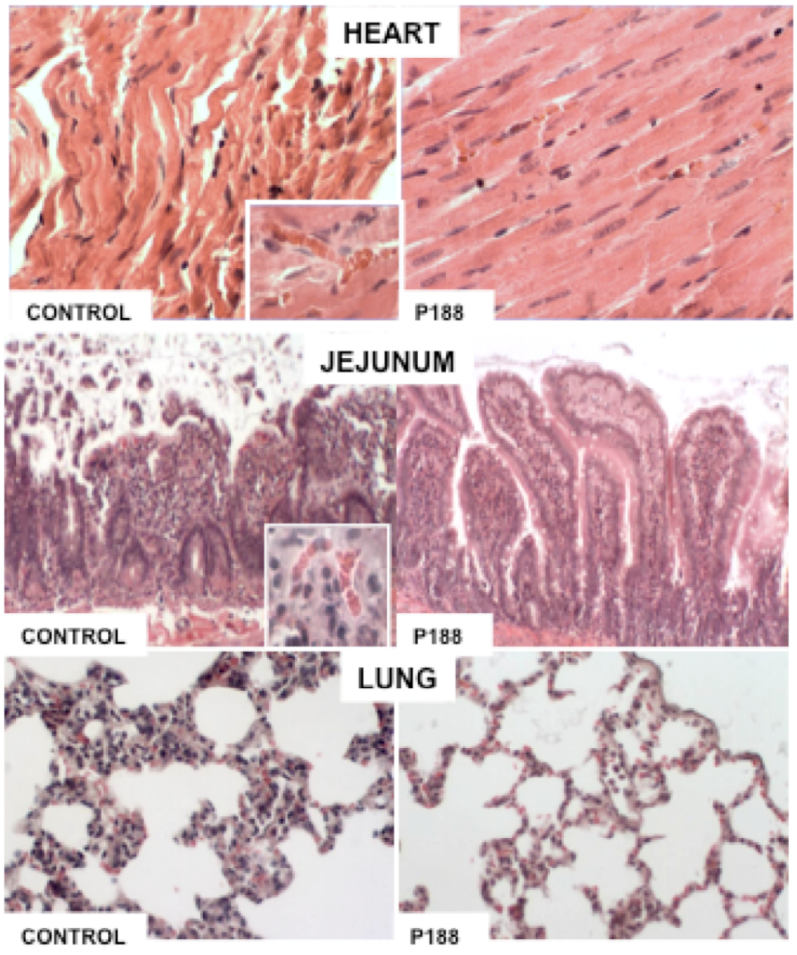

Figure 5: Histopathology of the effects of P188 following hypotensive and full resuscitation. Sections of the organs following severe hemorrhage for 0.5 hr followed by hypotensive resuscitation for 0.5 hr and then full resuscitation for 5 hours. The inserts demonstrating sludged vessels in heart, liver, kidney and jejunum are at 1 hour. P188 protected each of the organs from characteristic damage of ischemia. The control and P188 labels mark sections from animals that were treated identically except for the use of P188 in the resuscitation fluids. All H&E stain. Magnifications: Heart, kidney, spleen and liver are 400x, Lung is 200x, jejunum is 100x and inserts are 400x magnification.

P188 was originally used as a rheological agent to decrease whole blood viscosity without hemodilution and improve blood flow in damaged tissue. Using video microscopy, P188 was observed to improve mesenteric microcirculation in hemorrhagic shock within minutes. The ability of P188 to counter the no reflow phenomenon and improve microvascular perfusion was probably an essential component its efficacy in this study.

P188 in resuscitation fluid abrogated the characteristic lesions produced by shock. The lesions in control animals increased with time after initiating full resuscitation suggesting that they were due to reperfusion injury. P188 reduced acute tubular necrosis of the kidney, central lobular necrosis of the liver, acute lung injury, small bowel mucosal disruption and myocardial dysfunction (wavy fibers). The protective effects of P188 were supported by significant changes in biochemical markers of

inflammation (MPO) and apoptosis (caspases). Each of the organs from animals treated with P188 had less tissue damage and less inflammation than controls treated identically except for P188.

Finally, since hypotensive resuscitation typically is used before definitive hemorrhage control in possible, it is essential that it does not exacerbate bleeding. The effects of P188 on such bleeding were investigated in an uncontrolled hemorrhage model using 75% tail amputation. P188 did not cause increased bleeding, **Figure 6.** Both the mean volume of blood loss and distribution among animals were unchanged be P188. In fact, the mean blood loss was slightly smaller in the P188 treated group than in controls. This is consistent with previous studies that P188 has no effect on blood coagulation, platelet aggregation or bleeding time. P188 has been infused into nearly 4000 acutely ill patients with acute myocardial infarction in combination with thrombolytic enzymes, acute crisis of sickle cell disease with no reports of bleeding problems. It has also been used in cardiac bypass surgery and other surgery in hundreds of patients with no adverse effects on bleeding.

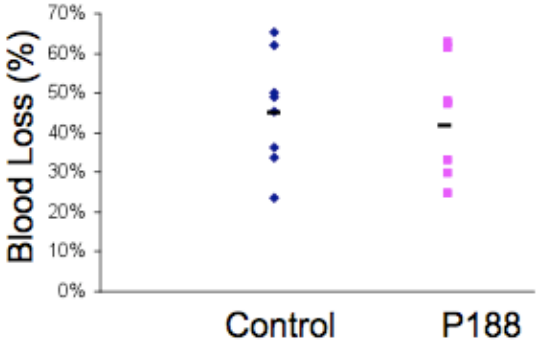

Figure 6. Effects of P188 on bleeding in uncontrolled hemorrhagic shock.
Rats were infused with P188 or saline prior to 75% tail amputation. They then received continuous infusion of the same fluid for one hour at a rate of 0.3 ml/min. No attempts at hemostasis were made. The amount of blood loss of individual animals is shown. The differences between the groups were not significant.

Lethal 2 phase volume-controlled hemorrhagic shock: The third hemorrhagic shock protocol was designed to study the effects of P188 in a highly lethal two-phase 50 % volume-controlled hemorrhagic shock. Animals were randomly pretreated with 1ml normal saline with or without P188. In the 1st phase 27 ml/kg blood was removed over 10 min to stimulate the initial brisk arterial hemorrhage that occurs in a traumatic limb amputation from a blast injury. This can quickly and largely be controlled by placement of a tourniquet(s). However, the significant ongoing oozing that occurs while waiting for evacuation and during transport (2nd phase). This was simulated by bleeding an additional 8 ml/kg over the following 75 min. The resuscitation was started in the 2nd phase of bleeding.

The results showed that 100% of the control animals died before 5hrs while 75% of the P188 treated survived and recovered ($p<0.001$), **Figure 7.** Examination of the relationship between blood loss and survival demonstrated that the two P188 treated rats that died had the highest (53% and 54%) blood loss of any animals in this study, yet survived longer than all but one of the controls who had less loss of blood (41%-50%). This demonstrates that P188 has a highly significant ability to facilitate survival and recovery from otherwise lethal hemorrhagic shock.

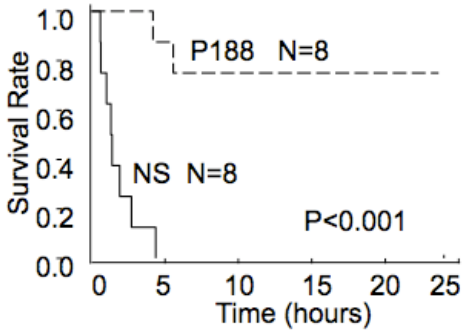

Figure 7. Volume-controlled hemorrhagic shock. Previous studies indicated that 50% hemorrhage would be uniformly lethal without rapid resuscitation. An experiment was conducted to learn the potential of P188 for treatment of this degree of hemorrhage. Rats were infused with P188 or NS in a volume of 1 ml 10 min prior to bleeding followed by 0.3 ml/min continuously infusion for 85min.

Suitability for Military Use. These studies were supported by USAMRMC Contract #W81XWH-06-1-0570 (UNCLASSIFIED). The RFA for this project listed 7 criteria for suitability for military use. Purified P188 meets or exceeds all of these requirements:

1) Shelf life: Threshold - 2 years; Objective – 3 years; at temperatures between 5 and 30 degrees Celsius. Poloxamer 188 formulated for clinical administration and stored for 5 years at refrigeration temperatures meets all specifications and has been approved by the FDA for use in new clinical trials assuming it continues to meet specifications. The agent is subject to slow oxidation. This can be retarded or prevented by formulation with an antioxidant (Citrate is currently used). Accelerated stability studies at elevated temperature suggest that it could be stable at 30^{o} C for a prolonged period.

2) Storage temperature: Threshold – 0 to 40 degrees Celsius; Objective – 20 to 55 degrees Celsius; for six months P188 in bulk form is stable at room temperature indefinitely if it is protected from oxygen. Stability studies at elevated temperature need to be done. We anticipate that in an appropriate formulation they will be successful.

3) Volume: Threshold – less than 500 ml per dose, P188 is currently formulated at 150mg per ml to produce a therapeutic dose in 100ml. It can be diluted with an appropriate intravenous fluid as needed. No drug interactions or incompatibilities have been identified.

4) Efficacy: Threshold – FDA approval for use in a trauma indication, sustain hemorrhaging casualty through 4 hours of hypotensive resuscitation; Objective – FDA approval for use in a trauma indication, sustain hemorrhaging casualty through 6+ hours of hypotensive resuscitation. The studies descried above using the proscribed rat model demonstrated efficacy in excess of the threshold. The mean survival time was 9.6 hours with a range of 5-20 hours. The mean for the low responding animals in this study was 3 hours longer that the controls treated with Hextend in a protocol designed to simulate current protocols in use by the military.

5) Ease of use: Threshold – risk high enough that a test is required to determine suitability of use in individual patient; Objective – combat lifesaver can make educated decision on suitability of use without additional tests or equipment. We anticipate that the ease of use will be comparable to that of current crystalloid solutions. P188 is not irritating following local injection and has a wide safety margin in studies conducted to date. Additional safety studies especially those with uncontrolled hemorrhage will to be done. However, the facts that P188 has been used in thousands of patients in combination with thrombolytic enzymes and during bypass surgery without reports of excessive bleeding are reasons for optimism in studies with uncontrolled hemorrhage.

6) Packaging: Recommended – packaging that is designed to survive transport by air, land or sea, is resistant to breakage, and does not present a health hazard when broken. These conditions are satisfied. The material could be packaged as a dry powder to be formulated with appropriate intravenous fluid or as an intravenous fluid in any one of a variety of containers. The major requirement is that it is protected from oxygen.

7) Disposal: Recommended – product does not create a biohazard or necessitate special disposal upon expiration, damage, or excess. There is extensive information and data that P188 can be disposed of as a nontoxic non-hazardous agent.

Key Research Accomplishments:

- More than doubled the 4 hour threshold for prolongation of survival during hypotensive resuscitation to 9.6 hours.
- Increased autoresuscitation
- Decreased fluid requirements for resuscitation.
- Prevented the no reflow phenomenon.
- Demonstrated tissue preservation in the heart, lungs, gut, liver, spleen and kidney following ischemia-reperfusion injury.
- Improved survival of 50% volume controlled hemorrhage from 0% to 70%.
- Did not increase bleeding from uncontrolled hemorrhage.

Reportable Outcomes:

References Published from this Project.

Peer Reviewed Papers

Gonzalez EA, Hunter RL, Kozar RA, Weisbrodt NW, Field E, Moore FA. Poloxamer 188 resuscitation after mesenteric ischemia/reperfusion abrogates neutrophil mediated ileal mucosal injury. J Trauma 2008. Submitted.

Harting MT, Jimenez F, Kozar RA, Moore FA, Mercer DW, Hunter RL, Cox CS, Gonzalez EA. Effects of poloxamer 188 on human PMN cells. Surgery. 2008 144(2):198-203.

Zhang, R., R. L. Hunter, E. A. Gonzalez, and F. A. Moore. 2009 Mar 13. Poloxamer 188 Prolongs Survival of Hypotensive Resuscitation and Decreases Vital Tissue Injury after Full Resuscitation. Shock (Epub ahead of print).

Abstracts

Gonzalez EA, Hunter R, Kozar RA, Moore FA. Poloxamer 188-Hypertonic saline provides optimal ileal protection after mesenteric ischemia/reperfusion. J Surg Res 2007;137;337

Gonzalez EA, Hunter R, Kozar RA, Weisbrodt N, Field E, Moore FA.Poloxamer 188 resuscitation after mesenteric ischemia/reperfusion abrogates neutrophil mediated ileal mucosal injury. Journal of Trauma 2006;61:514.

Presentations

Moore FA Poloxamer 188 (P188) as an Adjunct in Prolonged Hypotensive Resuscitation. Advanced Technology Applications for Combat Casualty Care (ATACC), St. Pete Beach, Florida, August 16, 2006

Moore FA Poloxamer 188 Abrogrates Neutrophil Mediated Mesenteric Ischemia/Reperfusion Ileal Mucosal Injury. American Association for the Surgery of Trauma-65th Meeting, New Orleans, LA, September 29, 2006

Gonzalez EA, Hunter RL, Mercer DW, Kozar RA. "Poloxamer 188 abrogates mesenteric ischemia/reperfusion-induced gut dysfunction." American College of Surgeons 94th annual clinical

congress. San Francisco, CA 10/2008

Gonzalez EA, Hunter RL, Kozar RA, Moore FA. "Poloxamer 188-Hypertonic Saline Provides Optimal Ileal Protection After Mesenteric Ischemia Reperfusion." 2nd Academic Surgical Congress, Phoenix, AZ 2/2007

Gonzalez EA, Zhang R, Kozar RA, Moore FA, Hunter RL: Poloxamer 188 improves survival after prolonged hypotensive resuscitation. International Shock Congress, Cologne, Germany, 6/2008

Robert L Hunter, Rongzhen. Zhang, Ernest A Gonzalez and Frederick A Moore. Poloxamer 188 Prolongs Survival of Hypotensive Resuscitation and Decreases Vital Tissue Injury after Full Resuscitation. Association of Clinical Scientists, May 16, 2009, Tampa FL.

Conclusion:

Since, P188 prolongs survival, decreases fluid requirements, reduces tissue damage, has a favorable toxicity profile and satisfies the military's logistical requirements, we believe that it deserves further consideration as an adjunct to hypotensive resuscitation. The next step it to verify and extend the studies in a relevant large animal model.

References

1. Handrigan, M. T., T. B. Bentley, J. D. Oliver, L. S. Tabaku, J. R. Burge, and J. L. Atkins. 2005. Choice of fluid influences outcome in prolonged hypotensive resuscitation after hemorrhage in awake rats. *Shock* 23:337-343.
2. Dubick, M. A., and J. L. Atkins. 2003. Small-volume fluid resuscitation for the far-forward combat environment: current concepts. *J Trauma* 54:S43-45.
3. Moore, F. A., B. A. McKinley, and E. E. Moore. 2004. The next generation in shock resuscitation. *Lancet* 363:1988-1996.
4. Hymes, A. C., M. H. Safavian, and T. Gunther. 1971. The influence of an industrial surfactant Pluronic F-68, in the treatment of hemorrhagic shock. *J Surg Res* 11:191-197.
5. Cadichon, S. B., M. Le Hoang, D. A. Wright, D. J. Curry, U. Kang, and D. M. Frim. 2007. Neuroprotective effect of the surfactant poloxamer 188 in a model of intracranial hemorrhage in rats. *J Neurosurg* 106:36-40.
6. Mezrow, C. K., M. Mazzoni, D. Wolfe, H. H. Shiang, R. S. Litwak, and R. B. Griepp. 1992. Poloxamer 188 improves neurologic outcome after hypothermic circulatory arrest. *J Thorac Cardiovasc Surg* 103:1143-1146.
7. Zhang, R., R. L. Hunter, E. A. Gonzalez, and F. A. Moore. 2009 Mar 13. Poloxamer 188 Prolongs Survival of Hypotensive Resuscitation and Decreases Vital Tissue Injury after Full Resuscitation. *Shock* [Epub ahead of print].

Appendices:

Effects of poloxamer 188 on human PMN cells

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Background. *Poloxamer 188 (P188), a nonionic block copolymer chemical surfactant known to have cytoprotective, rheologic, anti-inflammatory, and anti-thrombotic activity, has shown promise in the management of selected trauma patients. We studied human PMN oxidative burst and adhesion molecule expression when exposed to P188.*

Methods. *After RBC lysis of whole blood samples, white blood cell components were primed with phosphotidylcholine, primed and activated with fMLP, primed and activated with PMA, or left unstimulated. Each group was treated with vehicle or P188 (0.005–15 mg/ml concentrations). Flow cytometry quantified: (1) PMN superoxide anion production and (2) PMN marker expression of CD11b and L-selectin.*

Results. *Among non-PMA activated PMNs, P188 increased superoxide anion production. PMA-activated PMNs decreased superoxide anion production, proportional to P188 dose. Among fMLP-activated PMNs, the highest P188 dose increased the expression of CD11b. Among PMA-activated PMNs, decreased CD11b expression was seen for the mid-range doses.*

Conclusions. *PMNs altered their oxidative burst and marker expression after exposure to P188. When used at lower doses, P188 may increase the oxidative burst response and, when used at very high doses, increase CD11b expression. However, if PMNs are in a maximally activated state, a higher dose of P188 may decrease the oxidative burst response and decrease CD11b expression. (Surgery 2008;144:198-203.)*

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TRAUMATICALLY INJURED PATIENTS develop pathophysiologic responses including inflammation, coagulopathy, and acidosis, which lead to cell membrane breakdown, hypoperfusion, and microvascular disease. Subsequent to the morbidity related to acute trauma, these pathophysiologic immune responses lead to a cascade of events including the systemic inflammatory response syndrome, ischemia/reperfusion injury, and multiple organ failure (MOF).[1,2]

Polymorphonuclear cells (PMNs) mediate the global response to major trauma via activation, microvascular adherence, migration, and oxidative burst. While some of the proinflammatory response and PMN activity is protective, reducing the risk of sepsis, PMN adherence and oxidative burst is also known to damage endothelial cells, leading to vascular permeability, edema, and, ultimately, MOF.[3,4]

Poloxamer 188 (P188) is a nonionic block copolymer chemical surfactant known to have cytoprotective, rheologic, anti-inflammatory, and anti-thrombotic activity combined with minimal toxicity. P188 derives its wide range of therapeutic applications from its adherence to hydrophobic surfaces exposed after cell damage, restoring normal hydrated surfaces. P188 has shown promise in the management of many diseases, including sickle cell disease, vascular disease, stroke, and cancer; clinical trials, involving more than 4,000 patients, have shown it to be safe when administered intravenously.[5-7] P188 has shown the ability to improve the microcirculation,[8] minimize platelet aggregation,[9] and improve survival after hemorrhagic shock.[10] P188 has also shown the ability to mitigate reperfusion injury after myocardial infarction[11,12] and to improve hemodynamics, and subsequent oxygen delivery, after hemorrhagic shock

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in humans.[13] Preliminary in vitro and animal model work has shown that P188 limits PMN migration,[14,15] impairs PMN delivery to an inflammatory locus,[16] and attenuates enzyme release from PMNs.[17] Although these beneficial effects of P188 on PMNs have been previously seen, the direct effects of P188 on human PMNs are unknown. Therefore, we designed experiments to specifically examine whether P188 influenced human PMN oxidative burst or cell surface marker expression and hypothesized that it would.

MATERIALS AND METHODS

Materials. Phosphotidylcholine (PAF), N-Formyl-Met-Leu-Phe (fMLP), Phorbol 12-Myristate 13-Acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffered saline with glucose (7mM) (PBSG) was used to re-suspend the cells, along with the additional priming and activating agents, to a final volume of 100 μl. Dihydroethidium (DHE), phycoerythrin (PE) conjugated CD11b antibody, and fluorescein isothiocyanate conjugated CD62L (L-selectin) antibody were purchased from Invitrogen™ (Invitrogen Corporation, Carlsbad, CA). Purified poloxamer 188 formulated for clinical use was provided by SynthRx Corporation (Bellaire, TX).

Sample collection and red blood cell lysis. Venous blood was collected from six healthy, human volunteers through a sterile 21-gauge butterfly needle into a heparinized collection tube (143 USP units of sodium heparin/tube; ~8 ml blood/tube). Red blood cell lysis solution (Qiagen Sciences, Valencia, CA) was mixed with the collected blood (3:1 ratio) for ~5 min on a shaker at room temperature. After centrifugation at 2000 × g for 2 min, 100 μl of PBSG was added per every 100 μl of blood (~7.5×10^5 total WBCs). For each experimental sample, 50 μl of the PBSG+ cells solution was used. The final cell population was ~60% PMNs versus other white blood cell types by flow cytometry and was >99% viable by trypan blue exclusion.

Superoxide anion production assay. This assay was performed to assess the production of superoxide anion, a key component of the oxidative burst by PMNs in response to a stimulus. Superoxide anion production was measured by hydroethidine conversion to ethidium bromide, detected by flow cytometry (BD LSR II; BD Biosciences, San Jose, CA). The PMN + PBSG solution (100 μl) was incubated with DHE (10 μM final concentration) for 15 min at 37°C. Either the vehicle (sodium chloride, sodium citrate, and citric acid) or P188 was added to unprimed and unactivated experimental wells at final concentrations of 0.005 mg/ml (ultra low dose), 0.05 mg/ml (very low dose), 0.5 mg/ml (low dose), 2.0 mg/ml (medium dose*), 6.0 mg/ml (high dose), or 15 mg/ml (very high dose) and allowed to incubate for 5 min at 37°C.

**The 2 mg/ml was chosen as the mid-range dose based on an approximate average ~120 mg/kg (range: 50–200 mg/kg) in published pre-clinical and clinical work.[6,10] For a 120 mg/kg dose, in 250g rats, with 60 ml/kg blood per rat, the average dose is approximately 2 mg/ml.*

The 'priming' agent, PAF (10 μM final concentration), was added to certain control and experimental wells and allowed to incubate for 5 min at 37°C. P188 was added in the above concentrations to the appropriate experimental wells and allowed to incubate for 5 min at 37°C. The NADPH (nicotinamide adenine dinucleotide phosphate) oxidase dependent activating agent, fMLP (1 μM final concentration), or the NADPH-independent activating agent, PMA (10 μM final concentration), was added and allowed to incubate for 20 min at 37°C. The 96-well plate was placed on ice for 5 min to stop the reactions and immediately run on the high throughput system of the BD LSR II flow cytometer. The PMN population was gated on the FSC/SSC (forward scatter/side scatter) plot and the mean fluorescent intensity (MFI) was recorded. All samples were run in duplicate.

To ensure that decreased superoxide anion production was not the result of PMN cell death, the samples were checked for cell viability using the BD™ Cell Viability Kit (BD Biosciences) and viewed using Trypan blue exclusion.

CD11b or L-selectin surface marker expression. The adhesion molecules L-selectin and CD11b are important for PMN rolling, adhesion to the endothelium, and transmigration to an insult. Surface marker expression was measured by conjugated antibodies binding to the PMN cell surface, detected by flow cytometry. P188 was added to unstimulated (unprimed and unactivated) experimental wells at final concentrations of 0.5 mg/ml (low dose), 2.0 mg/ml (medium dose), 6.0 mg/ml (high dose), or 15 mg/ml (very high dose) and allowed to incubate for 5 min at 37°C. The 'priming' agent, PAF (10 μM final concentration), was added to certain control and experimental wells and allowed to incubate for 5 min at 37°C. P188 was added in the above concentrations to the appropriate experimental wells and allowed to incubate for 5 min at 37°C. The NADPH-dependent activating agent, fMLP (1 μM final concentration), or the NADPH-independent activating

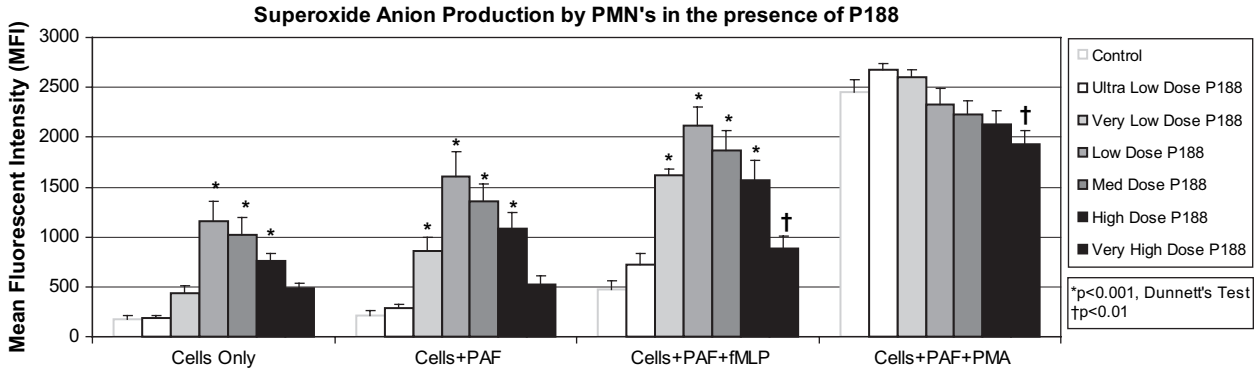

Fig 1. Superoxide anion production by PMNs in the presence of P188. After incubation with DHE and priming (PAF), P188 (in the following final doses: Ultra low dose: 0.005 mg/ml, very low dose: 0.05 mg/ml, low dose: 0.5 mg/ml, medium dose: 2.0 mg/ml, high dose: 6.0 mg/ml, very high dose: 15 mg/ml) was added to the human PMNs. Cells were then activated (fMLP or PMA). Mean fluorescent intensity (MFI), a surrogate for superoxide anion production, was measured by flow cytometry. Unstimulated, primed, and activated (with fMLP) PMNs exhibited increased superoxide anion production in the presence of P188 in a dose-dependent fashion. Among the maximally activated PMNs, addition of very high dose P188 reduced superoxide anion production.

agent, PMA (10 μM final concentration), was added and allowed to incubate for 20 min at 37°C. The conjugated antibodies (CD11b and L-selectin, 2 μl each antibody) were added to all wells and allowed to incubate for 30 min at 37°C. The 96-well plate was immediately run on the high throughput system of the BD LSR II flow cytometer. The PMN population was gated on the FSC/SSC plot and the MFI (mean receptor density) was recorded. All samples were run in duplicate.

Cell viability/osmolality assays. To ensure that there was no significant PMN cell death in the presence of P188, we performed two experiments to ensure cell viability. First, we placed the same number of cells in increasing doses of P188 (same doses as above) and identified cell death via trypan blue exclusion. Second, we ran a flow cytometric cell viability assay (BD cell viability kit) which uses thiazole orange (TO) to stain live cells and propidium iodide (PI) to stain dead cells. Finally, we determined the osmolality of the cell media, with the increasing doses of P188, to be sure that alterations in the osmolality of the cell suspension solution were not responsible for observed effects.

Statistical analysis. Data were compared using a repeated measures analysis of variance with a Dunnett's test versus a control for post-hoc comparisons. Data are reported as the mean ± standard error of the mean for each group. $P < .05$ was considered statistically significant. All groups contain six different human donors.

RESULTS

Superoxide anion production. Among control groups (no P188), PAF alone led to an insignificant increase in superoxide anion production (172 ± 38 vs 214 ± 44 MFI). When the cells were subsequently activated with fMLP or PMA, the MFI increased to 474 ± 80 ($P < .05$ vs. baseline and primed only) and 2446 ± 132 ($P < .05$ vs. other 3 groups), respectively.

Exposure to the ultra-low doses of P188 did not increase the superoxide anion production among the unstimulated cells (no priming/activating agents; hereafter *unstimulated*), primed cells (PAF), and primed and NADPH-dependently activated (PAF+fMLP) cells (Fig 1). When the PMNs were exposed to the very low dose, cells stimulated by PAF and PAF+fMLP began to increase their superoxide anion production. Exposure to the low dose of P188 led to a significant increase in superoxide anion production (Fig 1) among the unstimulated cells and cells stimulated by PAF and PAF+fMLP, but not among the primed and NADPH-independently activated (PAF+PMA) cells. Exposure to increasing doses of P188 caused an increased response proportional to the low doses. The response peaked with the low dose and an inversely proportional superoxide anion production response was noted for the higher doses (Fig 1). The low, medium, and high P188 dose exposures led to significant increases in superoxide anion production, compared to the baseline controls among unstimulated cells and cells stimulated with PAF or PAF+fMLP ($P < .001$ for all). The difference between superoxide anion production of cells exposed to the very high P188 dose and the baseline controls was not statistically significant among the unstimulated and PAF-stimulated PMN groups.

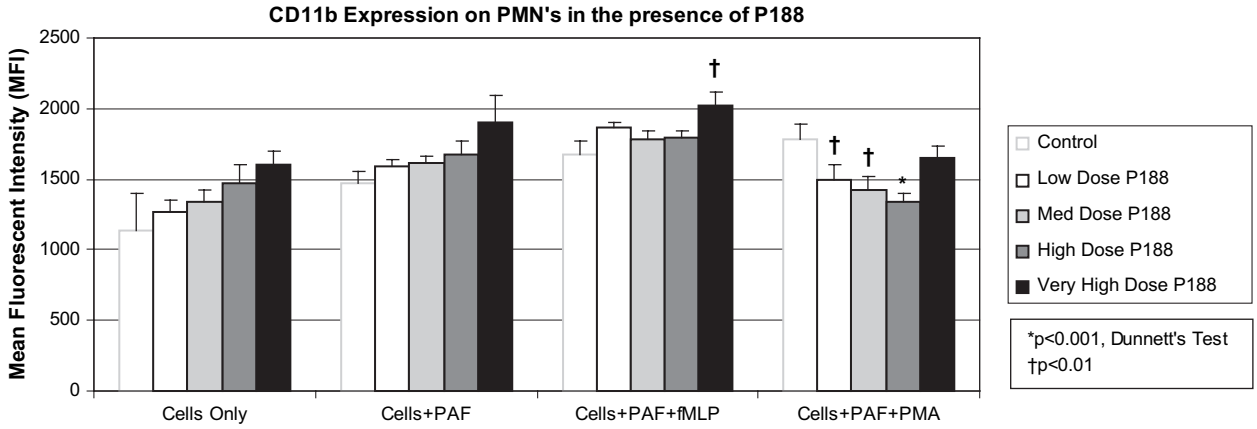

Fig 2. CD11b expression on PMNs in the presence of P188. After priming (PAF), P188 (in the following final doses: low dose: 0.5 mg/ml, medium dose: 2.0 mg/ml, high dose: 6.0 mg/ml, very high dose: 15 mg/ml) was added to the human PMNs. Cells were then activated (fMLP or PMA) and PE conjugated antibodies specific for human CD11b antibodies were added. Mean fluorescent intensity (MFI), a surrogate for CD11b marker expression, was measured by flow cytometry. Among unstimulated or primed PMNs, P188 did not alter CD11b marker expression. Primed and activated (fMLP) PMNs increased CD11b expression in the presence of very high dose P188. Primed and activated (PMA) PMNs decreased CD11b expression in the presence of low, medium, and high dose P188.

Among the cells stimulated with PAF+PMA, exposure to the ultra low, very low, low, medium, and high doses of P188 did not significantly change the superoxide anion production. Interestingly, exposure to the very high dose of P188 led to a statistically significant decrease in superoxide anion production ($P < .01$) (Fig 1). No difference in PMN cell death between the control and experimental groups was observed using the flow cytometric cell viability kit or via Trypan blue exclusion (>95% viability for all groups by both methods). The osmolality of the cell suspension media, with increasing doses of P188 ranged from 377 to 388 mmol/kg, with no differences observed between different concentrations of P188.

CD11b expression. Among control groups (no P188), PAF alone led to a mild increase in CD11b expression (1140 ± 254 vs 1468 ± 92 MFI). When the cells were activated subsequently with fMLP or PMA, the MFI increased slightly to 1673 ± 99 and 1779 ± 114, respectively (Fig 2).

Incubation of the cells with P188 did not lead to a significant change in the CD11b expression of unstimulated cells or PAF-stimulated cells. After PMN stimulation with PAF+fMLP, the very high dose of P188 increased the expression of CD11b (1673 ± 99 vs 2022 ± 92 MFI, $P < .05$). Among PMNs stimulated with PAF+PMA, the low, medium, and high doses of P188 decreased the CD11b expression relative to the control ($P < .05$) (Fig 2).

L-selectin expression. Among control groups (no P188), PAF did not alter L-selectin expression (81 ± 19 vs 75 ± 22 MFI). When the cells were activated subsequently with fMLP or PMA, the MFI decreased significantly to 28 ± 3 and 27 ± 2, respectively (Fig 3).

Incubation of the PMNs with P188 did not lead to any significant change in L-selectin expression, irrespective of dose used or stimulation status of the cells (Fig 3).

DISCUSSION

We have shown that PMNs alter their oxidative burst and surface marker expression when exposed to P188. When unstimulated, primed, or primed and NADPH-dependently activated, PMNs exposed to low, medium, and high doses of P188 have significantly increased superoxide anion production in an inversely proportional dose-dependent fashion. When PMNs are primed and NADPH independently activated, however, exposure to very high dose P188 leads to a significant decrease in superoxide anion production. Expression of CD11b among unstimulated and primed PMNs was unchanged in the presence of P188. Very high dose P188 increased CD11b expression among primed and NADPH dependently activated PMNs. After priming and NADPH independent activation of PMNs, low, medium, and high dose P188 significantly decreased CD11b expression. The expression of L-selectin on PMNs was unaffected by exposure to P188.

Lane and colleagues first identified P188 inhibition of neutrophil migration and adherence in vitro.[15] Tan and coworkers subsequently showed

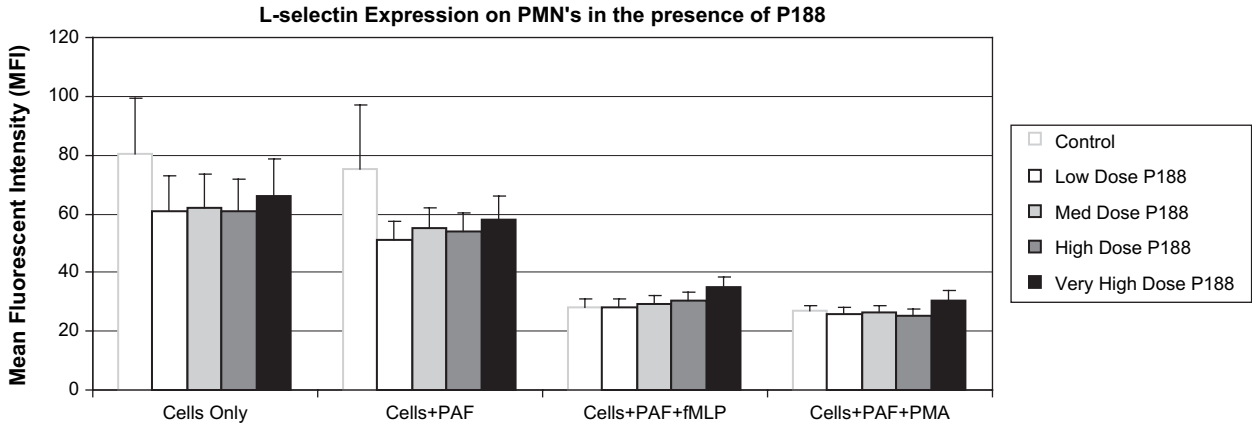

Fig 3. L-selectin expression on PMNs in the presence of P188. Incubation of the PMNs with P188 did not lead to any significant change in L-selectin expression, irrespective of dose used or stimulation status of the cells.

that neutrophil migration was inhibited by synthetic polymers in an in vitro, three-dimensional collagen gel.[14] Lane and colleagues followed their original work by assessing PMN migration in vivo after P188 treatment in a rodent model of bacterial sepsis.[16] They found a significantly increased mortality among rodents treated with P188 and attributed this to impaired neutrophil delivery to the inflammatory locus and a subsequent increased rate of sepsis. Animals that received P188 alone (without the bacterial injection) did not have increased mortality. Additionally, the effects of P188 on neutrophil-mediated injury to endothelial cells in an in vitro sheep model of microvascular injury revealed that neutrophil incubation with P188 significantly attenuated neutrophil adhesion, cytotoxicity, and proteolytic enzyme release.[17] Pretreatment of the endothelial cells with P188 did not inhibit neutrophil adherence, indicating that P188 may have direct effects on the cell membrane of the PMN.

Separating the effects of P188 on PMNs, in isolation from endothelium or activated endothelium interactions, required in vitro experimentation. Our study design is simultaneously a strength and a recognized limitation, because in vivo study could elucidate further physiologic relevance. Additionally, identifying the direct effects of P188 on isolated PMNs, as opposed to the lymphocyte-monocyte-PMN mixture we used, would require gradient separation, an action that is known to prime/activate PMNs. Given this, we chose to use a non-separated cell mixture and gate on the PMN population. We recognize that the effects of P188 on PMNs may be indirect effects, through interactions with other cell types.

PMNs appear to respond to P188 by increasing selectively their expression of CD11b and increasing generally the release of oxygen free radicals. Under maximally activated conditions, however, PMNs appear to respond to high dose P188 by reducing the oxidative burst response and decreasing their CD11b expression. The mechanism of action behind the latter response is unknown. Low doses may prime/activate PMNs, similar to any other foreign substance, while higher doses may prevent activation. P188 has been previously shown to attenuate neutrophil adhesion and transendothelial migration in vivo,[14,16,17] prompting the hypothesis that P188 may interact with the cell membrane or adhesion molecule, acting as a competitive inhibitor of adhesion molecule interactions. The poloxamer may be acting in a similar fashion at very high doses in our study, preventing some level of NADPH-independent activation by PMA and preventing interaction between the adhesion molecule and the antibody.

Our work may have important clinical implications. Among mild and moderately injured patients with unstimulated or primed PMNs, use of P188, particularly at low or moderate doses, may exacerbate the PMN oxidative burst response. This observation may have serious ramifications, such as eventual development of MOF. On the other hand, severely injured trauma patients or patients developing ARDS and/or MOF, whose PMNs are likely to be maximally primed and activated, may benefit from P188 therapy through reduced superoxide anion production and/or decreased CD11b expression.

Human PMNs alter their oxidative burst and surface marker expression after exposure to P188. These findings should be taken into consideration when considering optimal dosing and patient selection for future trials involving P188.

REFERENCES

1. Moore FA, Moore EE. Evolving concepts in the pathogenesis of postinjury multiple organ failure. Surg Clin North Am 1995;75:257-77.
2. Lenz A, Franklin GA, Cheadle WG. Systemic inflammation after trauma. Injury 2007;38:1336-45.
3. Botha AJ, Moore FA, Moore EE, Fontes B, Banerjee A, Peterson VM. Postinjury neutrophil priming and activation states: therapeutic challenges. Shock 1995;3:157-66.
4. Botha AJ, Moore FA, Moore EE, Kim FJ, Banerjee A, Peterson VM. Postinjury neutrophil priming and activation: an early vulnerable window. Surgery 1995;118:358-64; discussion 64–5.
5. Jewell RC, Khor SP, Kisor DF, LaCroix KA, Wargin WA. Pharmacokinetics of RheothRx injection in healthy male volunteers. J Pharm Sci 1997;86:808-12.
6. Orringer EP, Casella JF, Ataga KI, et al. Purified poloxamer 188 for treatment of acute vaso-occlusive crisis of sickle cell disease: A randomized controlled trial. JAMA 2001;286:2099-106.
7. Grindel JM, Jaworski T, Emanuele RM, Culbreth P. Pharmacokinetics of a novel surface-active agent, purified poloxamer 188, in rat, rabbit, dog and man. Biopharm Drug Dispos 2002;23:87-103.
8. Hymes AC, Safavian MH, Gunther T. The influence of an industrial surfactant Pluronic F-68, in the treatment of hemorrhagic shock. J Surg Res 1971;11:191-7.
9. Edwards CM, May JA, Heptinstall S, Lowe KC. Effects of pluronic F-68 (poloxamer 188) on platelet aggregation in human whole blood. Thromb Res 1996;81:511-2.
10. Mayer DC, Strada SJ, Hoff C, Hunter RL, Artman M. Effects of poloxamer 188 in a rabbit model of hemorrhagic shock. Ann Clin Lab Sci 1994;24:302-11.
11. Schaer GL, Hursey TL, Abrahams SL, et al. Reduction in reperfusion-induced myocardial necrosis in dogs by RheothRx injection (poloxamer 188 N.F.), a hemorheological agent that alters neutrophil function. Circulation 1994; 90:2964-75.
12. Schaer GL, Spaccavento LJ, Browne KF, et al. Beneficial effects of RheothRx injection in patients receiving thrombolytic therapy for acute myocardial infarction. Results of a randomized, double-blind, placebo-controlled trial. Circulation 1996;94:298-307.
13. Haneda K HK, Kanno M, Murakami S, Ohta M. Use of Fluosol DA (FDA) in hemorrhagic shock: Effects on oxygen carrying capacity and peripheral perfusion. New York: Alan R. Liss; 1983.
14. Tan J, Saltzman WM. Influence of synthetic polymers on neutrophil migration in three-dimensional collagen gels. J Biomed Mater Res 1999;46:465-74.
15. Lane TA, Lamkin GE. Paralysis of phagocyte migration due to an artificial blood substitute. Blood 1984;64:400-5.
16. Lane TA, Lamkin GE. Increased infection mortality and decreased neutrophil migration due to a component of an artificial blood substitute. Blood 1986;68:351-4.
17. Babbitt DG, Forman MB, Jones R, Bajaj AK, Hoover RL. Prevention of neutrophil-mediated injury to endothelial cells by perfluorochemical. Am J Pathol 1990;136:451-9.

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POLOXAMER 188 PROLONGS SURVIVAL OF HYPOTENSIVE RESUSCITATION AND DECREASES VITAL TISSUE INJURY AFTER FULL RESUSCITATION

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ABSTRACT—Hypotensive resuscitation prolongs survival of patients with severe bleeding until they can undergo hemorrhage control. However, its value is limited by continuing ischemic injury. Purified poloxamer 188 (P188), a copolymer with rheological and cytoprotective activities, was known to reduce mortality of hemorrhagic shock when used as an adjunct to full resuscitation with fresh whole blood and crystalloid. Studies were undertaken to determine if it could prolong survival and reduce reperfusion injury during prolonged hypotensive resuscitation when added to the best regimen currently available. Unanesthetized rats were bled to a MAP of 30 mmHg for 30 min under computer control. They then received hypotensive resuscitation with Hextend or Hextend + P188 to maintain a MAP of 60 mmHg until death. Poloxamer 188 improved autoresuscitation, reduced fluid requirements, and increased the survivable duration of hypotensive resuscitation by more than 3 h ($P < 0.01$). Additional studies assessed tissue damage after shock and hypotensive resuscitation with Hextend followed by full resuscitation with crystalloid. In these studies, P188 blunted the no-reflow phenomenon and largely prevented myocardial injury, pulmonary inflammation, small bowel damage, renal tubular necrosis, hepatic central lobular necrosis, and apoptosis of splenic germinal centers that occurred during full resuscitation. Additional studies demonstrated that P188 increased survival from 0% to 75% in 50% volume-controlled hemorrhage ($P < 0.001$). Finally, P188 did not increase bleeding in uncontrolled hemorrhage produced by 75% tail amputation. Because P188 prolongs survival, decreases fluid requirements, and reduces tissue damage, it deserves further consideration as an adjunct to hypotensive resuscitation.

KEYWORDS—Drug, hemorrhagic shock, survival, reperfusion injury, resuscitation, rat

INTRODUCTION

Despite tremendous efforts, death from bleeding remains a leading cause of death in both civilian and military trauma (1, 2). Some people die immediately from devastating wounds. Others survive with minimal therapy. A subset of injured people, however, survive the initial injury only to develop decompensated shock and die usually within 6 h. Even if first responders reach these patients within minutes, definitive hemorrhage control is often delayed because of difficulty with extrication, transport, diagnosing the source of bleeding, and/or controlling bleeding. Advanced trauma life support of such patients called for resuscitation with large volumes of fluid. However, it is now known that such therapy may lead to increased hemorrhage and mortality (3). Hypotensive resuscitation was developed to maintain perfusion of vital organs without increased bleeding until hemorrhage can be controlled (3). It is based on the observations that a blood pressure of 60 mmHg is sufficient to maintain perfusion of vital organs without producing further bleeding. Accumulating evidence demonstrates that hypotensive resuscitation does improve survival compared with traditional normotensive resuscitation (4). The success of hypotensive resuscitation varies with the type of fluid used. In one study, Hextend was identified as the best available fluid for hypotensive resuscitation (3). It has been adopted by the military such that the current battlefield standard of care for special operation medics is permissive hypotensive resuscitation with a small volume of colloid (i.e., Hextend 500 mL × 2) combined with site-specific hemorrhage control (i.e., direct pressure and limb tourniquets) (5). Hypotensive resuscitation is also becoming the standard of care for victims of penetrating trauma in urban trauma systems with rapid response time and probably has a role in the massively injured victims of blunt trauma (6).

Hypotensive resuscitation still has limitations. Blood pressure low enough to discourage bleeding from severed vessels is too low for adequate perfusion of all organs. Consequently, although perfusion is sufficient to sustain life for a period, it may not be able to prevent ischemia of the gut and other vulnerable organs, which results in the no-reflow phenomenon and/or reperfusion injury with full resuscitation (7, 8). This sets the stage for acute lung injury (ALI), abdominal compartment syndrome, and subsequent multiple organ failure (9). Further progress in increasing the duration and decreasing complications of hypotensive resuscitation will require novel interventions to protect cells from ischemia and prevent the no-reflow phenomenon and reperfusion injury.

Purified poloxamer 188 (P188) is a nonionic block copolymer of polyoxyethylene and polyoxypropylene that has rheological, cytoprotective, anti-inflammatory, and antithrombotic activities (10). It improved survival from hemorrhagic

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shock in rabbits and dogs that were fully resuscitated with fresh whole blood plus optimal amounts of crystalloid (11, 12). This article reports studies undertaken to determine if P188 could also improve the results of hypotensive resuscitation with Hextend. The first studies demonstrated that P188 could reduce the volume of fluid required to maintain MAP of 60 mmHg and prolong the period of survivable hypotensive resuscitation by more than 3 h. Further studies were designed to simulate the bleeding and hypotensive, full resuscitation likely to be encountered in the field. The results demonstrated that P188 could (*a*) maintain perfusion of vital organs during hypotensive resuscitation without exacerbating bleeding, (*b*) prevent the no-reflow phenomenon from developing after full resuscitation, (*c*) attenuate microvascular fluid loss in the gut and lung, and (*d*) reduce I/R injury in the heart, lungs, gut, and kidney after full resuscitation. The results suggest that P188 deserves further study as an adjunct to hypotensive resuscitation from hemorrhagic shock.

METHODS

Purified P188 is a synthetic, nonionic, block copolymer of ethylene oxide and propylene oxide that has been purified to reduce low-molecular polymeric impurities. The copolymer with an average molecular weight of 8,500 ± 1,000 daltons is composed of a single chain (block) of hydrophobic polyoxypropylene, flanked by 2 chains (blocks) of hydrophilic polyoxyethylene and has the following structural formula:

$$HO(CH_2CH_2O)_a(\underset{\displaystyle CH_3}{\underset{|}{C}}HCH_2O)_b(CH_2CH_2O)_aH$$

Current clinical supplies of P188 are formulated as a clear, colorless, sterile, nonpyrogenic solution intended for i.v. administration with or without dilution. Each 100-mL vial contains 15 g purified P188 (150 mg $\cdot$ mL^{-1}), 308 mg sodium chloride USP, 238 mg sodium citrate USP, 36.6 mg citric acid USP, and water for injection USP Qs to 100 mL. The pH of the solution is approximately 6.0 and has an osmolarity of 312 mOsm $\cdot$ L^{-1}. It contains no bacteriostatic agents or preservatives. Poloxamer 188 is not metabolized and is excreted intact by the kidney. Pharmacokinetic parameters have been calculated from plasma concentration values determined after i.v. administration in sickle cell patients and healthy volunteers. The clearance rate ranged from 1.0 to 1.5 mL $\cdot$ min^{-1} $\cdot$ kg^{-1} with a half-life approximating 4 h (10).

All procedures were reviewed and approved by the University of Texas–Houston Animal Welfare Committee. The experiments were conducted in compliance with the National Institutes of Health guidelines on the use of laboratory animals. All animals were housed at constant room temperature with a 12:12-h light-dark cycle with access to food and water *ad libitum*. All animals enrolled in the study were in good health and demonstrated appropriate daily weight gain.

Surgical preparation

Awake male Sprague-Dawley rats (Harlan Sprague Dawley Inc, Indianapolis, Ind) weighing 200 to 300 g were fasted for 18 h with free access to water. Under isoflurane anesthesia, they were placed on a heating blanket to maintain body temperature of 35°C to 37°C. Femoral arterial and venous catheters (Instech, Plymouth Meeting, Pa) were placed, tunneled, and secured to the back of the neck under sterile conditions. Immediately after placement, the catheters were flushed with 0.3 mL of 1% heparin. No further heparin was used. Bupivacaine (0.1%; VWR International, West Chester, Pa) was injected subcutaneously along the catheter track from the femoral triangle to an exit site at the posterior neck before incising the skin. The cannulas were secured at the posterior neck exit site via a flexible button cannula guide that was secured to the skin. The catheters were then connected to the corresponding fluid reservoir and blood pressure monitor (BPA-400; Micro-Med, Louisville, Ky) through a two-channel fluid swivel. This allows the animal to move freely about the cage. The animals recovered from anesthesia for a minimum of 1 h to be awake, alert, and without evidence of discomfort. No further anesthesia was administered.

The methods of hemorrhage and resuscitation were conducted as described previously (3). Briefly, a computer-controlled, low-flow peristaltic pump (model P720; Instech) was connected to the venous cannula through which blood was withdrawn and resuscitation fluids were given. Shed blood and resuscitation fluids were held in separate reservoirs placed on a balance with fluid weights recorded every 5 s using a LabVIEW program (National Instruments, Austin, Tex). Fluid volume was calculated from its weight based on measured fluid density. Animals were monitored until death or for 24 h. MAP, shed blood volume, and i.v. fluid volume were continuously monitored and recorded every 5 s. This methodology was used in each of the protocols of this study.

Vascular permeability (Evans blue extravasation and percentage of tissue water)

Evans blue (20 mg $\cdot$ kg^{-1}) was given i.v. 5 min before killing the rats. The chest was opened, a cannula was passed into the left ventricle with its tip in the aorta, the right atrium was cut open, and the circulatory system was perfused with 0.9% NaCl containing heparin (100 U $\cdot$ mL^{-1}) to remove intravascular dye. Four pieces (250 mg) of the heart, lung, liver, kidney, jejunum, ileum, and spleen were removed for study of vascular permeability, tissue water, histology, myeloperoxidase (MPO), and caspase assays.

Half of each tissue removed was rinsed in saline, gently blotted, and weighed. These tissues were then dried by incubation at 60°C for 48 h and reweighed. The wet-dry ratio was calculated as a measurement of tissue edema. Evans blue was then extracted from tissue by 2 mL formamide at room temperature for 48 h and was quantified by absorbance at 620 nm with reference to a standard curve and expressed as nanograms per milligram dry weight. Other pieces of tissue were fixed in 10% formalin, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin by routine procedures.

Tissue samples approximating 250 mg were homogenized in 1 mL of protein homogenization buffer (0.05 mol $\cdot$ L^{-1} Tris, 0.1 mmol $\cdot$ L^{-1} EDTA, 10 mmol $\cdot$ L^{-1} HEPES, and 0.1% NP-40; pH 7.40) containing 30 μL of protease inhibitor cocktail (Sigma Chemical Co, St Louis, Mo). The samples were centrifuged at 12,000 revolutions per min for 20 min, and the supernatant was used to assay total protein, MPO, and caspase activities. Total protein concentration was determined using the Bio-Rad microtiter plate assay protocol (Bio-Rad, Hercules, Calif).

Myeloperoxidase

For MPO activity, extracts were diluted 1:5 in buffer A (0.6% NP-40, 150 mmol $\cdot$ L^{-1} NaCl, 10 mmol $\cdot$ L^{-1} HEPES, 1 mmol $\cdot$ L^{-1} EDTA,

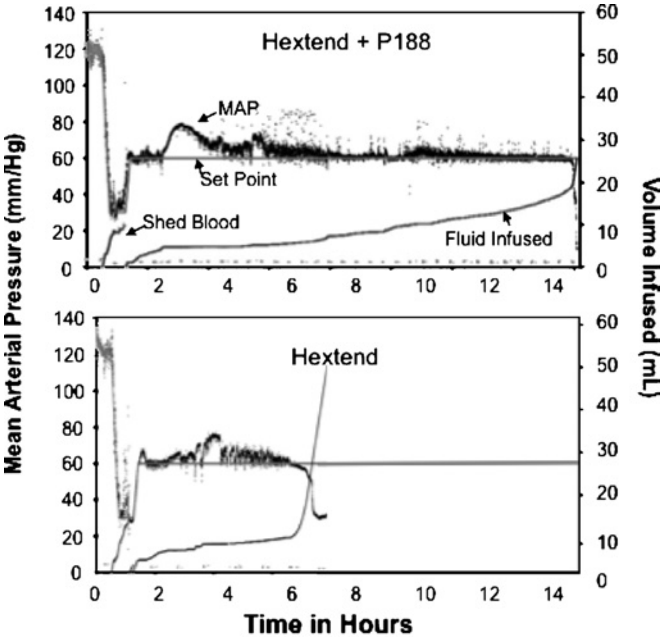

Fig. 1. **MAP responses to hemorrhage and hypotensive resuscitation**. Rats were bled to a MAP of 30 mmHg for 30 min followed by hypotensive resuscitation at 60 mmHg until death. Computer control was much more precise than our previous attempts at manually controlling resuscitation to hypotensive end points. Representative MAP and fluid infusions for one animal from each treatment group are shown. Set point is the computer target for MAP. The Hextend + P188–treated rat succumbed at 14 h, whereas the Hextend-treated animal died at 6 h. Note that the MAP overshoots the target of 60 mmHg (autoresuscitation), although fluid was administered only when MAP was less than 60 mmHg.

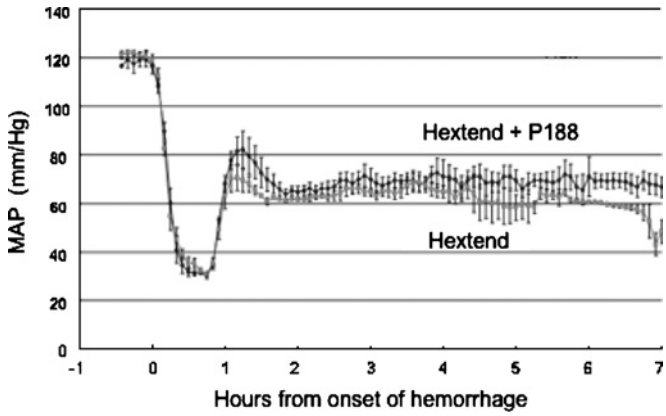

FIG. 2. **Mean MAP response to hemorrhage and hypotensive resuscitation**. The means ± SD of MAP of all animals in the study of Figure 1 are shown. After 30 min of hypotension (MAP of 30 mmHg), fluid was infused sufficient to increase blood pressure to 60 mmHg. MAP spontaneously increased to greater than 60 mmHg with no additional fluid. The MAP in rats infused with Hextend + P188 consistently improved more than those infused with Hextend alone, although fluid administration stopped when MAP reached 60 mmHg.

0.5 mmol · L^{-1} phenylmethylsulfonyl fluoride, and 30 μL · mL^{-1} protease inhibitor cocktail). Ten microliters of each sample was then added to 96-well plates and incubated with 100 μL tetramethylbenzidine microwell peroxidase substrate (KPL, Gaithersburg, Md) at room temperature for 20 min. The reaction was stopped with 100 μL of 1.8 mol · L^{-1} sulfuric acid. Optical density was measured at 450 nm. Assays were performed in duplicate, and the results were normalized for protein content.

Caspase

Caspases 3-, 6-, 8-, and 9-like activities were measured by following the cleavage of the fluorescent substrate analogs Ac-DEVD-AMC, Ac-VEID-AMC, Z-IETD-AFC, and Ac-LEHD-AFC, respectively (Calbiochem), in a fluorescent plate reader (PE Biosystems) as described previously (13). The rate of fluorescent change, an average of three replicate measurements, was normalized to the protein content.

Statistical analysis

Animals that died during the surgical preparation or before completion of the hemorrhage portion of the experiment were excluded from analysis. Kaplan-Meier product limit estimates of the survival functions and a survivorship function plot were produced using SigmaStat (version 3.0; SPSS Inc, Chicago, Ill). Individual fluid volume requirements were compared using one-way ANOVA in SigmaStat. Statistical tests resulting in $P \leq 0.05$ were considered significant. Data are presented as mean values ± SEM.

RESULTS

Pressure-controlled hemorrhagic shock with prolonged hypotensive resuscitation

An experiment was designed to determine if P188 could prolong survival of hypotensive resuscitation with a small volume of Hextend as currently used by the military. Rats were bled over 15 min to a MAP of 30 mmHg that was maintained for 30 min by withdrawal of additional blood as needed. Lactated Ringer's (LR) solution was infused as needed to maintain MAP of 30 mmHg. No blood was reinfused. The time that LR solution was started to be infused denoted shock decompensation, and amount of LR solution in the remaining 30 min to maintain MAP was recorded. After this shock insult, the rats were randomly assigned to two groups: hypotensive resuscitation with Hextend (n = 10) and hypotensive resuscitation with Hextend plus 20 mg · mL^{-1} P188 (Hextend + P188, n = 10). These fluids were continuously infused to elevate the MAP to 60 mmHg over 10 min and then as necessary to maintain the MAP at a minimum of 60 mmHg up to 24 h (Fig. 1). During this hypotensive resuscitation period, fluid was infused only if the MAP fell below 60 mmHg. No additional blood was withdrawn if the MAP rose above 60 mmHg. The MAPs of the groups treated with and without P188 demonstrated no difference until beginning of hypotensive resuscitation (Fig. 2). When MAP was increased from 30 to 60 mmHg by fluid infusions, it overshot the target to near 70 mmHg with Hextend compared with slightly more than 80 mmHg in those treated with Hextend + P188. Throughout the period of hypotensive resuscitation, the Hextend + P188–treated animals maintained higher MAPs with substantially less fluid than the Hextend-treated animals (Table 1). This is particularly interesting because Hextend was previously found to reduce the amount of fluid required for hypotensive resuscitation compared with other fluids tested (Fig. 3) (3). Eventually, all of the animals developed uncompensated shock and died, but the P188-treated group survived significantly longer ($P = 0.002$) (Fig. 4). Of note, roughly 70% of the animals treated with P188 survived to the time when 100% of the controls had died ($P < 0.01$ by Fisher exact test). Most survived approximately

TABLE 1. **Effects of P188 on hypotensive resuscitation with Hextend**

Group	Hextend	Hextend + P188	*P* (*t* test)
No. animals	10	10	
Animal weight, g	266 ± 4	270 ± 4	0.46
Shed blood to BP 30 mmHg, % total volume	64% ± 3%	61% ± 2%	0.29
Time of decompensated, min	13.1 ± 2.2	10.2 ± 2.4	0.37
Fluids to maintain BP at 30 mmHg	5.9 ± 1.2	3.9 ± 0.9	0.18
Initial resuscitation volume to BP 60 mmHg, mL · kg^{-1}	8.9 ± 0.6	7.0 ± 1.0	0.11
Volume to maintain BP at 60 mmHg for 6 h, mL · kg^{-1} · h^{-1}	7.7 ± 0.7	4.7 ± 0.4	0.0002
Total resuscitation volume until death, mL · kg^{-1} · h^{-1}	11.2 ± 1.4	4.7 ± 0.8	0.0005
Survival time from onset of hemorrhage, min	289 ± 37	589 ± 99	0.002

Rats were bled to a MAP of 30 mmHg for 30 min followed by hypotensive resuscitation at 60 mmHg until death. There were no significant differences in any of the parameters measured before the beginning of hypotensive resuscitation. During hypotensive resuscitation, there were highly significant differences in the volume of fluid required and length of survival.
BP indicates blood pressure.

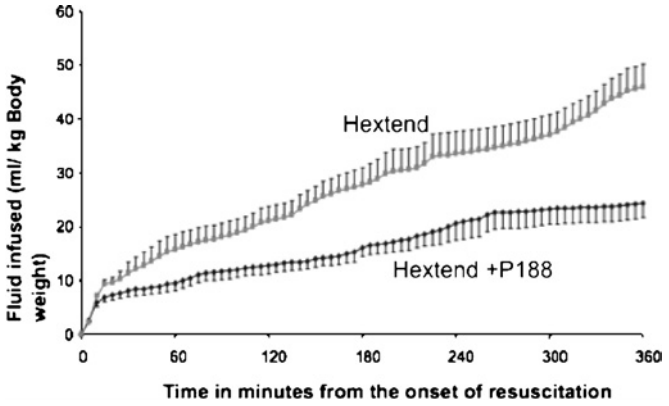

FIG. 3. **Fluid infusion requirements during hypotensive resuscitation**. The mean ± SD of fluid required to maintain MAP at 60 mm/Hg is shown. Poloxamer 188 decreased the amount of fluid required.

3 h longer than controls, whereas a subset of 30% had a much better response and survived more than 7 to 14 h after the last control had died.

Pressure-controlled hemorrhagic shock with hypotensive resuscitation followed by full resuscitation

A second model was developed to evaluate survival and organ injury under conditions likely to be observed in the field. It was assumed that hypotensive resuscitation would be initiated by a first responder 30 min after injury and that the injured patient would arrive at a treatment site where hemorrhage control and full resuscitation would be instituted 1 h after injury. Preliminary studies revealed that full resuscitation was best accomplished by raising blood pressure to 80 mmHg with LR solution and maintaining it at that level until the animals spontaneously improved. Using the same surgical preparation and methods of hemorrhage and resuscitation described above, the animals were bled at a rate sufficient to lower the MAP to 40 mmHg over a period of 15 min. This was maintained for 30 min. The animals were then randomly assigned to control or P188 treatment groups. The control group received 30 min of hypotensive resuscitation with Hextend followed by full resuscitation with LR solution. The P188-treated group was treated identically except that 20 mg · mL^{-1} P188 was added to both the Hextend and LR solutions. In the first study, groups of eight rats from each treatment group were observed for 24 h. Only one of the controls and four of the P188-treated animals survived 24 h. However, all animals had survived 5 h of full resuscitation. Consequently, this time point was chosen for further studies. A sham group that was prepared but not bled was included as a further control. Poloxamer 188 again reduced the amount of fluid required to maintain a MAP of 80 mmHg nearly threefold (35.5 ± 6.6 vs. 13.8 ± 3.32 mL · kg^{-1} · h^{-1}; $P < 0.05$).

Evans blue was used to measure extravasation of fluid into tissues of the heart, lung, liver, kidney, ileum, jejunum, and spleen (Fig. 5). As expected, fluid leakage was the largest in F5
the lung, ileum, and jejunum. The increased extravasation into these organs probably relates to their anatomic ability to hold more fluid rather than to differences in endothelial susceptibility. Surprisingly, P188 reduced fluid extravasation sufficiently in every organ that it was not significantly different from sham controls. This result was confirmed by measurements of tissue water (Fig. 6). The effects of P188 on tissues F6
were examined histologically (Fig. 7). Sections of the heart F7
at 1 h of reperfusion demonstrated relatively mild changes in myocardial cells. However, they did contain large aggregates of red blood cells (RBCs) in a pattern characteristic of reduced microvascular perfusion (sludging) (Fig. 7, insets). Sections of AQ4

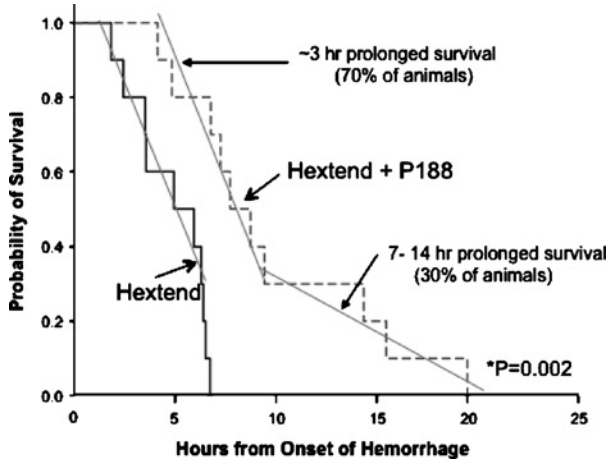

FIG. 4. **Effects of P188 on survival during prolonged hypotensive resuscitation after lethal hemorrhage**. Rats in the study of Figure 1 were bled to a MAP of 30 mmHg for 30 min followed by hypotensive resuscitation at 60 mmHg with either Hextend or Hextend + P188 until death.

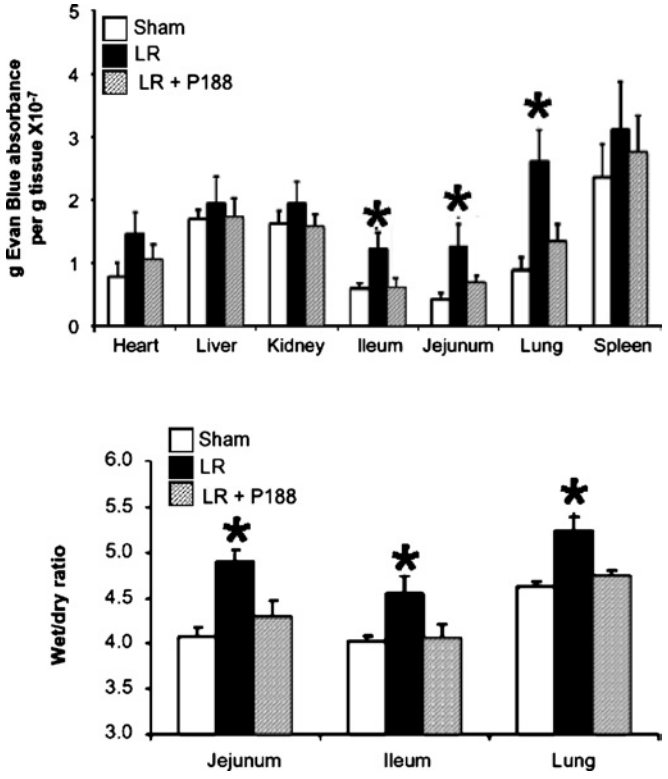

FIG. 5. **Tissue permeability assessment by Evans blue extravasation**. Rats were bled to a MAP of 40 mmHg for 30 min followed by hypotensive resuscitation at 60 mmHg for 30 min with Hextend or Hextend + P188 followed by full resuscitation with LR solution or LR solution + P188 as shown. The means ± SD for of Evans blue dye in tissues measured at 5 h of full resuscitation are shown for three groups of rats. The sham group was instrumented but not hemorrhaged or resuscitated. The other two groups differ only in the use of P188. Asterisk indicates statistical significance at $P <$ 0.01 of LR solution versus either the P188 or sham.

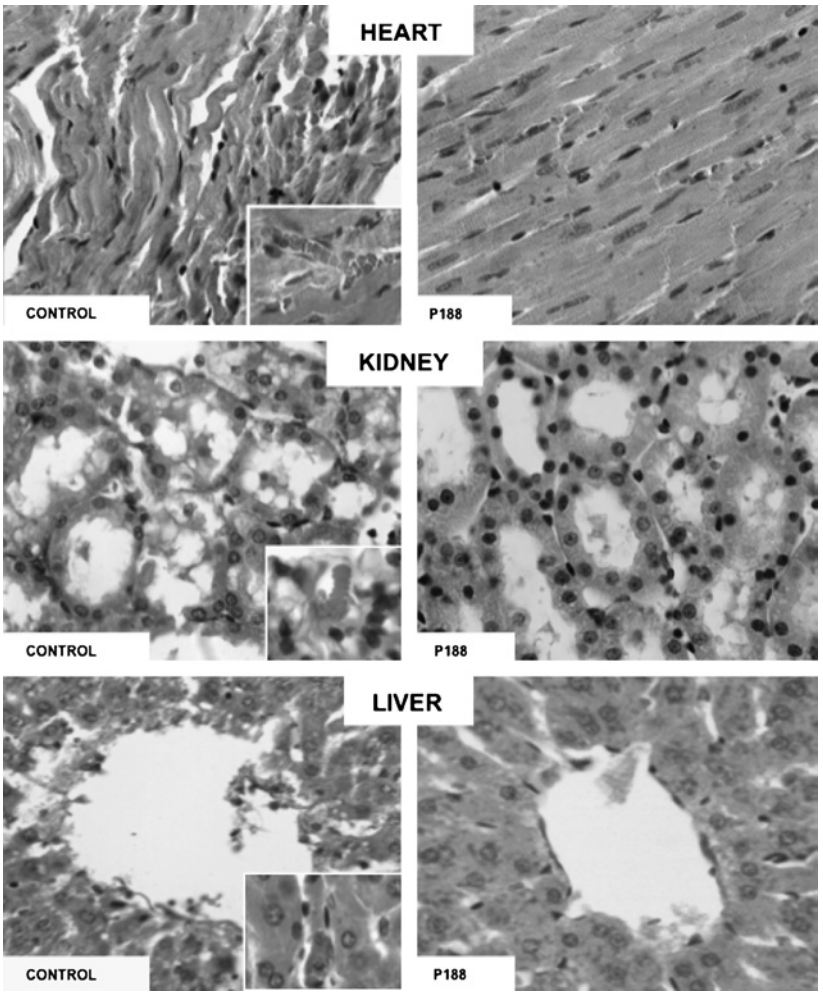

FIG. 6. **Assessment of water content of tissues**. The tissue water expressed as percentage of tissue weight was determined by measurement of wet weight and dry weight of each tissue using the same tissues as those in Figure 5. The results confirm that P188 reduces tissue edema. Asterisk indicates statistical significance at $P < 0.01$ of LR solution versus either P188 or sham.

the heart at 5 h in the LR solution–treated control animals contain very few RBCs, suggesting that tissue perfusion had been further compromised. The myocardial pathology had progressed to the point that heart sections demonstrated wavy fibers produced by stretching of ischemic noncontractile fibers and hypereosinophilic fibers typical of early coagulation necrosis (14). In addition, there were many pyknotic or apoptotic nuclei. The histology of the heart of the P188-treated animals, in contrast, was nearly normal. The myocardial fibers were intact, RBCs were plentiful in the capillaries in close association with myocytes, and there were few pyknotic nuclei. Sections of the heart, kidney, liver, and jejunum at 1 h of full resuscitation all demonstrated similar aggregation of RBCs within the microvasculature in a pattern consistent with severely impaired circulation (insets in Fig. 7). The lungs of control animals at 5 h demonstrated characteristic changes of early ALI and interstitial inflammation with neutrophils and macrophages, within alveolar walls. The P188-treated animals had few of these changes. Similar changes were observed in other organs. The mucosa of the jejunum of controls was necrotic near the tips and contained many pyknotic cells characteristic of apoptosis. Large numbers of cells had been sloughed into the lumen. In contrast, the P188 animals showed swelling of the mucosal villi, but much less loss of cells, necrosis, or evidence of apoptosis. The kidney at 5 h after resuscitation in controls demonstrated acute tubular necrosis or disruption and destruction of proximal tubular cells. Poloxamer 188 largely prevented this as well. Interestingly, the germinal centers of the spleens of control rats contained dense accumulations of apoptotic bodies, whereas the poloxamer-treated animals showed very few. Finally, the liver of the controls showed early evidence of central lobular necrosis with destruction of endothelial cells and adjoining hepatocytes. Both the endothelial cells and hepatic cells in liver sections from the P188-treated animals remained largely intact.

Inflammatory mediators

Myeloperoxidase, a marker of acute inflammation, and caspases 3, 6, 8, and 9 (mediators of apoptosis) were assayed on the samples of ileum of animals in this experiment (Table 2). Myeloperoxidase was significantly elevated in the T2 ileum of control LR solution resuscitation animals compared with the sham animals at 5 h of full resuscitation. Poloxamer 188 caused a reduction in MPO to a level even lower than the sham controls. Similarly, levels of each of the caspases 3, 6, 8, and 9 were markedly elevated in the LR solution resuscitation

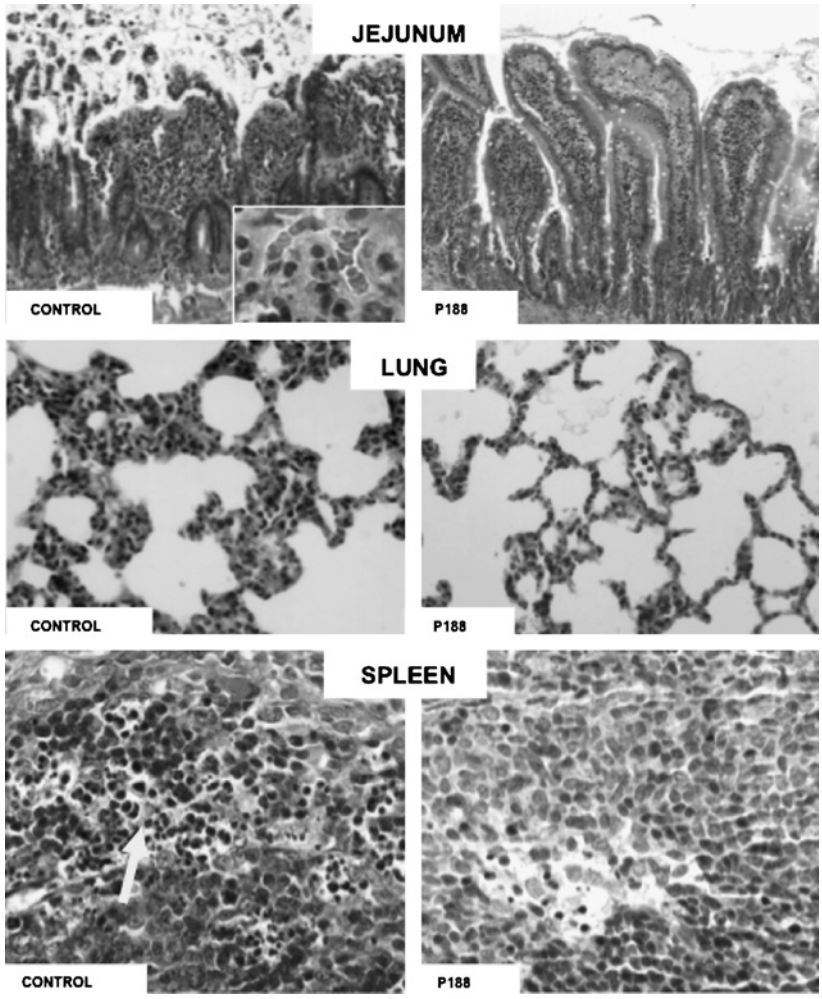

FIG. 7. **Histopathology of the effects of P188 after hypotensive and full resuscitation**. Protocol same as in Figure 5. Sections of the organs after severe hemorrhage for 0.5 h followed by hypotensive resuscitation for 0.5 h and then full resuscitation for 5 h. The insets demonstrating sludged vessels in the heart, liver, kidney, and jejunum are at 1 h. Poloxamer 188 protected each of the organs from characteristic damage of ischemia. The control and P188 labels mark sections from animals that were treated identically except for the use of P188 in the resuscitation fluids. Hematoxylin-eosin stain, original magnification ×400 for the heart, kidney, spleen, and liver; ×200 for the lung; and ×100 for the jejunum. Insets are 400× magnification.

animals at 5 h of full resuscitation. Poloxamer 188 produced a reduction in each caspase to a level not significantly different from sham controls. This is consistent with the histological evidence that P188 prevented apoptosis and with previous reports that P188 inhibits both apoptosis and necrosis of cells subjected to trauma (15). Collectively, these studies demonstrate a profound cytoprotective effect of P188 in preventing endothelial and other cell damage during hypotension and reperfusion. Interestingly, the major increase in each of these markers in the LR solution group took place between the 1- and 5-h time points of full resuscitation, suggesting that these were due to reperfusion injury.

TABLE 2. **MPO and caspase activity in ileal tissue**

				P (*t* test)	
	Sham	LR solution 5 h	P188 5 h	P188 vs. sham	P188 vs. LR solution
MPO	51 ± 9	97 ± 18	43 ± 2	0.4	0.02
Caspase 3	85 ± 13	220 ± 41	121 ± 18	0.1	0.04
Caspase 6	313 ± 57	474 ± 13	349 ± 49	0.6	0.03
Caspase 8	112 ± 19	186 ± 17	134 ± 17	0.4	0.04
Caspase 9	291 ± 54	408 ± 50	271 ± 44	0.8	0.04

Rats were bled to a MAP of 40 mmHg for 30 min followed by hypotensive resuscitation at 60 mmHg for 30 min with Hextend followed by full resuscitation with LR solution. Poloxamer 188 was added to the Hextend and LR solution as shown. MPO and caspases 3, 6, 8, and 9 were assayed on ileum of rats from each group at the 5-h time point of full resuscitation. The results are in units of nanograms per gram of protein for MPO and fluorescent units per hour per milligram of protein for caspase. They are shown as mean ± SD on groups of eight rats.

Lethal two-phase, volume-controlled hemorrhagic shock

The third hemorrhagic shock protocol was designed to study the effects of P188 in a highly lethal two-phase, 50% volume-controlled hemorrhagic shock. Animals were randomly pretreated with 1 mL normal saline (NS) without P188 (NS pretreated, n = 8) or with 20 mg · mL^{-1} P188 (P188 pretreated, n = 8) 10 min before hemorrhage. In the first phase, 27 mL · kg^{-1} blood was removed over 10 min to stimulate the initial brisk arterial hemorrhage that occurs in a traumatic limb amputation from a blast injury. This can quickly and largely be controlled by placement of a tourniquet(s). However, the significant ongoing oozing that occurs while

waiting for evacuation and during transport (second phase). This was simulated by bleeding an additional 8 mL · kg^{-1} over the following 75 min. The resuscitation was started in the second phase of bleeding with 4 mL · kg^{-1} of NS being administered in the NS-pretreated animals versus 75 mg · kg^{-1} P188 in 4 mL · kg^{-1} NS in the P188-pretreated animals over the first 15 min and then 10 mL · kg^{-1} of NS in the NS-pretreated animals versus 150 mg · kg^{-1} P188 in 10 mL · kg^{-1} NS in the P188-pretreated animals over the following 60 min. All animals were closely monitored up to 24 h until they died or were killed.

The results showed that 100% of the control animals died before 5 h, whereas 75% of the P188-treated survived and recovered ($P < 0.001$) (Fig. 8). Examination of the relationship between blood loss and survival demonstrated that the two P188-treated rats that died had the highest (53% and 54%) blood loss of any animals in this study, yet survived longer than all but one of the controls who had less loss of blood (41%–50%). This demonstrates that P188 has a highly significant ability to facilitate survival and recovery from otherwise lethal hemorrhagic shock.

Effects of P188 in a model of uncontrolled hemorrhage

It is important that an agent used during hypotensive resuscitation does not exacerbate hemorrhage. Consequently, an experiment was designed to evaluate the effects of P188 on bleeding and survival in a established model of uncontrolled hemorrhagic shock induced by amputation of a rat's tail (16). Experimental animals were randomly assigned to the control NS pretreatment group that received 4 mL · kg^{-1} NS infused at 5 min before 75% tail amputation, followed by 8-mL · kg^{-1} · h^{-1} infusion of NS over 60 min. The P188 pretreatment group received 75 mg · kg^{-1} P188 in 4 mL · kg^{-1} NS infused at 5 min before 75% tail amputation, followed by 150 mg · kg^{-1} · h^{-1} in 8 mL · kg^{-1} · h^{-1} of NS over 60 min. Both groups were observed for blood loss and time of death over 24 h until died.

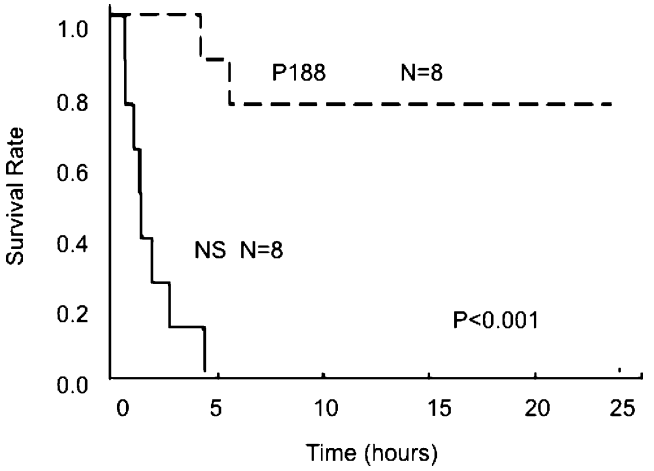

FIG. 8. **Volume-controlled hemorrhagic shock**. Previous studies indicated that 50% hemorrhage would be uniformly lethal without rapid resuscitation. An experiment was conducted to learn the potential of P188 for treatment of this degree of hemorrhage. Rats were infused with P188 or NS in a volume of 1 mL 10 min before bleeding followed by 0.3-mL · min^{-1} continuous infusion for 85 min.

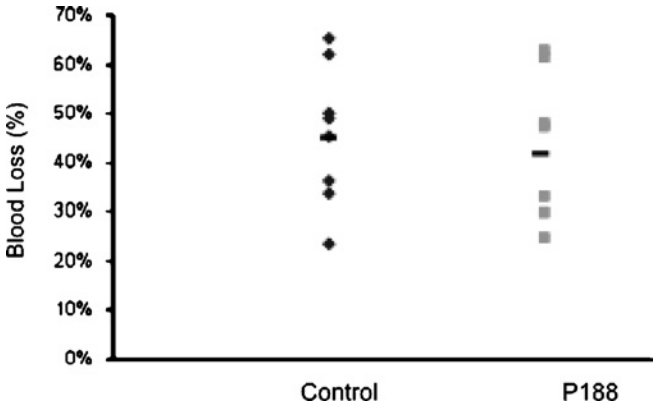

FIG. 9. **Effects of P188 on bleeding in uncontrolled hemorrhagic shock**. Rats were infused with P188 or saline before 75% tail amputation. They then received continuous infusion of the same fluid for 1 h at a rate of 0.3 mL · min^{-1}. No attempts at hemostasis were made. The amount of blood loss of individual animals is shown. The differences between the groups were not significant.

The amount of bleeding varied between animals, but both the mean and distribution of the control and P188-treated groups were similar (Fig. 9). In fact, the P188-treated group had slightly less bleeding. Animals who lost less than 40% of blood volume uniformly survived, whereas all of those that lost greater than 55% died. However, as seen in previous experiments, treatment with P188 allowed animals to survive longer with larger blood loss than the controls (data not shown).

DISCUSSION

Earlier studies demonstrated that P188 added to fresh whole blood and crystalloid during full resuscitation is able to prolong survival of animals with hemorrhagic shock. The results were significant, but the protocols were not representative of field conditions and left important questions unanswered. Hypotensive resuscitation is now used to buy time for control of hemorrhage before full resuscitation can begin. Current methodologies are beneficial, but far from optimal in that ischemic tissue damage progresses and contributes to reperfusion injury and the no-reflow phenomenon. The present studies were undertaken to determine if P188 could prolong the duration of hypotensive resuscitation and reduce its complications, facilitating better survival and recovery with full resuscitation.

The first experiment was designed to evaluate the effects of P188 in combination with Hextend on survival during hypotensive resuscitation. Hextend was chosen because it is being used clinically for hypotensive resuscitation. All of the animals had the same degree of hypotension documented by continuous computer monitoring. Nevertheless, 70% of P188 + Hextend–treated animals survived hypotension for 3 h longer than the Hextend-treated controls, and the remaining 30% survived 7 to 14 h after the last control had died. It is known that the blood pressure of animals undergoing hypotensive resuscitation spontaneously increases after cessation of fluid administration at MAP of 60 mmHg. This is known as spontaneous resuscitation (3). The combination of Hextend plus P188 facilitated greater and longer lasting spontaneous

resuscitation than Hextend alone documented by continuous computer monitoring.

Additional studies investigated the effects of P188 on the complications of hypotensive resuscitation in protocols designed to simulate clinical conditions of hemorrhagic shock. Most control animals survived hypotensive resuscitation but died after initiation of full resuscitation. Tissue damage progressed despite full resuscitation in control animals. Several studies suggested that this was due to the no-reflow phenomenon and reperfusion injury. The volume of resuscitation fluid required to maintain blood pressure was markedly reduced by P188. This was confirmed by measurements of Evans blue and tissue water. In both assays, P188 reduced leakage of fluid into the tissue of each organ studied. The reduction in fluid loss was especially significant in the lung and gastrointestinal tract that suffered the largest extravasation of fluid as a result of prolonged hypotension. These results suggest that P188 protected the integrity of the endothelial cells of the microvasculature. These results are particularly interesting because Hextend had previously been shown to reduce the volume of fluid required for hypotensive resuscitation more than any other agent tested (3).

The no-reflow phenomenon occurs when blood fails to flow adequately when blood pressure is restored after a period of ischemia (17). It has been studied extensively in ischemic heart disease and is probably related to microvascular collapse that has been extensively studied in hemorrhagic shock (18, 19). It is due to a combination of many factors including increased neutrophil adhesion, aggregation of RBCs, and damage to cells of the microvasculature. The finding of aggregated RBCs in the microvasculature of the heart 1 h into full resuscitation is evidence that no reflow occurred in our model. Similar findings were observed in the liver, kidney, and small intestine. We are not aware of previous reports of morphologic evidence of the no-reflow phenomenon. Congestion of vessels by uniformly packed RBCs is common, especially in autopsy specimens. However, the presence of dense aggregates of RBCs in an organ largely free of congestion is novel and consistent with observations of sludged blood flow in postischemic states (20, 21). By 5 h, RBCs had disappeared entirely from the large areas of myocardium. In contrast, RBCs remained present in the microvasculature of P188-treated animals in a nearly normal distribution throughout the study. Similar changes were observed in each of the organs studied.

Poloxamer 188 was originally used as a rheological agent to decrease whole-blood viscosity without hemodilution and improve blood flow in damaged tissue (22, 23). Using video microscopy, P188 was observed to improve mesenteric microcirculation in hemorrhagic shock within minutes (20). The ability of P188 to counter the no-reflow phenomenon and improve microvascular perfusion was probably an essential component to its efficacy in this study.

Poloxamer 188 in resuscitation fluid abrogated the characteristic lesions produced by shock. The lesions in control animals increased with time after initiating full resuscitation, suggesting that they were due to reperfusion injury. Poloxamer 188 reduced acute tubular necrosis of the kidney, central lobular necrosis of the liver, ALI, small bowel mucosal disruption, and myocardial dysfunction (wavy fibers). The protective effects of P188 were supported by significant changes in biochemical markers of inflammation (MPO) and apoptosis (caspases). Each of the organs from animals treated with P188 had less tissue damage and less inflammation than was observed in controls treated identically except for P888.

Finally, because hypotensive resuscitation typically is used before definitive hemorrhage control is possible, it is essential that it does not exacerbate bleeding. The effects of P188 on such bleeding were investigated in an uncontrolled hemorrhage model using 75% tail amputation. Poloxamer 188 did not cause increased bleeding. Both the mean volume of blood loss and distribution among animals were unchanged be P188. AQ5 In fact, the mean blood loss was slightly smaller in the P188-treated group than in controls. This is consistent with previous studies that P188 has no effect on blood coagulation, platelet aggregation, or bleeding time. Poloxamer 188 has been infused into nearly 4,000 acutely ill patients with acute myocardial infarction in combination with thrombolytic enzymes and acute crisis of sickle cell disease, with no reports of bleeding problems (24–26). It has also been used in cardiac bypass surgery and other surgery in hundreds of patients, with no adverse effects on bleeding (26).

The mechanisms of action of P188 deserve comment. Its first use as a pharmacological agent was to prevent hemolysis during cardiac bypass surgery (26). Subsequently, cytoprotective effects of P188 have been reported following heat injury, cold, freeze-thaw, physical trauma, electric shock, and a variety of other chemical and biologic insults (23, 27, 28). The mechanisms of the cytoprotective effects of P188 have been extensively studied (29). Poloxamer 188 binds by hydrophobic interaction to foci of damaged membranes like a Band-Aid, restores fully hydrated surfaces, and protects the cell. When the membrane is repaired, P188 dissociates and floats away. This membrane-sealing cytoprotective effect has been implicated in multiple conditions (28). The present studies provide histological confirmation of *in vitro* findings that P188 inhibits both necrosis and apoptosis induced by trauma (15).

Poloxamer 188 also has anti-inflammatory effects demonstrated in multiple models including acute myocardial infarction and bleomycin toxicity (30–32). It seems likely that the membrane-sealing cytoprotective effect contributes to reducing inflammation. In addition, P188 has been reported to inhibit phospholipase A_2 (PLA2) *in vitro* and *in vivo* (33, 34). Because PLA2 is emerging as a key mediator of I/R injury, inhibition of PLA2 probably contributes to protective effects of P188 in these studies (35). Collectively, these data suggest that P188 deserves further evaluation as an adjunct to hypotensive resuscitation.

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REFERENCES

1. Sauaia A, Moore FA, Moore EE, Moser KS, Brennan R, Read RA, Pons PT: Epidemiology of trauma deaths: a reassessment. *J Trauma* 38:185–193, 1995.
2. Holcomb JB, Jenkins D, Rhee P, Johannigman J, Mahoney P, Mehta S, Cox ED, Gehrke MJ, Beilman GJ, Schreiber M, et al.: Damage control resuscitation: directly addressing the early coagulopathy of trauma. *J Trauma* 62:307–310, 2007.
3. Handrigan MT, Bentley TB, Oliver JD, Tabaku LS, Burge JR, Atkins JL: Choice of fluid influences outcome in prolonged hypotensive resuscitation after hemorrhage in awake rats. *Shock* 23:337–343, 2005.
4. Dutton RP, Mackenzie CF, Scalea TM: Hypotensive resuscitation during active hemorrhage: impact on in-hospital mortality. *J Trauma* 52:1141–1146, 2002.
5. Dubick MA, Atkins JL: Small-volume fluid resuscitation for the far-forward combat environment: current concepts. *J Trauma* 54:S43–S45, 2003.
6. Moore FA, McKinley BA, Moore EE: The next generation in shock resuscitation. *Lancet* 363:1988–1996, 2004.
7. Rushing GD, Britt LD: Reperfusion injury after hemorrhage: a collective review. *Ann Surg* 247:929–937, 2008.
8. Lee KW, Norell MS: Management of 'no-reflow' complicating reperfusion therapy. *Acute Card Care* 10:5–14, 2008.
9. Balogh Z, McKinley BA, Cox CS Jr, Allen SJ, Cocanour CS, Kozar RA, Moore EE, Miller IC, Weisbrodt NW, Moore FA: Abdominal compartment syndrome: the cause or effect of postinjury multiple organ failure. *Shock* 20:483–492, 2003.
10. Grindel JM, Jaworski T, Piraner O, Emanuele RM, Balasubramanian M: Distribution, metabolism, and excretion of a novel surface-active agent, purified poloxamer 188, in rats, dogs, and humans. *J Pharm Sci* 91:1936–1947, 2002.
11. Hymes AC, Safavian MH, Gunther T: The influence of an industrial surfactant Pluronic F-68, in the treatment of hemorrhagic shock. *J Surg Res* 11:191–197, 1971.
12. Mayer DC, Strada SJ, Hoff C, Hunter RL, Artman M: Effects of poloxamer 188 in a rabbit model of hemorrhagic shock. *Ann Clin Lab Sci* 24:302–311, 1994.
13. Hickson-Bick DL, Buja ML, McMillin JB: Palmitate-mediated alterations in the fatty acid metabolism of rat neonatal cardiac myocytes. *J Mol Cell Cardiol* 32:511–519, 2000.
14. Fuster V, O'Rourke P, Poole-Wilson P, Walsh R: Pathology of acute myocardial infarction [chapter 57]. In: *Hutrst's The Heart*. 12th ed. McGraw-Hill, 2008. **AQ7**
15. Serbest G, Horwitz J, Barbee K: The effect of poloxamer-188 on neuronal cell recovery from mechanical injury. *J Neurotrauma* 22:119–132, 2005.
16. Capone AC, Safar P, Stezoski W, Tisherman S, Peitzman AB: Improved outcome with fluid restriction in treatment of uncontrolled hemorrhagic shock. *J Am Coll Surg* 180:49–56, 1995.
17. Barroso-Aranda J, Schmid-Schonbein GW, Zweifach BW, Engler RL: Granulocytes and no-reflow phenomenon in irreversible hemorrhagic shock. *Circ Res* 63:437–447, 1988.
18. Eeckhout E, Kern MJ: The coronary no-reflow phenomenon: a review of mechanisms and therapies. *Eur Heart J* 22:729–739, 2001.
19. Salazar Vazquez BY, Wettstein R, Cabrales P, Tsai AG, Intaglietta M: Microvascular experimental evidence on the relative significance of restoring oxygen carrying capacity vs. blood viscosity in shock resuscitation. *Biochim Biophys Acta* 1784:1421–1427, 2008.
20. Grover FL, Newman MM, Paton BC: Beneficial effect of Pluronic F-68 on the microcirculation in experimental hemorrhagic shock. *Surg Forum* 21:30–32, 1970.
21. Hinshaw LB: Sepsis/septic shock: participation of the microcirculation: an abbreviated review. *Crit Care Med* 24:1072–1078, 1996.
22. Hunter RL, Papadea C, Gallagher CJ, Finlayson DC, Check IJ: Increased whole blood viscosity during coronary artery bypass surgery. Studies to evaluate the effects of soluble fibrin and poloxamer 188. *Thromb Haemost* 63:6–12, 1990.
23. Knize DM, Weatherley-White RCA, Paton BC: Use of antisludging agents in experimental cold injuries. *Surg Gynecol Obstet* 129:1019–1026, 1969.
24. Investigators R: Effects of RheothRx on mortality, morbidity, left ventricular function, and infarct size in patients with acute myocardial infarction. Collaborative Organization for RheothRx Evaluation (CORE). *Circulation* 96:192–201, 1997.
25. Orringer EP, Casella JF, Ataga KI, Koshy M, Adams-Graves P, Luchtman-Jones L, Wun T, Watanabe M, Shafer F, Kutlar A, et al.: Purified poloxamer 188 for treatment of acute vaso-occlusive crisis of sickle cell disease: a randomized controlled trial. *JAMA* 286:2099–2106, 2001.
26. Nagata Y, Kakai T, Naike K, Kato R, Fukuta I, Yano T, Kobayashi M, Al E: Clinical evaluation on the effect of poloxamer 188 on the hemolysis during cardiopulmonary bypass. *J Aichi Med Univ Assoc* 11:48–54, 1983. **AQ8**
27. Murhammer DW, Goochee CF: Structural features of nonionic polyglycol polymer molecules responsible for the protective effect in sparged animal cell bioreactors. *Biotechnol Prog* 6:142–148, 1990.
28. Yasuda S, Townsend D, Michele DE, Favre EG, Day SM, Metzger JM: Dystrophic heart failure blocked by membrane sealant poloxamer. *Nature* 2005. **AQ9**
29. Lee RC, Hannig J, Matthews KL, Myerov A, Chen CT: Pharmaceutical therapies for sealing of permeabilized cell membranes in electrical injuries. *Ann N Y Acad Sci* 888:266–273, 1999.
30. Justicz AG, Farnsworth WV, Soberman MS, Tuvlin MB, Bonner GD, Hunter RL, Martino-Saltzman D, Sink JD, Austin GE: Reduction of myocardial infarct size by poloxamer 188 and mannitol in a canine model. *Am Heart J* 122:671–680, 1991.
31. Williams JH Jr, Chen M, Drew J, Panigan E, Hosseini S: Modulation of rat granulocyte traffic by a surface active agent *in vitro* and bleomycin injury. *Proc Soc Exp Biol Med* 188:461–470, 1988.
32. Tan J, Saltzman WM: Influence of synthetic polymers on neutrophil migration in three-dimensional collagen gels. *J Biomed Mater Res* 46:465–474, 1999.
33. Shakir KM, Williams TJ: Inhibition of phospholipase A_2 activity by fluosol, an artificial blood substitute. *Prostaglandins* 23:919–927, 1982.
34. Ballas SK, Files B, Luchtman-Jones L, Benjamin L, Swerdlow P, Hilliard L, Coates T, Abboud M, Wojtowicz-Praga S, Grindel JM: Safety of purified poloxamer 188 in sickle cell disease: phase I study of a non-ionic surfactant in the management of acute chest syndrome. *Hemoglobin* 28:85–102, 2004.
35. Jordan JR, Moore EE, Sarin EL, Damle SS, Kashuk SB, Silliman CC, Banerjee A: Arachidonic acid in postshock mesenteric lymph induces pulmonary synthesis of leukotriene B4. *J Appl Physiol* 104:1161–1166, 2008.