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14. ABSTRACT This study has rested on two basic hypotheses. The first is that agents given during and (for a short period of time) after resuscitation can favorably influence the response to hemorrhagic shock and injury. The second is that studies of gene response studies, specifically microarray and RT-PCR studies, could be used to assess the response of patients being treated for injuries to such therapeutic agents. To date, both hypotheses have been shown to be true in the animal model. Three therapeutic agents have been identified, to be used during and after resuscitation. Studies are ongoing to validate these hypotheses in patients, including the use of arginine, glutamine, and allopurinol in patients immediately following resuscitation.					
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2. Abstract. Gene Activation In White Blood Cells Following Arginine Administration In A Model Of Hemorrhagic Shock.	
3. Published Manuscript. Hemorrhagic Shock in the Rat: Comparison of Carotid and Femoral Cannulation.	

INTRODUCTION

Shock is a leading cause of death among American soldiers wounded in battle. If an injury is not immediately lethal, most deaths result from hemorrhagic shock or from its late sequelae, septic shock and multiple organ failure. The critical time in shock appears to be the period during which the patient is being resuscitated. Resuscitation is associated with a massive activation of the inflammatory reaction, producing immunosuppression, and rendering the patient vulnerable to sepsis and its sequelae. The *goal* of this research program is to develop new treatments for hemorrhagic shock which can be administered before or during initial resuscitation. These treatments are intended to be applied by front-line responders on the battlefield (and first responders in civilian life) as well as by fixed facilities, such as a Forward Surgical Team or a Combat Support Hospital. These agents must be non-toxic and have a very broad therapeutic ratio, so that they can be given safely to injured soldiers, and must be easy to administer under combat conditions. In previous work, the xanthine oxidase inhibitor allopurinol was found beneficial in a shock model (1). In current work, using an animal model of hemorrhagic shock (2), glutamine, glutamine-alanine dipeptide, and arginine have shown efficacy. (3,4) Studies with crocetin have also shown efficacy (5), but this agent is not available in a form which can be administered to patients. Others have advocated the use of DHEA, but studies in our laboratories have failed to show a useful effect (6). Studies with omega-3 fatty acids have shown promise, but are still at an early stage.

The *method* of the research is to measure the response of the genome to hemorrhagic shock using microarray studies. The effect of these agents in patients will be compared to their effect in the animal model, to assess whether they will be effective in the clinical setting.

BODY

Progress towards the four research objectives, while significant, has been slower than would be desired. The microarray studies in the animal models have been slowed by the relocation of the laboratory of the collaborating investigators to new quarters in a newly-built research building. Authorization for human studies was not obtained until June, 2007, and it has proven difficult to find suitable subjects. One of the key individuals, Dr. R. Yang, who carried out the animal studies, returned to China somewhat abruptly and unexpectedly. New personnel are being recruited. On the other hand, the PI (Dr. Van Way) was named to a research professorship (Soslund Chair of Trauma Research) effective July 1, 2008, and has been able to devote considerably more effort during the past year. Recruitment of patients has been expanded, and the therapeutic arm of the study (Objective #4) is about to begin. A no-cost extension of the grant until May, 2010, is pending.

KEY RESEARCH ACCOMPLISHMENTS

Research objective #1: Establish and validate the micro-array studies, using the animal model. *Deliverable:* A panel of genes that are reproducibly altered in white blood cells and in liver and muscle by shock and resuscitation.

1. These preliminary studies have been carried out, and presentation and/or publications are being prepared. (See list of publications, below). Collaboration continues with the laboratory of Dr. Peter Smith, Kansas University Medical Center, Kansas City, Kansas. This facility carries out micro-array analysis of both animal and human genomes.
2. To briefly summarize the work in this area, the four experimental groups noted below were studied. Even in the sham group, genomic response was quite marked. The microarray analysis used 31,099 probes. These data, which are typical of the entire data set, indicates that even the sham surgical procedure was a strong stimulant to the gene response in white blood cells. The overall pattern of the response to untreated shock and to shock with either form of resuscitation was qualitatively similar. This data supports previous work by many others indicating that so-called hemorrhagic shock procedures are, in fact, a combination of trauma and shock. As noted below, it is quite feasible to identify genes which are differentially responsive to sham, shock, and shock with resuscitation. Also refer to the pathway analysis, below.

Experimental group	Genes upregulated	Genes downregulated
Sham at 30 minutes vs sham baseline	4993	7178
Shock at 30 minutes vs shock baseline	4758	7579
Shock + LR at 30 minutes vs shock + LR baseline	5554	7510
Shock + LR + arginine at 30 minutes vs baseline	6143	6325

3. As noted in the previous-year report, a list of signature genes has been defined. The methodology for identifying these genes was as follows. All of the gene responses were uploaded into a web-based system maintained by Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com; see also below, #8). This system combines data analysis with an extensive genome knowledge base. Genes which showed a strong up-regulation or down-regulation (at least fivefold) to shock, as compares with sham controls, were selected. They were considered as possible signature genes if they showed a difference in their response to shock and to one of the two treatment arms, either fluid resuscitation (shock + LR) or fluid resuscitation plus arginine (shock + arginine). The genes meeting these criteria numbered 390. Selection among these was then carried out by eliminating genes which appeared to be irrelevant to the inflammatory reaction, such as oncogenes. Some genes were selected because they appeared to be related to the known components of the inflammatory reaction (cytokines). Several genes were kept on the list because their response in this shock model suggested that they

would be good markers of the response to the surgical preparation and to shock (B3GALNT, F3, GRID2, GRM5). The final list of 20 “signature” genes (below) are to be considered representative of genes which react strongly to the shock preparation, and which appear to be modified by treatment.

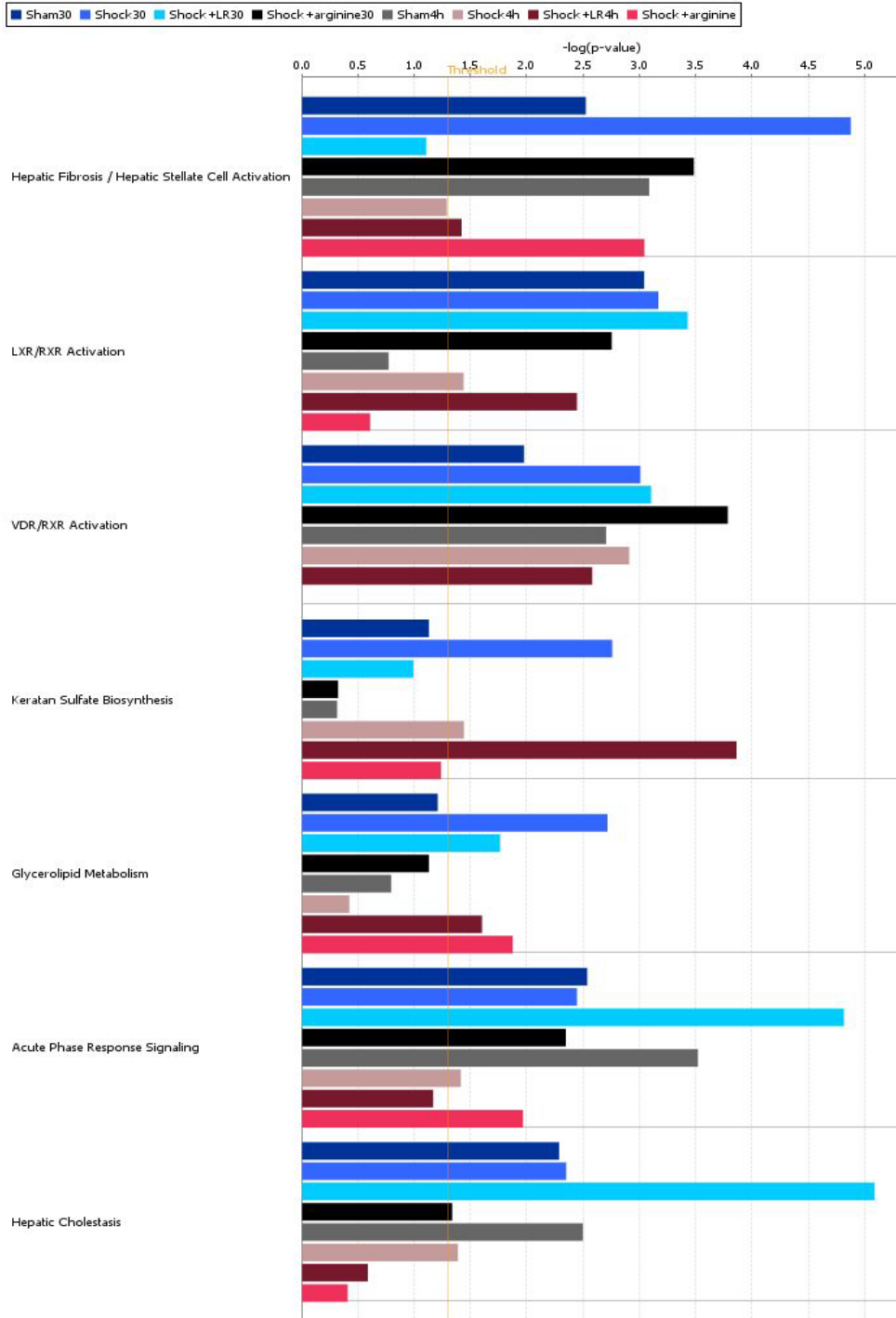
Signature Genes

<i>Gene</i>	<i>Protein and function</i>
ALOX5	Arachidonate 5-lipoxygenase (cytoplasmic enzyme)
B3GALNT1	Beta 1,3-N-acetyl/galactosaminyltransferase 2, membrane-bound galactotransferase enzyme
CXCL3	Chemokine (C-X-C motif) ligand 3, chemokine, cytokine
CXCL11	Chemokine (C-X-C motif) ligand 11, chemokine, cytokine
F3	Coagulation factor III, membrane receptor
GRID2	Glutamate receptor, ionotropic, delta 2 (ion channel)
GRM5	Glutamate membrane receptor
HSPA1B	Heat shock 70kDa protein 1b
HSPBAP	HSPB (heat shock 27kDa) associated protein 1, binds HSPB1
IL1A	Interleukin-1, alpha, cytokine
IL1R2	Interleukin 1 receptor, type II, membrane receptor
MSR1	Macrophage scavenger receptor 1, membrane receptor
NOS2A	Nitric oxide synthase 2A (inducible), cytoplasmic enzyme
NLGN1	Neuroigin 1, membrane receptor (neuronal)
NPY1R	Neuropeptide Y receptor Y1, membrane receptor (G-protein coupled)
NR4A3	Nuclear receptor (subfamily 4, group A, member 3), steroid-thyroid hormone receptor superfamily
PLAUR	Plasminogen activator, urokinase receptor, membrane receptor
RGS1	Regulator of G-protein signaling 1, membrane regulatory protein
ST3GAL3	ST3 beta-galactoside alpha-2,3-sialyltransferase 3, membrane glycosyltransferase enzyme
TNF	Tumor necrosis factor (TNF superfamily, member 2), cytokine

4. More detailed analysis of metabolic and signaling pathways has been carried out. The data has been presented in last year’s annual report.
5. To facilitate future work in this area, development continues on an assay system incorporating RT-PCR, using a microplate system. (TaqMan Gene Expression Assay, Applied Biosystems, Foster City, CA). In this system, 96-well plates will be made up, to incorporate all or some of the above genes, each replicated four times. There will be 8 genes used as standards (“housekeeping” genes), which will be genes un-affected by the surgical preparation, shock, or treatment. The response of the signature genes will be referenced to these standard genes. This system will allow four replications of a single sample to be determined. It is

anticipated that this system will provide validation of the microarray studies, but more importantly, will enable future studies to be done with considerably better accuracy and reproducibility.

Analysis: Sham30



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Figure 1: Relative activation of genes in the seven pathways most affected by shock., compared in each group with the baseline values for that group. The first four bar graphs for each pathway represent sham 30 minutes, shock 30 minutes, shock resuscitated with LR at 30 minutes, and shock resuscitated with LR and arginine at 30 minutes. The second four bar graphs represent the same experimental groups at 4 hours. Most of the pathways show an attenuated response at four hours.

Research objective #2: Define the effect on gene expression of the agents which will be used clinically, using the animal model. *Deliverable:* The effect of the agents upon the level of expression of the signature genes.

1. The agents to be used clinically agents will be allopurinol, glutamine, and arginine. Glutamine is an amino acid which has been approved for use in humans, Arginine is also an amino acid which strongly ameliorates the inflammatory reaction, and has shown benefit in the rat model. Allopurinol is available in an intravenous form, and will be used in the further studies. This drug was shown to provide survival benefit in the canine shock model 20 years ago, by the PI and others (1). It appears to act by inhibiting xanthine oxidase, and hence lowering the production of free radicals during reperfusion. Survival studies with each of these agents have been carried out. This was done in the rat model for glutamine and arginine. Previous work by the PI has shown survival benefit for allopurinol in both the canine shock model and the rat model.

Glutamine studies are presented in this table.

Survival with or without Glutamine in Resuscitation

	LR	Glutamine
Rat weight (gram)	295.83±3.03	293.00±3.84
Cannulating Time (minutes)	6.50±0.29	6.50±0.23
Bleeding (ml)	9.22±0.41	9.05±0.58
*Survival > 24 hours	4/12	11/12

Shock at MAP = 22±4 mmHg, 90 minutes. Resuscitated with Ringer's Lactate (LR, 21ml/kg) with/without glutamine (630mg/kg) over 30 minutes. Data expressed as mean ± SEM. *P< 0.05 (LR vs Glutamine).

Arginine studies are presented here. In this model, arginine was nearly as effective as glutamine.

Survival with or without Arginine in Resuscitation

	LR	Arginine
Rat weight (gram)	286.70±4.03	294.40±4.81
Cannulating Time (minutes)	6.35±0.13	6.80±0.17
Bleeding (ml)	9.32±0.31	10.06±0.35
*Survival < 24 hours	11/20	4/20
Survival > 72 hours	6/20	12/20

Shock at MAP = 20±4 mmHg, 90 minutes. Resuscitated with Ringer's Lactate (LR, 21ml/kg) with/without arginine (300mg/kg) over 30 minutes. Data expressed as mean ± SEM. *P< 0.05 (LR vs Arginine).

2. The genome data from the shock experiment described in Objective #1 will be analyzed further, specifically in the analysis of the two shock groups treated with and without arginine. The objective will be to determine the differential effect of adding arginine.

3. More studies are planned during the coming year to study the other two agents in the same way, to determine the differential effects of glutamine and allopurinol in the animal model.
4. A cell-culture based model has been developed, using a standard macrophage cell line (RAW 264.7). This model is based on exposing cultured macrophages to ischemia in a sealed chamber, and then re-exposing them to levels of oxygen found in room air (21%). Reducing the level of oxygen to 1.5% from the usual 21% produces a significant hypoxic insult. This corresponds to a partial pressure of oxygen of 10 mm Hg. Measuring the effect of therapeutic agents on hypoxia-challenged cell lines, will provide further evaluation of their possible therapeutic effectiveness. Several significant preliminary observations have been made.

- a. Cells grown in hypoxia grow at a rate about 15% less than cells in normoxia for 24 hours, and their growth slows markedly thereafter as compared with normoxic controls.
- b. Adenosine triphosphate (ATP) levels within the cells were measured using a luciferase assay. Data indicates that ATP in cells left chronically ischemic for up to 24 hours have the same ATP content per cell as cells grown in normoxia,
- c. Hypoxia produces a differential response to iNOS. Ischemic cells show an increase in mRNA for iNOS (by RT-PCR), but no increase in nitric oxide activity, and no increase in protein content (by Western blot).
- d. Studies strongly suggest that stress-induced modifications of host responses are associated with a change in the proteolytic subunits of the multifunctional cellular proteasome. This was confirmed in the hypoxic macrophage model, in which the chymotrypsin-like activity, but not the trypsin-like or post-glutamate activities, was increased in hypoxia. Further, this change in activity was found to be responsive to changes in arginine concentrations.
- e. In analyzing the protein subunits which comprise the proteasome, we found over-expression of LMP2 protease as compared with constitutively-expressed X protease. Moreover, this over-expression was responsive to changes in arginine concentrations. In the Western blot below, bands reflect relative levels of LMP2 protease and the numbers indicate the arginine concentration in culture media. Levels of LMP2 appears to be highly responsive to the concentration of arginine.



- f. The proteasome is central to many of the cell's immune functions, and to its response to inflammatory stimuli, including MHC-directed antigen presentation and regulation of cytokine production. Arginine concentration appears to be important in regulation of proteasomal activity. This underscores the concept which we expect to develop to maturity in further research, namely that changes in levels of arginine and/or glutamine at the level of the cell can profoundly affect the inflammatory response at the cellular level through changes in the proteasome.

- g. Further studies on glutamine are being carried out. In addition, expansion of this work to the study of intestinal epithelial cells (Caco2) is planned.

Research objective #3: Assess gene expression in patients treated with current initial resuscitation protocols. Deliverable: Coordinated with objective #1, A panel of genes that are reproducibly altered in white blood cells and in liver and muscle by shock and resuscitation.

1. The experimental protocol was approved by the Institutional Review Board of the University of Missouri – Kansas City. It was reviewed and approved by the Human Research Protection Office (HRPO), Office of Research Protections (ORP), U.S. Army Medical Research and Materiel Command (USAMRMC).
2. Under this protocol, blood samples have been taken from patients arriving in the trauma center who are injured severely enough to exhibit blood loss or signs of hypovolemia. Blood samples have been collected within one hour following resuscitation, again at 24 hours, and at 7 days or time of discharge from hospital, whichever comes first. These samples are then processed to isolate the white blood cells and extract the RNA, and then submitted for microarray analysis. Of 15 patients projected for this phase of the study, 13 have completed the protocol, with successful sample processing, and their data is presently being analyzed. Data on the first 5 patients is complete.
3. While full analysis will await availability of more of the patient data, several conclusions can be drawn. First, the genomic response is maximal at the first hour following resuscitation, with progressively less response at 24 hours and at 7 days or discharge day. Second, there is broad response across the genome, but large responses (four-fold up or down) is confined to a relatively smaller group of genes. Third, many of the genes involved in inflammation are highly up-regulated following trauma.
4. There have been a number of limitations to this study. First, the requirement for obtaining informed consent for the study has limited enrollment to patients with only mild to moderate trauma and blood loss. A further limitation is that only those patients whose mental faculties are intact can participate in the consent process. It has not been possible to enroll patients with severe shock, because there is no time for an adequate informed consent before the patient must be taken to the operating room. Typical time from emergency department admission to operating room is around 10 minutes in severely injured patients. Second, sample processing has been difficult, because the method initially used for white blood cell isolation was time-consuming and technically difficult. Since many patients arrive at night or on weekends, it has been difficult to carry out the processing. However, a commercially available white blood cell isolation system has been introduced. (LeukoLOCK Total RNA Isolation System, Ambion, Austin TX). This system allows the investigators to extract the white blood cells using a simple filter technique, inactivate the cells using a commercial hypertonic preservation agent (RNAlater, Ambion, Austin, TX), and keep the sample refrigerated for several days without degradation or alteration of the

mRNA. This has greatly simplified initial processing, and has resulted in good enrollment to the study.

5. Data analysis has been in terms of the initial and 24-hour samples being compared with the 7-day (or discharge) samples. While this has been a very useful way to analyze the data – each patient is, basically, his or her own control – we propose also to obtain samples on a group of 10 normal volunteers, to act as a population control. This group will be selected from hospital and university personnel, from volunteers, and will be selected to match the age and sex distribution of the trauma patients – i.e., age 20 to 40, 80% men. This has been included in the latest modification of the study protocol (see below).

Research objective #4: Assess the response of patients to administration of modified resuscitation procedures. Deliverable: The effect of the experimental agents upon signature genes, cellular response, and cellular damage in patients.

1. This objective is ready to begin. On the basis of work carried out so far, glutamine, arginine, and allopurinol are the agents selected for human use. All three have shown improvement in survival in whole-animal models of shock, all can be given intravenously, and all are safe when given to humans in the doses contemplated. The study has been approved by the Institutional Review Board of the University of Missouri - Kansas City, and is undergoing the Department of Defense review process, specifically by the Human Research Protection Office (HRPO), Office of Research Protections (ORP), U.S. Army Medical Research and Materiel Command (USAMRMC).
2. To complete this portion of the study, we have requested a no-cost extension of the grant for 1 year. While we are awaiting final documentation, we have been told that this extension will be approved.

REPORTABLE OUTCOMES

PUBLICATIONS AND PRESENTATIONS (Italized items in previous reports)

1. Yang R, Tan X, Thomas AM, Shen J, Qureshi N, Morrison DC, Van Way CW 3rd. *Crocetin inhibits mRNA expression for tumor necrosis factor-alpha, interleukin-1-beta, and inducible nitric oxide synthase in hemorrhagic shock. JPEN J Parenter Enteral Nutr. 2006 Jul-Aug;30(4):297-301.*
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3. Yang R, Van Way CW III, Tan X, Thomas A, Shen J, Steppacher R, Qureshi N, Morrison DC. *Alanine-glutamine dipeptide inhibits expression of inflammation-related genes in hemorrhagic shock. Clinical Nutrition Week of the American Society for Parenteral and Enteral Nutrition, Dallas. February 12-15, 2006. (Oral presentation). Clinical Nutrition week,. 2006, p145. (Abstract of Distinction)*
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8. Yang R, Martin L, Woodall C, Thomas AM, Qureshi N, Morrison DC, Van Way CW III. *Administration of glutamine following hemorrhagic shock restores cellular energy, reduces cell apoptosis and damage, and increases survival. JPEN J Parenter Enteral Nutr. 31:94-100,2007.*
9. Pontes-Arruda A, Van Way CW III. *Feeding During Sepsis: What Works, What Doesn't. Clinical Nutrition Week of American Society of Parenteral and Enteral Nutrition, Jan 27-31, 2007 (Phoenix, Arizona). (Presentation and abstract)*
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11. *Rallabhandi P, Awomyl A, Thomas KE, Phalipon A, Fujimoto Y, Fukase K, Kusumoto S, Qureshi N, Sztejn MB, Vogel SN. Differential activation of human TLR4 by Escherichia coli and Shigella flexneiri 2a lipopolysaccharide: Combined effects of lipid A acylation state and TRL4 polymorphisms on signaling. J. Immunol Jan 180:1139-1147, 2008.*
12. *Yang, R, Tan X, Kenney RJ Jr, Thomas AM, Landis M, Qureshi N, Morrison DC, Van Way CW III. Hemorrhagic Shock in the Rat: Comparison of Carotid and Femoral Cannulation. Journal of Surgical Research, 144:124-126, 2008.*
13. *Qureshi N, Reis J, Shen J, Monaco JJ, Papasian CJ, Qureshi AA, Vogel SN, and Morrison DC. Cutting Edge: Differential regulation of LPS-induced TNF and NO by proteasomes. (Shock, reviewed, acceptable after changes) 2008.*
14. *Rockwell CE, Morrison DC, and Qureshi N. Lipid A mediated Tolerance and Cancer therapy. In Tolerance in Cancer Therapy. (Invited Chapter, in press) 2008.*
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17. *Van Way, CW Yang R, Vernon K, Qureshi N, Morrison D, Smith P. Gene Activation In White Blood Cells Following Arginine Administration In A Model Of Hemorrhagic Shock. American Society for Parenteral and Enteral Nutrition, Nutrition Week, Feb 1-4, 2009. (New Orleans, LA) (Presented by C. Van Way)*
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CONCLUSION

This study has rested on two basic hypotheses. The first is that agents given during and (for a short period of time) after resuscitation can favorably influence the response to hemorrhagic shock and injury. The second is that studies of gene response studies, specifically microarray and RT-PCR studies, could be used to assess the response of patients being treated for injuries to such therapeutic agents.

To date, both hypotheses have been shown to be true in the animal model. Work continues to determine optimum therapeutic agents to be used during and after resuscitation. In addition, development has begun on a multiple-gene RT-PCR assay incorporating a group of signature genes which have been identified as uniquely responsive to shock and therapy. Development of this assay will greatly simplify future work in this area, both in our laboratories and in those of other investigators.

Studies are ongoing to validate these hypotheses in patients. During the next year, which will be a no-further-cost extension of the grant, we will continue this work. The gene studies will be subjected to validation, and the use of three specific therapeutic agents will be subjected to preliminary clinical trial.

While this work has not gone as rapidly as hoped, and while no research in the area of shock proceeds without setbacks, we anticipate considerable progress in this research over the next year.

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3. Yang R, Yan X, Thomas AM, Steppacher R, Qureshi N, Morrison DC, Van Way CW 3rd. Alanine-glutamine dipeptide inhibits expression of inflammation-related genes in hemorrhagic shock. *JPEN J Parenter Enteral Nutr*. 2007 (Jan-Feb) 31(1):32-6.
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5. Yang R, Tan X, Thomas AM, Shen J, Qureshi N, Morrison DC, Van Way CW 3rd. Crocetin inhibits mRNA expression for tumor necrosis factor-alpha, interleukin-1-beta, and inducible nitric oxide synthase in hemorrhagic shock. *JPEN J Parenter Enteral Nutr*. 2006 Jul-Aug;30(4):297-301.
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American Society for Parenteral and Enteral Nutrition, Nutrition Week, 2009

TITLE: GENE ACTIVATION IN WHITE BLOOD CELLS FOLLOWING ARGININE ADMINISTRATION IN A MODEL OF HEMORRHAGIC SHOCK

ABSTRACT BODY:

Introduction: The addition of arginine to a standard resuscitation regimen was found in a previous experimental study to increase survival following lethal hemorrhagic shock. The present study tested the hypothesis that the use of arginine would alter gene activation as sampled in circulating white cells.

Methods: A sublethal model of shock was used. A total of 14 Sprague-Dawley rats (300 gm) were divided into 4 groups: sham, shock alone, shock resuscitated with Ringer's lactate, and shock resuscitated with supplemental arginine. Animals were anesthetized with fluothane. The femoral artery and vein were cannulated. After stabilization, blood was withdrawn, and a mean blood pressure of 25 mm Hg was maintained for 30 minutes (except for the sham group). In the two resuscitation groups, 28 ml/kg Ringer's lactate was given over 30 minutes. In the arginine group, arginine 300 mg/kg was added to the resuscitation solution. Blood for analysis was drawn at baseline, 30 minutes, and 4 hours. The white cells were isolated, RNA extracted, and microarray analysis was carried out using the Affymetrix system, using 31,099 probes. Data analysis was done with canonical pathway analysis (Ingenuity Systems, Inc).

Results: Significant gene activation within 26 separate canonical pathways was seen in shock resuscitated with Ringer's lactate. Supplemental use of arginine was associated with upregulation of genes in 5 pathways: hepatic fibrosis/stellate cell activation, PPAR/RXR activation, androgen/estrogen metabolism, TGF-beta signaling, and IL-10 signaling. Arginine produced decreased activation in 6 pathways: hepatic cholestasis, FXR/RXR activation, PPAR activation, acute phase response signaling, synaptic long term potentiation, and xenobiotic metabolism signaling. Analysis of the changes within pathways indicate that arginine did not change the overall pattern of gene activation, but that it affected a number of signalling elements within the involved pathways. Arginine had highly selective effects. For example, while IL-1 was up-regulated in both resuscitation groups, IL-1 and IL-1 receptor accessory protein were further activated in the arginine group. MAP-kinase 11 was de-activated, while MAPK9 was further activated. Significantly, the gene SOCS2 (suppressor of cytokine signalling 2), was more activated following arginine as compared with Ringer's lactate alone.

Conclusions: Administration of arginine in this model differentially affected 11 canonical pathways. The pattern suggests that its effects on survival is associated with a broad range of effects at the gene activation level on both signaling and metabolic pathways.

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FISH OIL FEEDING MODULATES INFLAMMATION IN A RAT MODEL OF HEMORRHAGIC SHOCK

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Background: In the rat model of hemorrhagic shock, the inflammatory reaction plays a critical role.

Objective: We hypothesized that pre-feeding rats with fish oil rich in omega-3 fatty acids (EPA+DHA) would modulate the inflammatory response following experimental shock.

Procedures: Male rats (350±30g; n=48) were randomized to either control or fish oil diets. The latter contained 600 mg fish oil/kg diet/day; 150 mg EPA+DHA /kg/day, about 1% energy needs) whereas the former contained corn oil. Under fluothane anesthesia, shock was initiated by bleeding, and resuscitation carried out by reinfusing the shed blood and lactated Ringer's solution (21 ml/kg). Half of each group (n=12) was sacrificed at 30 minutes and half at 4 hours post-resuscitation. The effect on inflammation was determined in two ways. First, liver samples were assayed for mRNA for iNOS and IL-1-beta mRNA as indicators of inflammation, and for heat shock protein 25 (Hsp25). Second, at 4 hours, the lung tissue edema index was evaluated by measuring the ratio between the wet weight and dry weight of the whole lungs.

	Corn Oil	Fish Oil
iNOS mRNA (30 minutes) delta-delta-C _t	6.16±2.2	1.57±0.73*
IL-1beta mRNA (4 hours) delta-delta-C _t	3.39±1.56	1.83±0.75
Hsp25 mRNA (4 hours) delta-delta-C _t	1.14±0.22	2.11±0.74*
Lung edema index (4 hours)	5.28±0.28	4.69±0.14*
Mean±SEM; *p<.05		

Results: In the fish oil group, iNOS mRNA was reduced at 30 minutes. At 4 hours, IL-1-beta mRNA was reduced (p=0.08), while Hsp25 mRNA was increased. The lung edema index was reduced at 4 hours.

Conclusion: Fish oil pre-feeding reduced several markers of inflammation in a rat model of hemorrhagic shock. Whether higher tissue omega-3 levels would improve outcomes in humans with severe blood loss should be explored.

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Hemorrhagic Shock in the Rat: Comparison of Carotid and Femoral Cannulation

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Background. The reservoir model of rat hemorrhagic shock is widely used. In this model, either the carotid or femoral artery can be cannulated to withdraw blood and measure pressure. In animals undergoing hemorrhage using the carotid approach, we observed seizure activity during the post-shock period, suggesting some degree of brain damage. The hypothesis of the present study is that survival in a model of severe hemorrhagic shock would be higher with femoral cannulation than with carotid cannulation.

Materials and methods. All animals ($n = 90$) were anesthetized with isoflurane using an anesthesia vaporizer while breathing spontaneously. In group 1, the left carotid artery and jugular vein were cannulated; in group 2, the left femoral artery and vein were cannulated. Following a period of hemorrhagic shock (20 to 30 mmHg for 30, 60, or 50–90 min), resuscitation was performed through the venous cannula by giving L-lactated Ringer's (21 mL/kg) and returning the shed blood.

Results. In the carotid cannulation group, nearly 50% of the animals had seizures after resuscitation, and most of those animals died following the seizures. The 24-h survival rate in the femoral artery cannulation group was significantly higher than in the carotid artery cannulation group. Femoral cannulated animals had no seizures following reperfusion.

Conclusions. Femoral artery cannulation was associated with considerably better survival than carotid artery cannulation in this rodent model of hemorrhagic shock. The occurrence of seizures in animals undergoing carotid cannulation suggests brain dam-

age from inadequate cerebral perfusion or subsequent reperfusion damage © 2008 Elsevier Inc. All rights reserved.

Key Words: hemorrhagic shock; femoral cannulation; carotid cannulation; survival.

INTRODUCTION

The reservoir model of hemorrhagic shock using the rat is widely used in shock research. Cannulation techniques have used either the carotid or femoral artery. Carotid cannulation in the normal rat appears to produce no defects in cerebral perfusion, and the artery can be ligated at the conclusion of the procedure without apparent ill effects. However, this is not necessarily true during severe hemorrhagic shock. In a number of animals undergoing severe hemorrhage, we have observed seizure activity during the post-shock period, suggesting some degree of brain damage.

The hypothesis of the present study is survival in a rat model of severe hemorrhagic shock is higher with femoral cannulation than with carotid cannulation.

MATERIALS AND METHODS

Male Sprague Dawley rats ($n = 90$, 350 ± 30 g) were randomly assigned to two groups. In group 1 ($n = 70$), the right carotid artery and jugular vein were cannulated. In group 2 ($n = 20$), the left femoral artery and vein were used. All animals were anesthetized with isoflurane using an anesthesia vaporizer while breathing spontaneously. Following a period of hemorrhagic shock (20 to 25 or 25 to 30 mmHg for 30, 60, or 50–90 min), resuscitation was done through the venous cannula by giving L-lactated Ringer's solution (21 mL/kg) and returning the shed blood. Animals were monitored closely, and the survival rate was recorded at the end of 24, 48, and 72 h.

All animal care and experimental procedures were carried out according to the Guidelines of the Animal Care and Use Facility of the University of Missouri-Kansas City.

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TABLE 1

Cannulated Carotid Artery and Jugular Vein			
Survival time	>72 h	24–48 h	<24 h
Weight (gram)	359 ± 13	363 ± 17	359 ± 16
Bleeding (ml)	11.7 ± 0.7	12.1 ± 0.5	12.9 ± 0.4
Seizures	1/7	2/7	10/13*

Notes. Shock at 20–25 mmHg and 30 min. Data expressed as mean ± SEM.

* $P < 0.05$ (survival <24 h versus survival >24 h).

RESULTS

As shown in Table 1, rats ($n = 27$) were cannulated using the carotid arteries and were subjected to hemorrhagic shock, mean arterial pressure (MAP) = 20 to 25 mmHg, for 30 min. The survival results showed that seizures were more common in animals surviving <24 h than in those surviving >24 h ($P < 0.05$). That is, seizures were associated with earlier death following shock.

In comparing carotid ($n = 39$) and femoral ($n = 15$) cannulation, rats were subjected to MAP of 20 to 30 mmHg for 60 min. The 24 h survival rate (Table 2) in femoral cannulated animals (15/15) was significantly higher than in carotid cannulated animals (16/39). The carotid cannulation group had a significant occurrence of seizures (18/39), while the femoral cannulation group had none (0/15). Again, seizure rate was significantly higher in the group of animals that died within 24 h (Table 3, $P < 0.05$).

Table 4 shows the comparison of carotid ($n = 4$) and femoral ($n = 5$) cannulation in animals subjected to MAP of 20 to 25 mmHg for 50–90 min. All of the four rats in the carotid artery cannulation group had seizures and died within 24 h. In contrast, all five rats belonging to the femoral artery cannulation group survived longer than 24 h without seizure activity.

DISCUSSION

These survival studies have shown an advantage to femoral artery cannulation in the rat model of hemor-

TABLE 2

Comparison of Carotid and Femoral Cannulation		
Cannulated artery	Carotid	Femoral
Survival at 24 h	16/39	15/15*
Weight (gram)	355 ± 6	359 ± 12
Cannulation time (min)	7.2 ± 0.2	9.6 ± 0.6
MAP (mmHg)	25–30	20–30
Blood removed (mL)	13.6 ± 0.2	14.3 ± 0.6
Seizures	18/39	0/15*

Notes. Shock at 25–30 mmHg pressure, 60 min. Data expressed as mean ± SEM.

* $P < 0.05$ (femoral cannulation vs carotid cannulation).

TABLE 3

Cannulated Carotid Artery and Jugular Vein		
Survival time	>24 h	<24 h
Weight (gram)	360 ± 9	352 ± 7
Cannulated time (min)	7.3 ± 0.2	7.1 ± 0.4
Bleeding (mL)	13.8 ± 0.4	13.4 ± 0.3
Seizures	4/16	14/23*

Notes. Shock at 25–30 mmHg pressure, 60 min. Data expressed as mean ± SEM.

* $P < 0.05$ (survival < 24 h versus survival >24 h).

rhagic shock. Investigators have noticed that overweight and older rats have a markedly higher mortality rate following hemorrhagic shock. The present results show rats cannulated through the carotid artery in a hemorrhagic shock model also have increased mortality compared with rats cannulated through the femoral artery.

Cannulation technique is within the control of the investigator, and appears to represent a confounding factor in the rodent reservoir shock model. In studies by others, using carotid cannulation, MAP was maintained at 20 to 25 mmHg during hemorrhagic shock in the rat model. There was a high mortality following a 30 min shock period [1]. Previous studies from our laboratory showed that with femoral artery cannulation and shock at MAP = 20 to 25 mmHg for 30 min, survival was nearly 100%, even in animals receiving no resuscitation (shocked, nonresuscitated, controls; unpublished data). Lethal rat hemorrhagic models from different investigators have shown that MAP was lower and the shock time was longer using femoral cannulation than with carotid cannulation [2–5]. In one study, no functional or histological brain damage was noted in rats cannulated through the femoral artery and bled to a MAP of 40 mmHg for 60 min, or a MAP of 30 mmHg for 45 min [5].

CONCLUSION

Femoral artery cannulation was associated with considerably better survival than carotid artery can-

TABLE 4

Comparison of Carotid and Femoral Cannulation		
Cannulated artery	Carotid	Femoral
24 hours Survival	0/4	5/5*
Weight (gram)	353 ± 14	346 ± 10
Blood removed (mL)	12.8 ± 1.5	13.6 ± 1.4
Seizures	4/4	0/5*

Note. Shock at MAP = 20–25 mmHg, 50–90 minutes.

* $P < 0.05$ (femoral cannulation vs carotid cannulation).

nulation in this model of severe hemorrhagic shock. The occurrence of seizures in animals undergoing carotid cannulation suggests cerebral damage from inadequate cerebral perfusion combined with reperfusion.

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