Flaherty, DC. Pyruvate-Enhanced Fluid Resuscitation for Hemorrhagic Shock and Hindlimb Ischemia. Doctor of Philosophy (Integrative Physiology), March 25, 2009, 127 pp, 5 tables, 24 figures, 223 references, 136 titles.

Traumatic blood loss often necessitates the use of resuscitative fluids to replenish blood volume and stabilize blood pressure. The use of tourniquets to achieve hemostasis imposes ischemia-reperfusion on wounded limbs after release. Resuscitation with the physiological antioxidant and natural intermediary metabolite pyruvate may abrogate reperfusion injury of muscle by scavenging oxyradicals and stabilizing cytoprotective proteins. This study was designed to determine the effects of pyruvate in the setting of hemorrhagic shock with resuscitation and hindlimb ischemia-reperfusion. All experiments were conducted on isoflurane-anesthetized male goats. A controlled hemorrhage was performed to lower mean arterial pressure (MAP) to *c.* 50 mmHg, then the right femoral artery and vein were occluded for 90 min. Lactate Ringer's (LR) or pyruvate Ringer's (PR) was infused intravenously (10 ml/min) for 90 min, from 30 min occlusion until 30 min after reperfusion. At 4 h reperfusion, the right gastrocnemius muscle and left ventricular myocardium were biopsied and flash-frozen for analyses of metabolites, enzymes, pro- and anti-apoptotic proteins and markers of oxidative and inflammatory stress.

During the first phase of experimentation we hypothesized that controlled resuscitation with PR *vs.* LR more effectively stabilizes MAP and attenuates myocardial inflammation post-resuscitation. MAP (mmHg) was increased in PR (59 ± 4) vs. LR (47 ± 3) resuscitated goats ($p<0.05$) at 4 h post-occlusion. In addition, PR more effectively

augmented circulating HCO_3^- and total base excess, thus counteracting metabolic acidosis caused by systemic hypoperfusion. Marked tyrosine nitration, a footprint of nitrosative stress, was detected in myocardium 4 h after LR resuscitation, but was suppressed by PR. Finally, PR prevented the increase in circulating neutrophils that occurred during and following LR resuscitation.

During the second phase of experimentation, we hypothesized that PR resuscitation would protect ischemic hindlimb muscle in the setting of hemorrhagic shock and limb reperfusion. Lactate dehydrogenase and creatine kinase activities fell by 36 and 20%, respectively in LR-resuscitated *vs.* sham muscle ($p<0.05$). PR infusion preserved lactate dehydrogenase activity and more than doubled activity of the antioxidative enzyme glutathione reductase *vs.* the sham value. NADPH oxidase activity in LR-resuscitated muscle had an increased activity compared with PR-treated and sham muscle ($p = 0.056$). Poly(ADP-ribose) polymerase-2 (PARP-2) cleavage, a measure of apoptosis, was increased in the LR-resuscitated muscle but PR resuscitation prevented this pro-apoptotic effects. Moreover, LR-treated muscle had decreased content of the antiapoptotic protein Bcl-xL *vs.* the PR-treated and sham muscle. Nitrotyrosine content, a measure of nitrosative stress, more than doubled in LR-treated *vs.* sham muscle, but PR prevented the increase in nitrotyrosine. Finally, muscle water content (ml/100g) increased from 74.7 ± 1.2 in sham to 81.4 ± 2.2 4 h after LR resuscitation, indicating tissue edema; PR attenuated the increase in water content (78.1 ± 1.0).

We conclude that 1) Systemic hypotension and hindlimb ischemia-reperfusion with conventional LR treatment imposed pro-oxidative and pro-inflammatory stress both systemically and locally, thus preventing stabilization of MAP during recovery and

initiating apoptotic mechanisms in the hindlimb musculature; 2) Pyruvate-fortified Ringer's effectively stabilized hemodynamics and dampened systemic inflammation after hemorrhagic shock with resuscitation and hindlimb ischemia-reperfusion; 3) PR-fortified resuscitation blunted oxidative and inflammatory stress within the ischemic hindlimb and suppressed pro-apoptotic signaling. These investigations demonstrate the anti-oxidative and anti-inflammatory effects of pyruvate in a system exposed to hemorrhagic shock with fluid resuscitation, as well as identify the cytoprotection pyruvate affords tissue experiencing ischemia-reperfusion.

PYRUVATE-ENHANCED FLUID RESUSCITATION FOR HEMORRHAGIC SHOCK AND HINDLIMB ISCHEMIA

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas Health Science Center in Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

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Fort Worth, TX

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The ladder of success is best achieved by
stepping on the rungs of opportunity. ~ Ayn Rand

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ABBREVIATIONS

Bad	Bcl-2 antagonist of cell death
Bax	Bcl-2-associated X protein
Bcl-xL	B-cell Cll/lymphoma
BE	base excess
BL	baseline
CK	creatine kinase
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
G6PDH	glucose-6-phosphate dehydrogenase
GP	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulfide
H	hemorrhage
HR	heart rate
Hct	hematocrit
Hgb	hemoglobin
HIF	hypoxia inducible factor
HSP	heat shock protein
ICAM	intracellular adhesion molecule
LDH	lactate dehydrogenase
LR	lactate Ringer’s

MAP	mean arterial pressure
MPO	myeloperoxidase
NAD^+	nicotinamide adenine deaminase
NADPH	nicotinamide adenine dinucleotide phosphate
PARP	poly (ADP-ribose) polymerase
PI3K	phosphatidylinositol-3-kinase
PR	pyruvate Ringer's

PEER-REVIEWED PUBLICATIONS

Flaherty DC, Hoxha B, Sun J, Simecka JW, Olivencia-Yurvati AH, Mallet RT. Pyruvate-enriched resuscitation protects hindlimb musculature from ischemia-reperfusion injury in a goat model of hemorrhagic shock. Submitted to: *J Trauma*, April 2009.

Flaherty DC, Hoxha B, Sun J, Simecka JW, Mallet RT, Olivencia-Yurvati AH. Pyruvate-fortified fluid resuscitation suppresses systemic myocardial inflammation after hemorrhagic shock. Submitted to: *Mil Med*, April 2009.

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AWARDS

- 2009 Associate Member - Sigma Xi, The Scientific Research Society

- 2008 Outstanding Graduate Student – Department of Integrative Physiology, University of North Texas Health Science Center

- 2008 Medical Student Government Association Travel Award

- 2008 UNTHSC Student Affairs Scholarship

- 2008 UNTHSC Research Appreciation Day: 1st Place TCOM, poster presentation – 'Improved Fluid Resuscitation with Pyruvate-Fortified Ringer's for Hemorrhagic Shock and Hindlimb Ischemia'

- 2008 UNTHSC Research Appreciation Day: 1st Place Alcon Research, Ltd., poster presentation – 'Improved Fluid Resuscitation with Pyruvate-Fortified Ringer's for Hemorrhagic Shock and Hindlimb Ischemia'

- 2007 UNTHSC Research Appreciation Day: 2nd Place GSA, poster presentation – 'Antioxidant Effects of Pyruvate in Cardiopulmonary Bypass in Swine'

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INTRODUCTION

"The single major cause of death in potentially salvageable battlefield casualties is hemorrhage…"[63]

Hemorrhage is the leading cause of death associated with combat-related casualties in modern warfare.[15,63] During Operation Iraqi Freedom III, advances in Kevlar body armor worn by soldiers in the battlefield afforded a significant decrease in head and torso trauma, but left the extremities unprotected.[69] Trauma to the arms and legs has become the most common battlefield injury, and is often accompanied by significant blood loss, which may lead to hypovolemic shock.[63] Systemic shock causes underperfusion of the body's organ systems, which may culminate in multiple organ failure and death.

Clinical presentation of hypovolemic shock

The renowned American surgeon and researcher Alfred Blalock defined shock in the following manner: "Shock is a peripheral circulatory failure, resulting from a discrepancy in the size of the vascular bed and the volume of the intravascular fluid."[14] Dr. Blalock, with the help of his adept surgical technician Vivien Thomas, discovered this volume-based etiology of shock while serving as Professor and Chief of surgery at Vanderbilt University (Figure 1).[9,48] The Sabiston Textbook of Surgery defines

uncompensated hemorrhagic shock as an intravascular volume reduction of 20% or greater.[58] Patients losing this much blood present as hypotensive, pale and diaphoretic, with a variable heart rate, diminished capillary refill, decreased urine production and ultimately impaired consciousness.[14,58] The Berne and Levy Textbook of Physiology illustrates potential clinical outcomes of physiologically compensated (A) *vs.* uncompensated (B) states of hypovolemic shock (Figure 2).[41] Physiologic compensation is accomplished through peripheral vasoconstriction serving to centralize the body's blood supply, as well as a sympathetically-mediated increase in heart rate secondary to diminished baroreceptor activity in response to decreased mean arterial pressure (MAP).[41,63] Both mechanisms serve to increase MAP, thus optimizing end-organ perfusion during states of reduced extracellular volumes. Without these compensatory mechanisms, patients experience further decompensation and eventual cardiac arrest.[41,58]

Tourniquet application

Application of tourniquets has been confirmed as an effective battlefield technique in achieving hemostasis.[36,44,69] Although tourniquets are essential to staunch bleeding from wounded limbs, the tourniquet must eventually be released, which often initiates a systemic inflammatory cascade that injures internal organs recovering from hypovolemic shock.[2,7,13,29,33,80] Reintroduction of oxygenated blood to the ischemic limb causes explosive formation of reactive oxygen and nitrogen derivatives including hydrogen peroxide (H_2O_2), superoxide ($\cdot O_2^-$), nitric oxide ($\cdot NO$) and peroxynitrite ($ONOO^-$). These toxic compounds are central to the initiation and progression of systemic inflammation.[30,33]

"… and the greatest opportunity for reducing mortality and morbidity of battlefield casualties involves fluid resuscitation and treatment of hypovolemia."[63]

Current approaches to fluid resuscitation

Traumatic injury often requires immediate resuscitation to bolster MAP. Volume expansion increases venous return, which, consequently, improves stroke volume (SV) and, thus, cardiac output (CO). Ohm's law states MAP = total peripheral resistance (TPR) * CO.[41] Therefore, changes in MAP are directly proportional to changes in CO and TPR. Resuscitation through intravascular volume expansion affords the trauma patient an increased CO, which bolsters MAP, allowing for sustained end-organ perfusion, and ameliorates the dire systemic consequences of prolonged shock.

The resuscitative vehicle of choice for the treatment of hemorrhagic shock is an ongoing scientific debate. Factors to consider when choosing a resuscitative fluid include early hemodynamic effects, hemostasis, pH buffering potential, oxygen carrying capacity, modulation of the inflammatory response and capillary leak, safety, practicality and cost.[64] While treatment with hemoglobin-based oxygen carriers (HBOC) supplies the trauma patient with much needed oxygen carrying capacity, remote access to blood products in combat zones and rural civilian settings necessitates the use of alternative resuscitative measures.[59,67,69]

Since the work of trauma surgeon Tom Shires in burn patients, crystalloid resuscitation for hypovolemic shock has been viewed as an effective substitute for whole

blood.[9,73] Crystalloid solutions are composed of small non-ionic or ionic particles.[59] Examples of common crystalloid infusates include 0.9% saline, Hartmann's solution, Ringer's solution, PlasmaLyte 148, 5% Dextrose, 4% Dextrose in 0.18% Saline and 7.5% Saline.[59] In the setting of rapid fluid resuscitation, Ringer's solution is preferred to conventional saline solutions, due to the potential development of hyperchloremic acidosis accompanying significant saline-based volume expansion.[59]

Detrimental effects of fluid resuscitation with lactate Ringer's following hemorrhage

Since its introduction in 1883, lactate Ringer's solution has conventionally been applied as a volume expanding resuscitative fluid in the setting of severe hemorrhage.[16] Current perspectives concerning hemorrhagic shock with reperfusion are beginning to champion the idea that resuscitative measures play a large role in the associated detrimental local and systemic effects accompanying hypovolemic shock. Concern regarding lactate Ringer's toxicity dates back to observations recorded by Harvey Cushing in 1901, specifically noting lactate Ringer's "poisonous" effects on nervous tissue.[4] More recent reports indicate reperfusion with lactate Ringer's contributes significantly to oxy-radical formation and systemic inflammation.[3,5,18,65,67] Specifically, lactate Ringer's increases tissue neutrophil activation, which in turn potentiates superoxide formation.[3,10,19,43,65] This effect is expressly seen in racemic mixtures of DL-lactate Ringer's solution, the commonly used formulation in clinical settings.[43] Expression and content of the endothelial adhesion molecules E-selectin and P-selectin are also increased in the setting of lactate Ringer's resuscitation, thus enhancing neutrophil tissue infiltration.[5] Finally, •NO levels are depressed in hemorrhagic animal models reperfused

with lactate Ringer's, concordant with increased superoxide condensation with nitric oxide to produce peroxynitrite. Oxidative effects result in impaired vascular endothelium resulting in diminished endothelin-1 (ET-1) production, and, thus, a diminished systemic potential to increase blood pressure through vasoconstriction.[24,67] Impaired vasoconstriction, in concert with diminished cardiac Gibb's free energy of ATP hydrolysis secondary to reduced oxygenation, culminates in impaired end-organ perfusion and further propagation of tissue ischemia.[56,60]

Resuscitation with lactate Ringer's solution also acutely increases cellular apoptosis. Deb *et al.* demonstrated increased apoptosis in small intestine and liver tissue harvested from previously hemorrhaged rats treated with lactate Ringer's *vs.* hypertonic saline or whole blood resuscitation.[19] Other studies have shown racemic lactate Ringer's promotes apoptosis specifically by restricting phosphorylation of Bad and endothelial nitric oxide synthase (eNOS) and upregulating the pro-apoptotic protein Bax after traumatic hemorrhage with resuscitation (Figure 3).[4,18,35] In fact, many argue whole blood imposes fewer detrimental side-effects post-resuscitation than crystalloid solutions. However, traumatic events occurring in remote settings and prolonged transport times often preclude administration of whole blood, underscoring the need for effective volume expansion with proven crystalloid solutions.[67,73] The central hypothesis of this investigation is that pyruvate can be substituted for lactate in the conventional Ringer's solution, thereby ameliorating pro-inflammatory or pro-oxidant effects during and after resuscitation through its pleiotropic metabolic properties.

Molecular and systemic effects of exogenous pyruvate

Pyruvate's unique energy-yielding and antioxidant properties and its ability to protect the tissue from ischemia-reperfusion and oxyradical injury makes this metabolite a promising alternative to lactate in conventional Ringer's solution.[49,50] Pyruvate is a naturally occurring, aliphatic α-keto carboxylate that readily crosses plasma membranes via a high-capacity, high-affinity monocarboxylate transport mechanism, thereby making it available as an intracellular metabolic fuel.[12,52] As a physiologic fuel in the heart and skeletal muscle, pyruvate exerts its effects through conventional decarboxylation to acetyl CoA as well as through carboxylation-based pathways termed anaplerosis, which replenish the citric acid cycle intermediates malate and oxaloacetate.[25] Indeed, by increasing mechanical efficiency without increasing myocardial demand, pyruvate has been shown to have a positive inotropic effect on cardiac tissue in the clinical setting of cardiopulmonary bypass.[60]

Studies in isolated guinea-pig hearts demonstrated parallel enhancement by pyruvate of myocardial contractile performance, energy reserves and endogenous antioxidant defenses, the latter indexed by glutathione (GSH):glutathione disulfide (GSSG) ratio,[68] following ischemia-reperfusion injury[76] or H_2O_2-induced oxidative stress.[51] Subsequent research extended the use of pyruvate to larger animals, including dogs subjected to cardiac arrest and resuscitation[70,71] and pigs undergoing cardioplegic arrest on cardiopulmonary bypass.[38,39] In these large mammals, pyruvate enhanced GSH/GSSG in plasma and myocardium, suppressed lipid peroxidation, and protected oxidant-sensitive myocardial enzymes.[38,39,70] Finally, pyruvate-enhanced cardioplegia has been shown to be remarkably efficacious in enhancing cardiac recovery in patients

undergoing cardiopulmonary bypass surgery.[23,60] Importantly, treatment with pyruvate has never been linked to detrimental side-effects. In summary, administration of pyruvate bolstered endogenous antioxidant defenses and preserved myocardial function and metabolism during cardiac arrest-resuscitation[71] and cardiopulmonary bypass surgery.[23,38,39,60]

Pyruvate Ringer's solution in the setting of hypovolemic shock

Studies investigating resuscitation with pyruvate Ringer's have revealed an exceptional response when applied in the setting of hemorrhagic shock. Mongan *et al.* observed enhanced vascular stability and prolonged elevation of MAP after hemorrhage and resuscitation with pyruvate Ringer's vs. NaCl infusion. This group surmised the observed pyruvate-induced maintenance of vascular stability was due to diminished metabolic acidosis via pyruvate's known antioxidant effect as well as through pyruvate's establishment of a more favorable buffering capacity within the circulation.[56]

Pyruvate's cytoprotective effects on cardiac tissue during states of severe hypoxia / ischemia contribute directly to the maintenance of cardiac function during hemorrhagic shock. Upregulation of cardiac heat shock proteins (HSP) following trauma and hemorrhage protects the myocardium from the detrimental effects of systemic shock via a diminished systemic inflammatory cascade.[53] Pyruvate stabilizes the transcription factor hypoxia inducible factor – 1 (HIF-1), thereby increasing expression of such cytoprotective proteins as HSP-70 and eNOS and allowing for optimal cardiac functioning after hemorrhagic shock w/ resuscitation.[26,47]

Endothelial dysfunction is a known consequence of prolonged hypovolemic shock secondary to massive systemic hemorrhage. Mongan *et al.* reported that pyruvate delays the onset of vascular failure in a pig model of hypovolemic shock.[56] They postulated that, by reducing metabolic acidosis and stabilizing cytoplasmic phosphorylation potentials, pyruvate attenuated the opening of K_{ATP} channels, thereby preventing a decrease in intracellular Ca^{++} and preserving the contractile function of vascular smooth muscle.[1,56] Pyruvate's antioxidative and anti-inflammatory effects may also diminish vascular endothelial damage that would result in vascular failure.[74] As noted above, lactate Ringer's solution impairs vasoconstriction indirectly through production of the free radical, peroxynitrite, which weakens endothelial-dependent vascular function.[67]

Apoptosis in the setting of severe shock with concomitant pyruvate treatment

The use of a tourniquet for up to 90 minutes while a wounded soldier is being transported to a field hospital imposes prolonged ischemia on limb musculature. Among the most serious consequences of ischemia is cell death by apoptosis and necrosis.[8,42] Increases in pro-apoptotic proteins indicate amplification of cellular apoptotic signaling cascades. Current literature indicates pyruvate-fortified Ringer's solution, when administered in the setting of hemorrhagic shock, directly suppresses apoptosis in the lung and liver by preventing formation of oxyradicals, by increasing the amounts of protective proteins such as bcl-2, and by lowering pro-apoptotic proteins, such as bax, in these organs.[34,42,46]

Although the exact molecular mechanism through which pyruvate suppresses apoptosis is unknown, studies have elucidated a host of mechanisms that may contribute

to pyruvate's anti-apoptotic character (Table 1). It is well established that pyruvate enhances cellular energy states through its anaplerotic bolstering of oxidative metabolism during ischemia.[25,50,52] Ischemia-related apoptosis is initiated by mitochondrial permeability transition and leakage of cytochrome c, a pro-apoptotic activator of caspase-3.[22] Through preservation of cellular energetics, pyruvate attenuates the uncoupling of the electron transport chain that accompanies severe, prolonged ischemia. By promoting inner mitochondrial membrane integrity, pyruvate prevents the release of cytochrome c, a potent activator of caspase-3, from the inner-membrane space.[46,70] High rates of apoptosis are also associated with a decrease in the intracellular bcl-2/bax ratio. Bcl-2 and bcl-xl are anti-apoptotic proteins localized to the cytoplasmic side of the outer mitochondrial membrane where they prevent the efflux of cytochrome c.[28] In the setting of hemorrhage with resuscitation, pyruvate Ringer's enhances cellular levels of the anti-apoptotic proteins bcl-2, bcl-xl and diminishes the expression of the pro-apoptotic mediators cytochrome c, bax, bad and cleaved caspase-3 in the cytosol.[34,42,46,55,72] Pyruvate's exact anti-apoptotic signaling pathway has yet to be elucidated. This signaling pathway is proposed to involve the activation of PI3K/Akt, yet this idea is controversial.[22,34] HIF-1 preservation by pyruvate may also yield anti-apoptotic effects through increased transcription of cytoprotective proteins.[47] Studies involving inhibition of eNOS, a cytoprotective protein whose expression is upregulated by HIF-1, demonstrated activation of bax and increased apoptotic events.[22,26]

Poly(ADP-ribose) polymerase (PARP) has also been implicated as a mediator of apoptosis.[21,54,55] PARP, specifically the isoforms PARP-1 and PARP-2, is involved in DNA nick and break repair and is activated in response to the nuclear fragmentation that

occurs during apoptosis.[6] PARP activation causes NAD^+ reduction to NADH, and the over-activation of PARP severely depletes the intracellular NAD^+/NADH cytosolic redox potential. When this reduction occurs, cells will regenerate NAD^+ through an ATP-dependent process. In the setting of ischemia and apoptosis, further reduction in cellular levels of ATP will lead to enhanced translation and modification of pro-apoptotic proteins and propagation of programmed cell death.[21] Pyruvate, through its reduction to lactate via lactate dehydrogenase (LDH), converts NADH to NAD^+, thus increasing the cellular NAD^+/NADH level previously depleted by PARP over-activation. This allows diminished *de novo* synthesis of NAD^+ and preservation of cellular ATP stores, all of which interrupts the vicious, low energy intracellular cycle that promotes further apoptosis in an ischemic setting.

Pyruvate-fortified Ringer's treatment of hemorrhagic shock has exerted anti-apoptotic effects in several organs. Figure 4 illustrates the proposed mechanisms through which pyruvate Ringer's attenuates cellular apoptosis.[46] It is well established that the presented mechanism of cellular apoptosis is ubiquitous to most body tissues. Similarities include pro- and anti-apoptotic mediators, the PI3K/Akt signaling pathway and cellular responses to oxidative stress.[17,27,32,77,78] In skeletal muscle, apoptosis plays a major role in embryogenesis, muscle atrophy and regeneration, and in the setting of certain muscle pathologies, specifically muscular dystrophy.[37] Skeletal muscle does contain mildly increased creatine phosphate content, indicating increased metabolic reserves during ischemia. Compared directly to other tissues, this increase has been shown not to affect predicted apoptotic end-points.[79]

Molecular mechanism of inflammation during hemorrhagic shock and hindlimb ischemia-reperfusion

Tourniquet release initiates a systemic inflammatory cascade that injures internal organs recovering from hypovolemic shock.[2,7,29,33,80] Reintroduction of oxygenated blood to the ischemic limb causes explosive formation H_2O_2, $\cdot O_2^-$, $\cdot NO$ and $ONOO^-$, toxic compounds that are central to the initiation and progression of systemic inflammation which can lead to multiple organ failure.[11,30,33] Although pharmacological antioxidants have been advocated for resuscitation,[63] antioxidant-enhanced resuscitative fluids are not currently used in clinical and military settings. Moreover, the poor membrane permeability of most pharmacological antioxidants limits their access to the intracellular sites of oxyradical production, especially mitochondria.

Experimental hindlimb ischemia elicits increased neutrophil-mediated matrix metalloproteinase (MMP) activity in the hindlimb muscle.[62] MMP's are zinc and calcium-dependent, pro-inflammatory enzymes that are released from activated neutrophils.[31] MMP activation through cleavage results in degradation of extracellular matrices and basement membranes, as well as enhanced reactive oxygen species generation.[62,70] Extracellular matrix degradation permits increased neutrophil tissue infiltration. Specifically, MMP-2 and MMP-9 cleavage and, thus, activation are reported in ischemic gastrocnemius muscle following femoral artery ligation.[57,62] The oxyradical-mediated activation of the pro-inflammatory MMP enzymes may lead to further inflammatory damage via circulating neutrophils and eventual systemic inflammation and end-organ damage.[62] In cardiopulmonary resuscitation studies conducted by Sharma *et*

al., pyruvate administration attenuated MMP activity in hippocampus after cardiac arrest *vs*. NaCl resuscitation (Figure 5).[70]

Circulating pro-inflammatory cytokines also activate neutrophils which in turn, as a consequence of continued generation of reactive oxygen species, upregulate inducible NOS (iNOS) expression.[63] The combined effect of neurophil ROS production and iNOS generation of ·NO leads to the generation of $ONOO^-$.[75] Experimental hindlimb ischemia-reperfusion leads to the seeding of end organs with nitrotyrosine, a marker of inflammation and tissue damage by NO-derived free radicals.[40] During hypovolemic shock, the persistent production of $ONOO^-$ and other ROS lead to single- and double-strand breaks within nuclear DNA.[74,75]

The cumulative effects of reactive oxygen species produced during inflammation causes DNA cleavage, which in turn activates PARP. Activation of PARP consumes NAD^+, thus diminishing the NAD^+/NADH ratio and promoting further ROS production and subsequent inflammation.[74,75] The inflammatory marker and neutrophilic enzyme myeloperoxidase (MPO) has been found to be upregulated in experimental hindlimb ischemia-reperfusion studies.[20] Specifically, inhibition of PARP overactivation in the setting of hindlimb ischemia-reperfusion attenuates the systemic inflammatory response and remote seeding of end-organs as evidenced by diminished MPO activities in those organs.[40]

Mongan *et al*. demonstrated pyruvate's prevention of PARP over-activation in a swine model of hemorrhagic shock. Through elevation of the cytosolic redox potential, pyruvate decreases overactivation of PARP in the setting of ischemia, thus augmenting its already robust anti-oxidative effects.[56] Pyruvate's free radical scavenging properties

may abrogate further upregulation of the inflammatory transcriptional factor NF-kB. Skeletal muscle reperfusion injury is linked to the upregulation of NF-kB, which, by activating transcription of pro-inflammatory cytokines and chemokines, leads to complement activation and inflammatory injury of the tissue parenchyma.[61,74] By diminishing oxidative stress and, thus, systemic inflammatory responses, pyruvate Ringer's, in the setting of hindlimb ischemia, diminishes the activation of NF-kB and dampens systemic inflammatory events.

Treatment with pyruvate-fortified resuscitation introduces a broad-spectrum, membrane-permeable antioxidant into the reperfused limb that is capable of neutralizing reactive oxygen and nitrogen species, and thus, suppressing inflammation.[23] Underlying molecular mechanisms of the direct anti-inflammatory effects of pyruvate remain to be elucidated. Preinduction of heat shock proteins before traumatic hemorrhage markedly attenuates circulating activities of the pro-inflammatory cytokines TNF-α and interleukin-6.[53] Pyruvate's stabilization of HIF-1, and the enhanced gene expression of such cytoprotective proteins as HSP-70, may comprise a direct anti-inflammatory molecular pathway.[45]

Summary

Pyruvate, being a natural energy fuel and antioxidant,[51,52,76] is uniquely capable of suppressing oxidative stress, systemic inflammation and reperfusion injury following tourniquet release. Substituting pyruvate for lactate in Ringer's solution allows application of pyruvate to hemodynamically unstable victims of hemorrhagic shock. This study seeks to demonstrate pyruvate's positive hemodynamic, antioxidative and cyto-

protective contributions, as well as its local and systemic anti-inflammatory capabilities in the setting of controlled hemorrhagic shock with resuscitation and hindlimb ischemia.

Specific Aims

The first aim of this investigation was to determine the hemodynamic and systemic anti-inflammatory effects of pyruvate-fortified Ringer's volume expansion after severe, controlled hemorrhage. The experiments were designed to compare, in a setting of hemorrhagic shock, the effects of systemic administration of resuscitative solutions containing 130 mM Na^+, 109 mM Cl^-, 4 mM K^+, 3 mM Ca^{2+} and either 28 mM lactate or 28 mM pyruvate. Sham animals that were neither hemorrhaged nor resuscitated also were studied to control for effects of anesthesia, surgical trauma and insensible water loss. MAP, heart rate and circulating neutrophils were measured at multiple predetermined time points. Plasma and tissue lactate/pyruvate also were measured. Nitrotyrosine, an indicator of nitrosative stress, was measured in cardiac tissue. The study tested the hypothesis that pyruvate-fortified Ringer's resuscitative fluid stabilizes systemic arterial pressure more effectively than conventional Ringer's lactate solution. The study also tested the hypothesis that pyruvate Ringer's solution suppresses systemic inflammation during recovery from hemorrhagic shock and hindlimb ischemia. Pyruvate-fortified Ringer's bolstered MAP during the recovery period more effectively than lactate Ringer's. The PR group also exhibited an increased circulating whole blood HCO_3^- concentration and plasma lactate/pyruvate ratio at the end of experimentation. Further, pyruvate Ringer's-treatment served to protect myocardial function after hemorrhagic shock and during the recovery phase by mitigating harmful free radicals and establishing a more

favorable cytosolic redox potential. Finally, PR dampened any increase in circulating systemic neutrophils. Thus, PR prevents overt systemic inflammation and enhances hemodynamic recovery in a goat model of hemorrhagic shock with resuscitation.

The second aim of this investigation was to determine the ability of pyruvate-fortified Ringer's resuscitation to ameliorate reperfusion injury following tourniquet release through pyruvate's natural oxyradical scavenging properties and its ability to stabilize cytoprotective proteins in ischemically-challenged hindlimb musculature. Isoflurane-anesthetized male goats underwent controlled hemorrhage to lower MAP to *c.* 50 mmHg, and then the right femoral artery and vein were occluded for 90 min. Lactate Ringer's solution (LR) or PR was infused *iv* (10 ml/min) for 90 min, from 30 min occlusion until 30 min after release of occlusion. At 4 h post-occlusion, gastrocnemius of the ischemic hindlimb was biopsied for analyses of antioxidative, energy shuttling and pro-inflammatory enzymes, and pro- and anti-apoptotic proteins. The study tested the hypothesis that volume resuscitation with PR suppresses cellular apoptosis after hindlimb ischemia and hypovolemia. Pyruvate administration created a more favorable cytosolic redox state within previously ischemic skeletal muscle. PR also bolstered antioxidative enzymes and preserved the activity of energy shuttling enzymes. PR stabilized muscle content of the anti-apoptotic protein Bcl-xL and the ratio of cleaved/uncleaved PARP-2. Finally, PR prevented the accumulation of nitrotyrosine within previously ischemic muscle. It is concluded that PR effectively protects hindlimb musculature after tourniquet application in a goat model subjected to hemorrhagic shock with resuscitation.

Significance

This investigation, for the first time, demonstrated pyruvate-fortified Ringer's resuscitative effects in a goat model of hemorrhagic shock with resuscitation and hindlimb ischemia. PR introduces a potent, membrane-permeable antioxidant to the reperfused limb, capable of neutralizing reactive oxygen and nitrogen species, and, thus, suppressing both local and systemic inflammation.[23] Importantly, pyruvate had no detrimental side effects. This investigation will serve to support the development of novel fluid resuscitation strategies aimed at controlling harmful systemic inflammation and end-organ failure in persons suffering hemorrhage and hypovolemic shock in combat and civilian settings.

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Table 1. *Research involving both apoptosis and pyruvate Ringer's*

End-Target Organ	Model	Proposed mechanism through which **pyruvate Ringer's** diminishes cellular apoptosis	Reference
Liver	Rats: 60 min. hemorrhage (40 mmHg) w/ 60 min infusion	Enhanced cellular ATP prevents uncoupling of electron transport chain and subsequent release of cytochrome-c	Sharma *et al.*[72]
	Rats: 10 min. hemorrhage (40 mmHg) w/ 45 min infusion	Increased histone acetylation allows enhanced transcription of acute survival genes that promote the upregulation of anti-apoptotic proteins (bcl2)	Jaskille *et al.*[34]
	Swine: 240 min hemorrhage (40 mmHg) w/ 210 min infusion	Through maintenance of the GSH/GSSG ratio, decreased lactate / pyruvate ratio (indicating NADH/NAD), upregulation of bcl-xl and decreased PARP fragmentation	Mongan *et al.*[55]
Lung	Rats: 10 min. hemorrhage w/ 45 min infusion	Optimal post-translational modifications (increased bcl2/bax), altered degradation (diminished PARP activation and enhanced tissue levels of ATP promote tissue survival	Koustova *et al.*[42]
	Rats: 10 min. hemorrhage w/ 45 min infusion	Decreased phosphorylation of the pro-apoptotic protein Bad allows the binding of this protein to the anti-apoptotic protein bcl-2, thus preventing bcl-2's sequestration of the pro-apoptotic protein cytochrome-c within the mitochondria	Jaskille *et al.*[35]
Human endothelial cells	Cell culture treated w/ H_2O_2	Pyruvate's antioxidative effect suppresses p53 induction, thus minimizing upregulation of bax, downregulation of bcl-2 and cleavage of caspase-3.	Lee *et al.*[46]

Research reports of the anti-apoptotic effects of pyruvate Ringer's infusion in the setting of hemorrhagic shock with resuscitation.

Figure 1. *Dr. Alfred Blalock and Vivien Thomas.* Dr. Blalock, with his research assistant Vivien Thomas (as portrayed by Alan Rickman and Mos Def in the film, *Something the Lord Made*), in his hypovolemic shock research laboratory at Vanderbilt University. Image from Sargent J. HBO Films, 2004.[66]

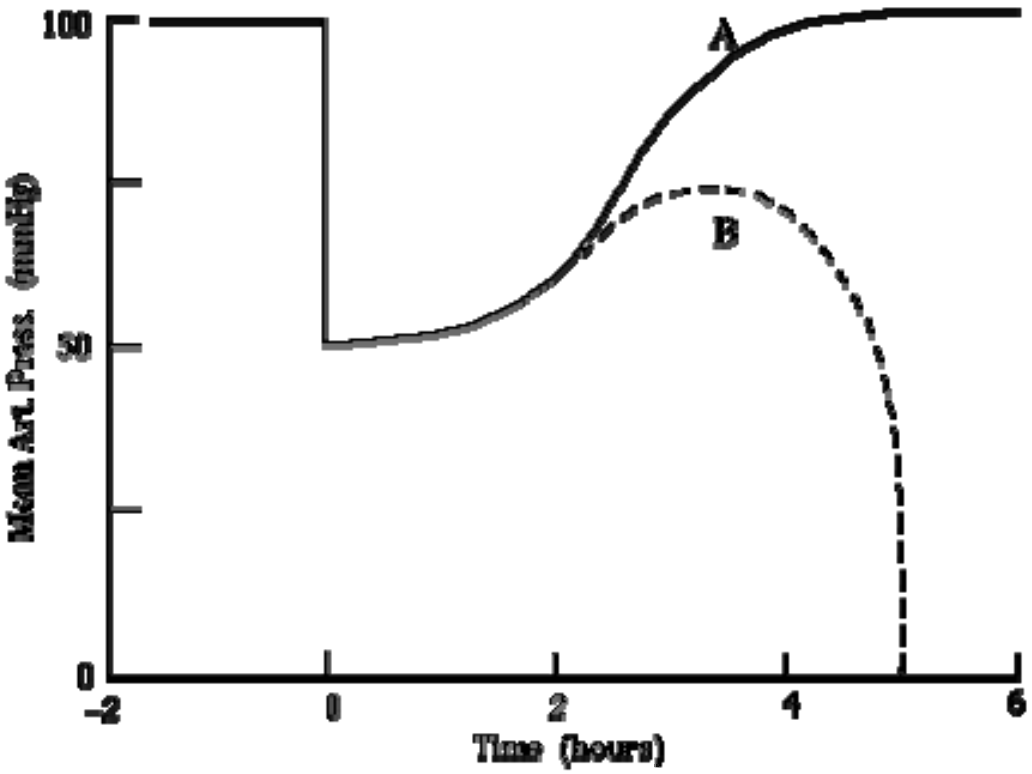

Figure 2. *Physiologic compensation during hypovolemic shock.* Diagram illustrating the difference between physiologically compensated (A) *vs.* noncompensated (B) hypovolemic shock over the course of 6 h. Figure from Koeppen *et al. Berne and Levy Physiology*. Mosby Elsevier, 2008.[41]

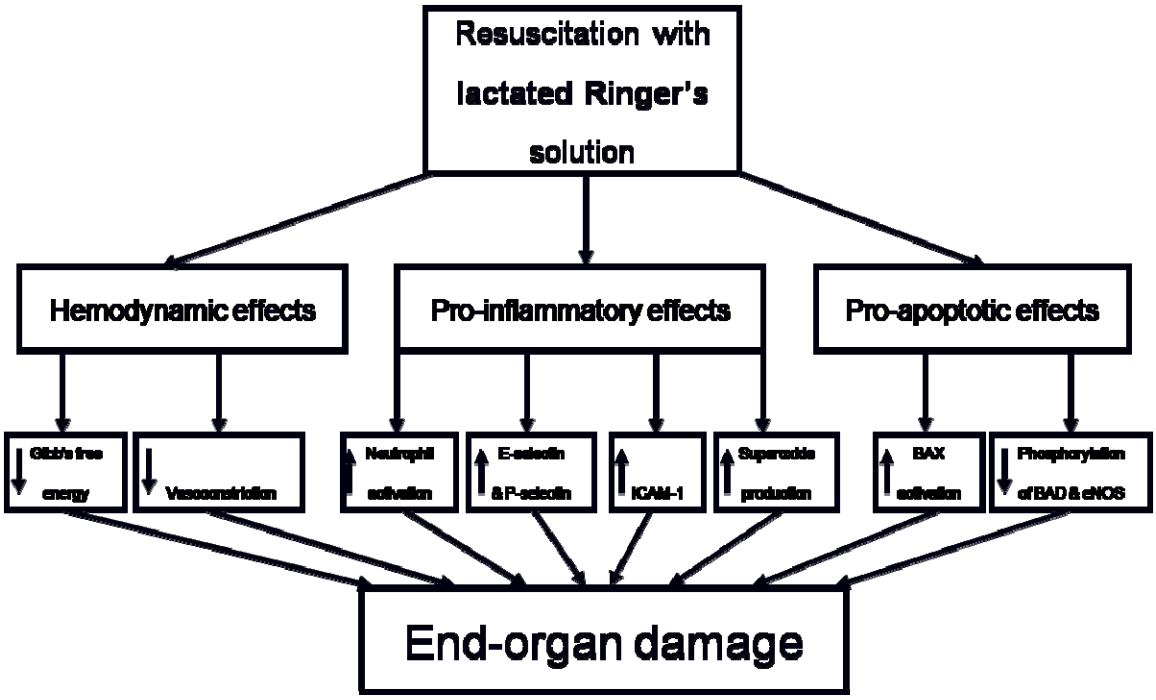

Figure 3. *Detrimental effects of lactate Ringer's.* Flow diagram illustrating the detrimental effects of LR in the setting of hypovolemic shock with resuscitation. These effects have driven researchers to seek alternative crystalloid solutions that minimize the pro-oxidative, -inflammatory and –apoptotic resuscitative environment that LR promotes.

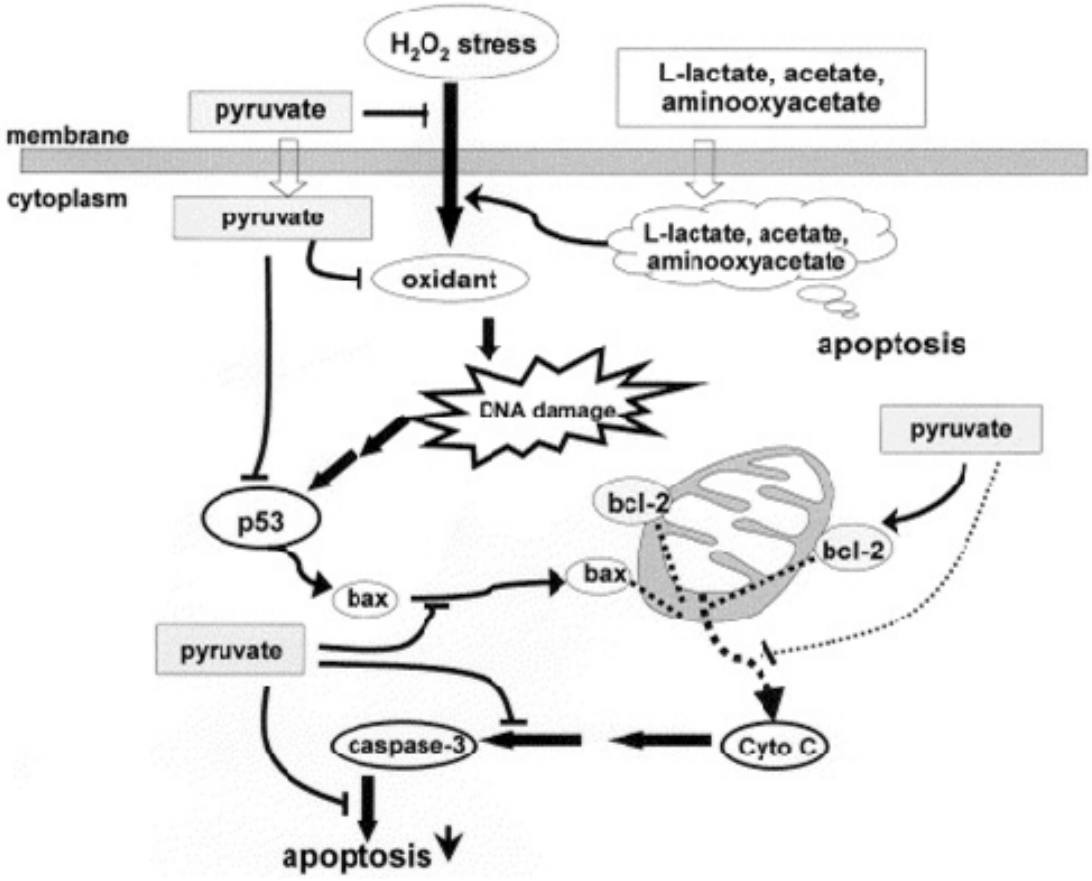

Figure 4. *Proposed mechanisms through which pyruvate attenuates cellular apoptosis.* Cellular apoptosis is induced with extracellular stress. Pyruvate, through its unique anti-oxidative and –inflammatory effects, ameliorates cellular apoptosis by directly inhibiting the release of cytochrome c from the inner-mitochondrial space. This is accomplished in part by pyruvate's stabilization of Bcl-2, a protein which serves to limit cytochrome c release. Thus, downstream pro-apoptotic signaling is interrupted. Figure from Lee *et al.* *Microvasc Res* 2003;66:91-101.[46]

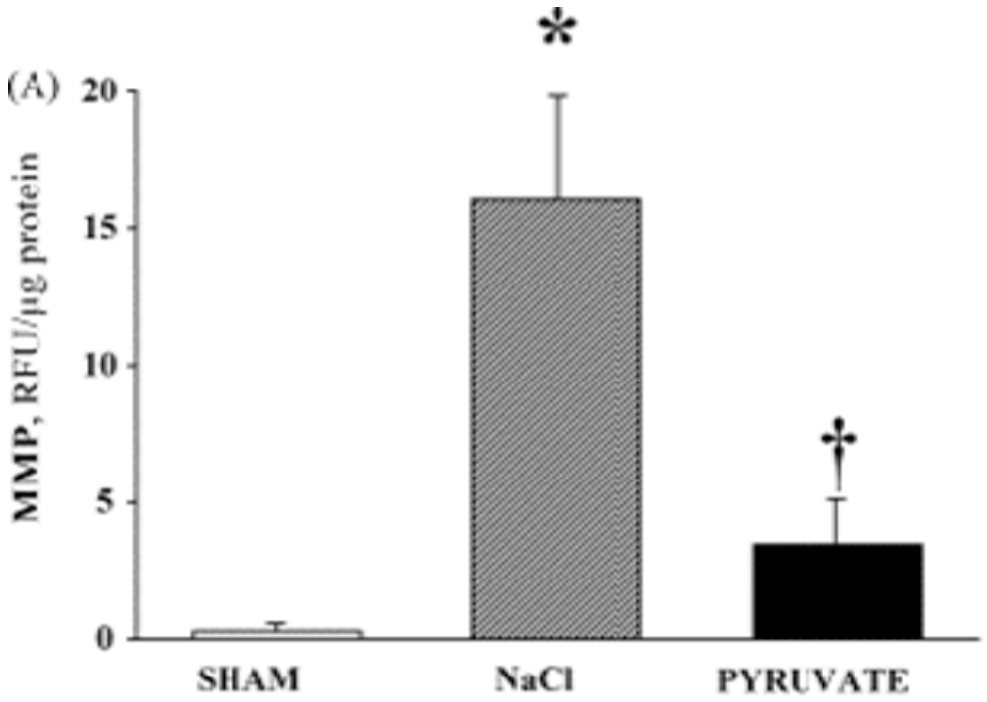

Figure 5. *Anti-inflammatory effect of pyruvate*. Elevated tissue matrix metalloproteinase activity indicates the presence of local inflammation. Canine hippocampal tissue subjected to cardiac arrest with subsequent resuscitation and 3-day recovery revealed elevated MMP activity after open-chest cardiac compressions with concurrent infusion of NaCl. Conversely, infusion of pyruvate during resuscitative efforts significantly blunted the pro-inflammatory effects of cardiac arrest with resuscitation. Reference from Sharma *et al. Resuscitation* 2008;76:108-119.[70]

CHAPTER II

PYRUVATE-FORTIFIED FLUID RESUSCITATION IMPROVES HEMODYNAMIC STABILITY WHILE SUPPRESSING SYSTEMIC INFLAMMATION AND MYOCARDIAL OXIDATIVE STRESS AFTER HEMORRHAGIC SHOCK

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ABSTRACT

Background: Traumatic blood loss often necessitates the use of resuscitative fluids to stabilize blood pressure. Tourniquet use imposes ischemia-reperfusion injury on wounded limbs, which can initiate systemic inflammation, ultimately impacting the heart. Resuscitation with pyruvate, an anti-oxidative and -inflammatory metabolite, may stabilize hemodynamics and dampen systemic inflammation after hindlimb ischemia-reperfusion. **Hypothesis:** Resuscitation with pyruvate-fortified Ringer's (PR) solution *vs.* conventional lactate Ringer's (LR) more effectively stabilizes mean arterial pressure (MAP) and suppresses systemic inflammation post-resuscitation. **Methods:** Goats were hemorrhaged (255 ± 22 ml) to lower MAP to 48 ± 1 mmHg. Next, the femoral vessels were occluded for 90 min to model tourniquet application. Beginning at 30 min occlusion, LR or PR was infused *iv* at 10 ml/min for 90 min. The femoral occlusions were released at 60 min infusion. **Results:** At 4 h post-occlusion, MAP (mmHg) was increased in PR (59 ± 4) vs. LR (47 ± 3) resuscitated goats ($p<0.05$). PR also more effectively augmented circulating HCO_3^- and total base excess, thus counteracting metabolic acidosis caused by systemic hypoperfusion. Marked tyrosine nitration, a footprint of nitrosative stress, was detected in myocardium 4 h after LR resuscitation, but was suppressed by PR. Finally, PR prevented the increase in circulating neutrophils that occurred during and following LR resuscitation. **Conclusions:** Relative to LR, resuscitation with PR more effectively stabilizes MAP, suppresses nitrosative stress in myocardium and minimizes systemic inflammation in a caprine model of hemorrhagic shock complicated by hindlimb ischemia-reperfusion.

INTRODUCTION

Traumatic hemorrhage is responsible for 50% of combat-related deaths.[13] To stabilize blood pressure and achieve hemostasis, intravenous resuscitative fluids are administered, and tourniquets are applied to wounded extremities. Both techniques have proven reliable and effective, but are not completely benign. Tourniquets impose ischemia on distal skeletal muscle and with tourniquet release the reentry of oxygenated blood into the ischemic tissue initiates a systemic inflammatory cascade that threatens internal organs recovering from hypovolemic shock.[24] Moreover, fluid resuscitation may contribute to systemic oxidative stress and inflammation, especially if lactate Ringer's (LR) solution is the primary vehicle of volume expansion.[2,36,41]

Concern regarding LR's toxicity dates back to observations recorded by Cushing in 1901, specifically noting LR's "poisonous" effects on nervous tissue.[3] More recent reports indicate resuscitation with LR contributes to oxyradical formation and systemic inflammation.[2,4,14,36,37] Specifically, LR increases neutrophil activation and invasion of tissue parenchyma, where the neutrophils release superoxide ($\bullet O_2^-$).[2,8,22] Nitric oxide ($\bullet$NO) concentrations are depressed in hemorrhaged animal models administered LR, concordant with increased $\bullet O_2^-$ condensation with $\bullet$NO to produce peroxynitrite ($ONOO^-$).[37] $\bullet$NO depletion is especially severe with application of LR containing racemic mixtures of DL-lactate, a commonly used formulation in clinical settings.[22]

Crystalloid solutions fortified with pyruvate, an intermediary metabolite, energy substrate and antioxidant,[26,28,30] suppress oxyradical formation and inflammatory cascades and improve cardiac recovery from cardiopulmonary bypass surgery[19,35] and cardiac arrest.[39] Resuscitation with pyruvate after hemorrhagic shock introduces a

potent, membrane-permeable antioxidant to the systemic circulation and the reperfused limb, potentially capable of neutralizing the reactive oxygen and nitrogen species that initiate systemic inflammatory cascades.[15,26,28,29] Accordingly, this study tested the hypothesis that controlled resuscitation with pyruvate-fortified Ringer's (PR) solution stabilizes post-resuscitation systemic arterial pressure and alleviates systemic inflammation and the resulting oxidative stress initiated by hindlimb reperfusion more effectively than conventional lactate-based fluid resuscitation.

MATERIALS AND METHODS

All experimentation was approved by the University of North Texas Health Science Center's Institutional Animal Care and Use Committee, and was performed in accordance with the *Guide to Care and Use of Laboratory Animals* (NIH publication 85-23, revised 1996). Eighteen male goats weighing 21.8 ± 1.2 kg were randomly assigned to three groups of six experiments: LR resuscitation; PR resuscitation; sham controls. Animals in the LR group received conventional lactate Ringer's resuscitation, while those in the PR group were resuscitated with Ringer's solution that contained pyruvate substituted for lactate. Sham animals were subjected to surgical and experimental protocols but did not undergo hemorrhage, hindlimb ischemia-reperfusion or resuscitation.

Surgical Preparation

After 24 h fast, goats were sedated with diazepam (0.25 mg/kg) and ketamine (5 mg/kg) via the left external jugular vein. After intubation goats were mechanically ventilated with room air supplemented with 100% O_2 and 1-2% isoflurane to maintain a

surgical plane of anesthesia. Body temperature was maintained at 38 – 39.5°C throughout the protocol with electric heating pads.[5]

The left internal jugular vein was exposed via an 8 cm neck incision and catheterized to permit controlled exsanguination and fluid resuscitation. The left common carotid artery was isolated, catheterized and connected to an inline CDXress 3cc arterial pressure transducer (Maxxim Medical) for continuous monitoring of blood pressure and heart rate and for sampling arterial blood. A right femoral dissection was performed to expose hindlimb vasculature just inferior to the inguinal ligament.

Experimental Protocol

Controlled hemorrhage was effected by withdrawing blood from the left internal jugular vein at a rate of 20 ml•min^{-1} until mean arterial pressure (MAP) fell to approximately 50 mmHg. Once MAP stabilized at the target range for 5 min, the right femoral artery and vein were simultaneously occluded with a nontraumatic vascular clamp applied just inferior to the inguinal ligament. 30 min after femoral occlusion Ringer's solution was infused into the internal jugular vein at a rate sufficient to deliver 3 times the hemorrhaged volume over a period of 90 min. Infusion was either conventional mixed racemic DL-lactate Ringer's (B. Braun Medical Inc.) or pyruvate Ringer's prepared by equimolar substitution of 28 mM sodium pyruvate (Sigma, St. Louis, MO) for sodium lactate. After 90 min occlusion the vascular clamp was released to initiate hindlimb reperfusion. Thirty minutes later, i.e. 90 min after starting infusion, LR or PR resuscitation was terminated, and a 3.5 h recovery period ensued.

Blood sampling

Blood was sampled from the left common carotid artery at 11 predetermined time points (baseline, post-hemorrhage, 30 min ischemia, 60 min ischemia / 30 min Ringer's infusion, 90 min ischemia / 60 min Ringer's infusion, 90 min Ringer's infusion, and 30, 60, 120, 180 and 210 min post-infusion). Arterial pO_2, pCO_2, hemoglobin, lactate, HCO_3^-, base excess and glucose concentrations were measured in an Instrumentation Laboratory model 1730 blood gas analyzer and model 682 Co-Oximeter. Alveolar ventilation was adjusted to maintain arterial pCO_2 within physiological limits.

Plasma samples were obtained after whole blood centrifugation (Eppendorf 5415R table-top centrifuge) and stored at -80°C. After deproteinization with 0.6 M $HClO_4$, lactate and pyruvate were measured by colorimetric assays[7] in a Shimadzu Instruments model UV-1601 spectrophotometer.[20,39]

Complete blood count with differential

Approximately 2 ml of whole blood was transferred to a purple-stoppered collection tube containing ethylenediaminetetraacetatic acid (EDTA) and stored at room temperature. Peripheral blood smears were prepared on a glass slide, examined by light microscopy and white blood cells counted manually with a hemocytometer and automatically with a Coulter Counter. The white cell differential was determined manually after Wright staining.

Extraction and analyses of myocardial proteins and metabolites

At 3.5 h post-infusion, the heart was exposed via left-sided thoracotomy and pericardiotomy. Samples of left ventricular myocardium were snap-frozen *in situ* with

Wollenberger tongs pre-cooled in liquid N_2. The frozen tissue was pulverized to a fine powder in a porcelain mortar under liquid N_2. Myocardial proteins were extracted by homogenizing *c.* 100 mg powdered tissue in 1.8 ml phosphate buffer, as previously described.[19] Total protein concentrations were determined colorimetrically with a Coomassie Plus Kit (Pierce, Rockford, IL) by the Bradford method[9] in a Shimadzu Instruments model UV-1601 spectrophotometer (Columbia, MD).[19]

Nitrotyrosine content was measured in myocardial protein extracts by enzyme-linked immunosorbent assay (ELISA) (Northwest Life Sciences, Vancouver, WA) according to the manufacturer's instructions. Optical densities were measured at 450 nm on a MX80 plate reader (Dynatech, Chantilly, VA). Nitrotyrosine contents were normalized to the respective tissue extract protein concentrations. Myocardial lactate and pyruvate were extracted as previously described[20] and spectrophotometrically assayed.[7]

Statistical analysis

Values are means ± SEM. Intra-group comparisons of values at different time points were accomplished by two-way repeated measures ANOVA. Comparisons among the 3 groups (sham, LR, PR) employed one-way ANOVA. Values reported as percentages of baseline were analyzed by prospective two-way ANOVA. When ANOVA detected statistical significance a Student-Newman-Keuls *post hoc* test was applied to detect specific differences. Direct comparisons between 2 groups were accomplished by student's T-test. P values <0.05 were taken to indicate statistically significant differences. Statistical analyses were performed with Sigma Stat software version 3.1 (Aspire Software Int., Ashburn, VA).

RESULTS

Arterial pressure

Mean arterial pressure (MAP) was monitored throughout the experiment. There were no significant differences among the groups at pre-hemorrhage baseline (Figure 1). The controlled hemorrhage sharply lowered MAP (mmHg) in both the LR (48 ± 1) and PR (49 ± 2) groups *vs.* sham (101 ± 19). MAP partially recovered in the LR and PR groups during the 30 min of hindlimb ischemia preceding fluid resuscitation, and did not differ among the groups during hindlimb ischemia and fluid resuscitation. During post-resuscitation recovery MAP was better maintained in PR than the other groups, and these differences were statistically significant at 150, 180, 240, 300 and 330 min after hemorrhage, i.e. 30, 60, 120, 180 and 210 min post-resuscitation (Figure 1).

Arterial hemoglobin and acid-base chemistries

Arterial hemoglobin content fell by *c.* 1 g/dL during both LR and PR fluid resuscitation, and then remained stable for the remainder of the experiment (Figure 2A). Hemoglobin content gradually increased by 0.93 g/dL over the course of the sham protocol ($p<0.05$ vs. baseline), and was nearly 3 g/dL above that of the LR and PR groups by the end of the experiment (Figure 2A). HCO_3^- concentrations remained stable throughout the sham experiments and increased slightly, by *c.* 2.5 mEq/L, in the LR group (Figure 2B). Resuscitation with PR increased HCO_3^- more substantially; HCO_3^- was significantly increased in PR *vs.* LR at 60 min post-infusion, and in PR *vs.* sham throughout the post-occlusion period (Figure 2B). The time-courses of arterial base

excess generally paralleled those of HCO_3^- concentration in the 3 groups (Figure 2C). Arterial glucose did not differ among the groups at any time point, and trended downward throughout the protocol (Figure 2D).

Plasma pyruvate and lactate

Plasma lactate concentrations were significantly increased in the LR group *vs.* sham throughout hindlimb ischemia and LR infusion, and increased to a more modest extent during PR infusion (Figure 3A). Infusion of PR doubled arterial plasma pyruvate concentration (Figure 3B). Pyruvate concentrations returned to baseline after PR infusion. Lactate Ringer's infusion also increased plasma pyruvate concentration modestly during and 2 h after resuscitation, the result of circulating lactate dehydrogenase activity acting on the increased plasma lactate concentrations during the LR protocol. Plasma lactate/pyruvate ratios were similar among the groups during baseline sampling, immediately after hemorrhage and after 30 min hindlimb ischemia. As expected, plasma lactate/pyruvate concentration ratio, a measure of $NADH/NAD^+$ redox state in the circulation, increased during LR infusion, and then returned to baseline after LR treatment (Figure 3C). In contrast, PR infusion lowered the plasma lactate/pyruvate ratio *vs.* that of the LR and sham groups, but this effect subsided 30 min after PR infusion (Figure 3C). Plasma lactate/pyruvate ratio again increased during the last 90 min of recovery in the LR group, but remained stable and lower in the PR group ($p<0.05$, LR *vs.* PR).

Myocardial lactate and pyruvate

The residual effects of hemorrhage, hindlimb ischemia-reperfusion and LR or PR treatment on myocardial lactate and pyruvate contents were examined 3.5 h after resuscitation was completed. Both the LR- and PR-treated myocardium had a greater than two-fold increase in lactate content *vs.* sham myocardium (Figure 4). The post-LR myocardium also showed a significant decrease in pyruvate *vs.* sham myocardium (1.3 ± 0.2 *vs.* 2.3 ± 0.2 μmol/g dry, respectively), while pyruvate content was 2.9 ± 1.1 μmol/g dry in the post-PR myocardium. The ratio of lactate/pyruvate, a measure of cytosolic [NADH]/[NAD^+] via the LDH equilibrium,[31] increased three-fold in the LR group and two–fold in the PR group *vs.* sham (Figure 4).

Myocardial nitrotyrosine content

Chemical attack by reactive nitrogen metabolites, including $ONOO^-$, causes nitration of tyrosine residues in proteins. Thus, nitrotyrosine in the protein fraction is a stable marker of oxidative and nitrosative stress.[42] Although nitrotyrosine content showed a modest upward trend in LR-treated *vs.* sham myocardium, PR resuscitation lowered myocardial nitrotyrosine content by 53% *vs.* LR. Thus, severe hemorrhage with hindlimb ischemia-reperfusion and LR resuscitation increased nitrosative stress, but PR resuscitation prevented this deleterious effect.

Circulating neutrophils

Figure 6 presents circulating neutrophils as percentages of baseline values to normalize for individual differences among the goats. Neutrophil counts in the LR group

trended downward at 30 min hindlimb ischemia, then increased 16% above baseline by 90 min resuscitation, and remained elevated for the remainder of the protocol. In contrast, neutrophil counts in the PR experiments remained at or below baseline throughout hindlimb ischemia-reperfusion and resuscitation, and only increased at the end of the protocol. Circulating neutrophils also increased at the end of the sham experiments. Thus, LR resuscitation increased circulating neutrophils during the post-resuscitation period, but PR administration suppressed this pro-inflammatory response.

DISCUSSION

Hemostasis and restoration of intravascular volume in an effort to stabilize arterial pressure are the primary therapeutic goals for treating hemorrhagic shock.[33] Failure to maintain an arterial pressure sufficient to perfuse all end organs may be due to a weakened cardiac pump, diminished vasomotor tone, and/or a reduction of blood volume below the minimal capacity of the vascular system.[12] Rapid volume expansion after hemorrhage carries a risk of increasing hemorrhage volumes and potentiating the detrimental effects of hemorrhagic shock.[23] The objectives of this study were to define pro-oxidant and inflammatory responses impacting myocardium in a large mammal model of hemorrhagic shock complicated by hindlimb ischemia-reperfusion, and to test novel, pyruvate-enriched resuscitation as a means of stabilizing systemic hemodynamics and suppressing inflammation in this model.

Post-shock hemodynamic stabilization with pyruvate

This investigation is the first to document the effects of PR infusions in the setting of combined hemorrhagic shock and hindlimb ischemia-reperfusion. While no

differences in hemodynamics were expected during fluid resuscitation,[34] PR effectively stabilized MAP during the recovery period in a manner superior to LR. These results complement the findings of Mongan *et al.*, who demonstrated that pyruvate stabilized cardiac function and prolonged survival of pigs subjected to severe hemorrhage.[31] Pyruvate's antioxidant and anti-inflammatory capabilities, unique among metabolic substrates, may have served to protect the myocardium and possibly the vascular endothelium, thus preserving systemic arterial pressure. Pyruvate readily traverses plasma membranes via a high-capacity monocarboxylate transport mechanism, enabling it to deliver antioxidant protection and oxidizable fuel to the cell interior.[17,43]

As expected, PR lowered circulating lactate/pyruvate ratio, but this effect unexpectedly persisted for 3.5 h post-resuscitation. This persistent redox effect can be taken to indicate oxidation of NADH within the perfused tissues. Increased intracellular NAD+/NADH would help sustain energy-yielding glycolytic flux and decrease availability of NADH for $\bullet O_2^-$ generating NAD(P)H oxidase, further protecting tissue from increasing oxidative stress.[6] Circulating pyruvate may also have helped maintain vascular performance by elevating circulating HCO_3^-, thereby affording a more favorable acid-base profile to offset acidemia resulting from end-organ hypoxia.[33] Enhancement of cytosolic NAD^+/NADH redox state bolsters ATP phosphorylation potential and Gibbs free energy of ATP hydrolysis via the equilibrium between redox and phosphorylation states catalyzed by the glyceraldehyde-3-phosphate dehydrogenase / phosphoglycerate kinase system.[10,31,35] Mongan *et al.* proposed that pyruvate, by reducing metabolic acidosis and stabilizing ATP phosphorylation potentials, may suppress the opening of K_{ATP} channels,[31] thereby preventing a decrease in intracellular Ca^{2+}, preserving the

contractile function of vascular smooth muscle and stabilizing vasomotor tone.[1,34] On the other hand, lactate Ringer's solution is known to impair vasoconstriction indirectly through production of peroxynitrite, thus negating the beneficial effects of increased blood volume.[37]

Anti-oxidative and –inflammatory effects of pyruvate

Reintroduction of oxygenated blood to the ischemic limb causes explosive formation and release of reactive oxygen and nitrogen derivatives including hydrogen peroxide (H_2O_2), $\bullet O_2^-$, •NO and $ONOO^-$.[16,18,25] Hindlimb ischemia-reperfusion provokes tyrosine nitration throughout the body, the consequence of inflammation and tissue damage by •NO and $ONOO^-$.[21] In this study, PR resuscitation markedly lowered myocardial nitrotyrosine *vs.* the LR group. This result suggests that pyruvate, which among its diverse beneficial is a scavenger of $ONOO^-$,[27] is in fact diminishing nitrosative stress within myocardium while suppressing the systemic inflammatory response to hemorrhagic shock.[15,38] Alternatively, reperfusion with LR exacerbates oxidative and nitrosative stress and systemic inflammation.[2,4,14,36,37]

Limitations

The limitations of this study must be acknowledged. First, mean arterial pressures in the sham group trended downward throughout the entire experimental protocol. This effect can be attributed to two factors: 1) extensive insensible fluid loss without volume replacement in this group which, at a rate of 3-4 ml/kg/h, would total nearly 500 ml in a 20 kg goat over the 6 h protocol;[32] 2) withdrawal of 11 blood samples totaling

approximately 150 ml; 3) prolonged exposure to isoflurane general anesthesia throughout the protocol.[40] Second, the most effective pyruvate concentration to protect tissues and stabilize arterial pressures in the setting of severe hemorrhage and hindlimb ischemia-reperfusion has yet to be determined. A pyruvate concentration of 28 mEq/L in Ringer's was tested, representing an equimolar substitution of pyruvate for lactate. At an infusion rate of 10 $ml \bullet min^{-1}$, i.e. 0.4-0.5 $ml \bullet kg^{-1} \bullet min^{-1}$, plasma pyruvate increased to approximately 0.5-0.6 mM. This value is below the range of 3-5 mM known to afford maximal protection to isolated[11] and in situ[19,20,38,39] hearts subjected to ischemia-reperfusion. However, resuscitation with more extreme, hypertonic pyruvate concentrations did not afford appreciable improvement over hypertonic NaCl resuscitation in a sheep model of hemorrhagic shock,[34] suggesting there may be an upper limit to the effective range of pyruvate concentrations.

Conclusions

In conclusion, pyruvate-fortified Ringer's stabilized arterial pressure more effectively than lactate Ringer's in goats subjected to severe hemorrhage and hindlimb ischemia-reperfusion. Pyruvate-enhanced fluid resuscitation also augmented circulating HCO_3^- concentration and base excess, suppressed nitrosative damage to myocardial proteins, and dampened systemic inflammation in this setting. These findings support the concept that fluid resuscitation with the natural antioxidant and anti-inflammatory agent, pyruvate, may afford superior acute stabilization of trauma victims.

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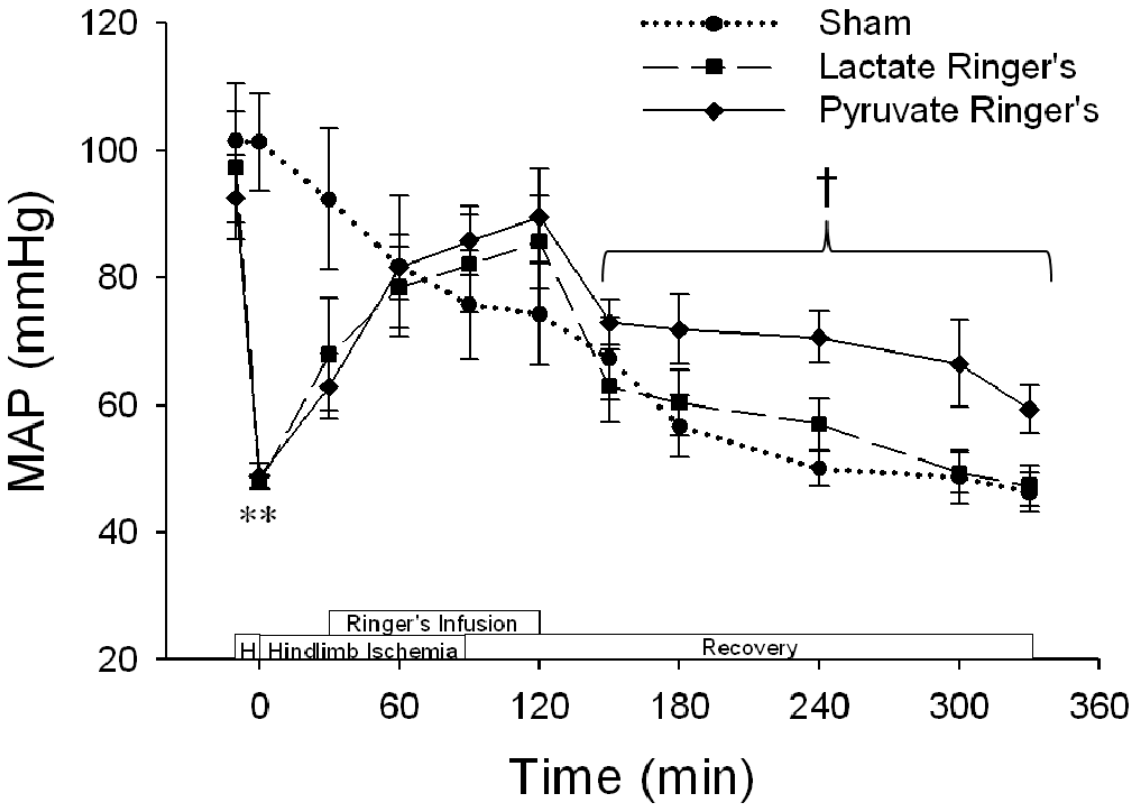

Figure 1. *Mean arterial pressure.* Arterial blood pressure was measured in each of three experimental groups: sham (circles, n=6), lactate Ringer's (squares, n=6) and pyruvate Ringer's (diamonds, n=6). Values in this and the other figures are means ± SEM. * $p<0.05$ *vs.* sham; † $p<0.05$ *vs.* LR.

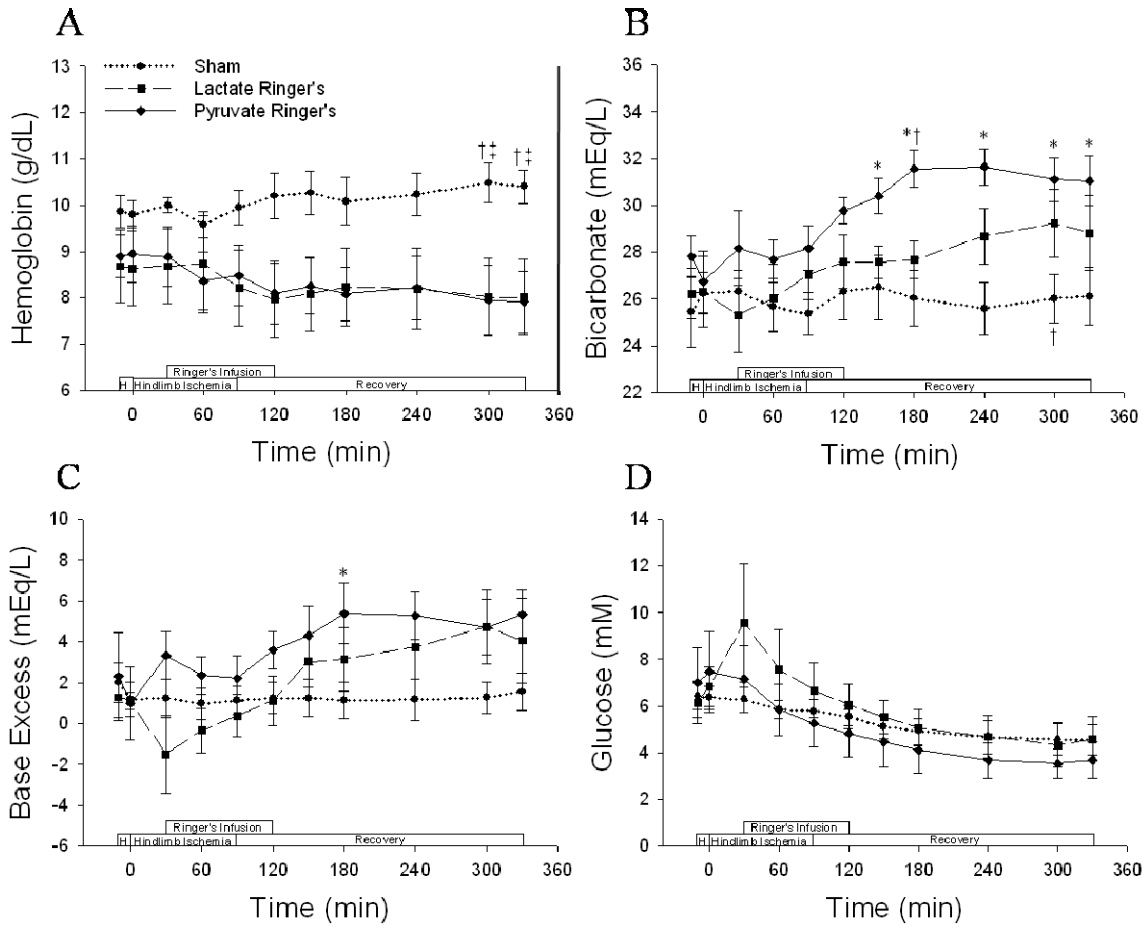

Figure 2. *Arterial hemoglobin content, acid-base chemistries and glucose.* Panel A: arterial whole blood hemoglobin content; Panel B: whole blood HCO_3^- concentrations. Panel C; whole blood base excess; Panel D: whole blood glucose concentrations. * $p<0.05$ *vs.* sham; † $p<0.05$ *vs.* LR; ‡ $p<0.05$ *vs.* PR.

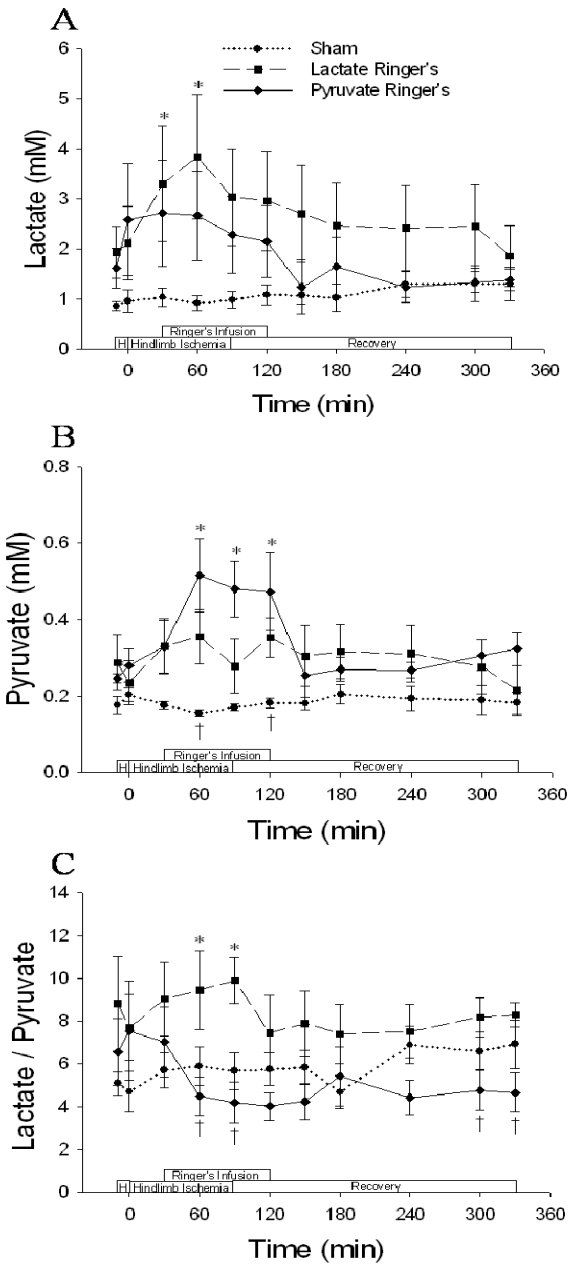

Figure 3. *Arterial plasma lactate and pyruvate concentrations and lactate/pyruvate ratio.* Panel A: plasma lactate concentrations in sham (circles), lactate Ringer's (squares) and pyruvate Ringer's (diamonds) groups; Panel B: plasma pyruvate concentrations; Panel C: plasma lactate/pyruvate ratio. * $p<0.05$ *vs.* sham; † $p<0.05$ *vs.* LR.

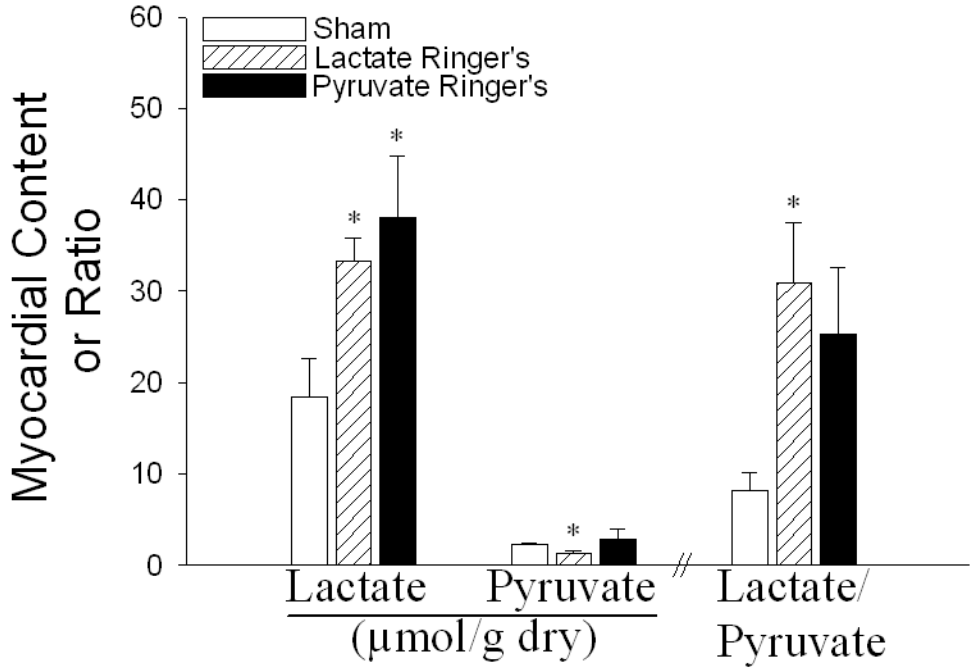

Figure 4. *Myocardial lactate/pyruvate content ratio.* Lactate and pyruvate contents and lactate/pyruvate ratio in left ventricular myocardium of sham (open bar), LR (hatched bar) and PR (solid bar) groups. * $p<0.05$ vs. sham.

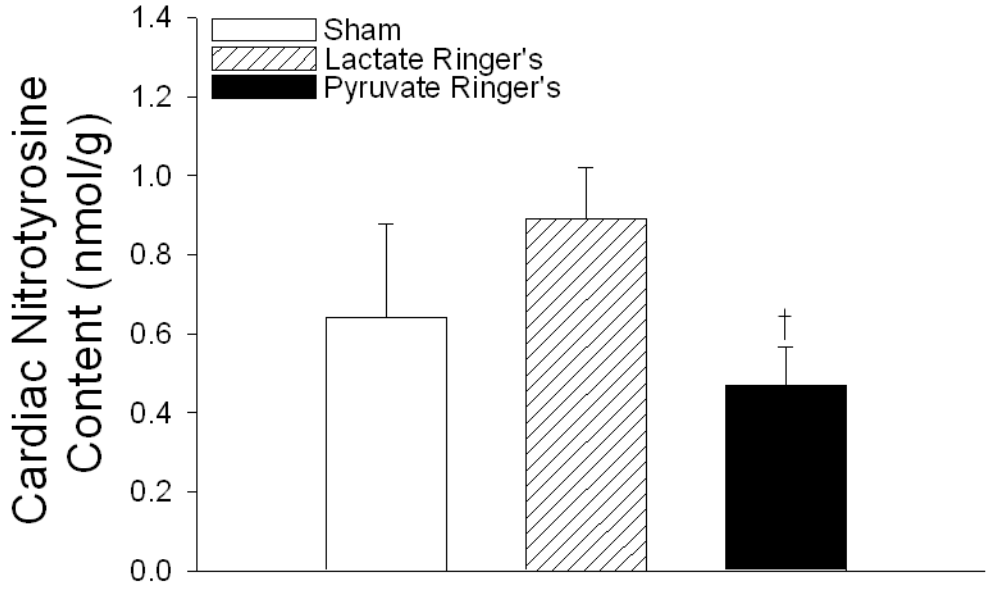

Figure 5. *Myocardial nitrotyrosine*. Nitrotyrosine content (nmol/g) in left ventricular myocardium of sham (open bar), LR (hatched bar) and PR (solid bar) groups. † $p<0.05$ *vs.* LR.

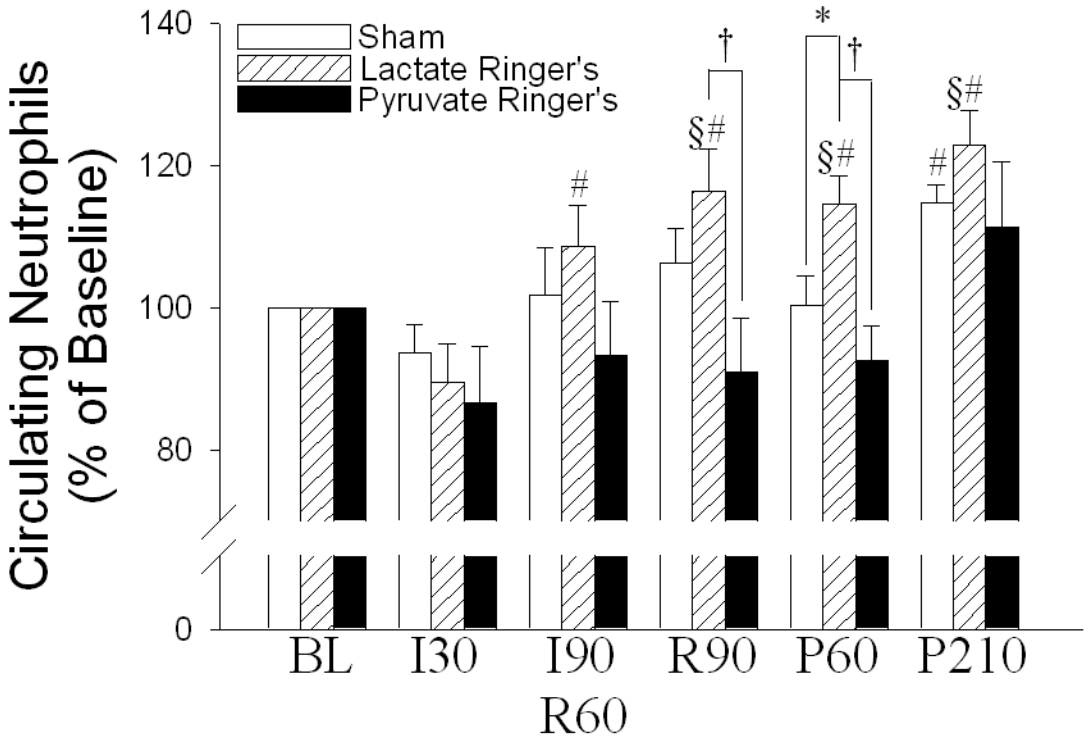

Figure 6. *Circulating neutrophils.* Arterial neutrophils in sham (open bar), LR (hatched bar) and PR (solid bar) groups are expressed as percentages of baseline measurements obtained immediately before initiating hemorrhage. BL: baseline; I: ischemia; R: resuscitation; P: post-infusion. Numbers on the abscissa indicate minutes of each phase. * $p<0.05$ vs. sham; † $p<0.05$ *vs.* LR; § $p<0.05$ *vs.* BL; # $p<0.05$ *vs.* I30.

CHAPTER III

PYRUVATE-ENRICHED RESUSCITATION FOR HEMORRHAGIC SHOCK PROTECTS HINDLIMB MUSCLE FROM ISCHEMIA-REPERFUSION INJURY

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ABSTRACT

Background: Temporary application of tourniquets to achieve hemostasis imposes ischemia-reperfusion on wounded extremities. Resuscitation with pyruvate may blunt muscle reperfusion injury by scavenging oxyradicals and stabilizing cytoprotective proteins. **Methods:** Goats underwent controlled hemorrhage to lower mean arterial pressure to *c*. 50 mmHg. A femoral vasoclamp was placed, and lactate Ringer's (LR) or pyruvate Ringer's (PR) was infused *iv* (10 ml/min) for 90 min, from 30 min femoral occlusion until 30 min after reperfusion. At 4 h reperfusion, gastrocnemius was biopsied for analyses of metabolites, enzymes, apoptotic proteins and markers of oxidative and inflammatory stress. **Results:** In LR *vs.* sham muscle, lactate dehydrogenase and creatine kinase activities fell by 36 and 20%, respectively ($p<0.05$). PR preserved lactate dehydrogenase activity and doubled the activity of the antioxidative enzyme glutathione reductase *vs.* sham. Pro-oxidant NADPH oxidase activity doubled in LR-resuscitated muscle *vs.* PR-treated and sham muscle ($p = 0.056$). Poly(ADP-ribose) polymerase-2 cleavage was increased in LR-resuscitated *vs.* sham muscle. PR prevented this pro-apoptotic effect. Moreover, LR-treated muscle had decreased anti-apoptotic Bcl-xL content *vs.* PR and sham muscle. Nitrotyrosine content doubled in LR-treated *vs.* sham muscle, while PR prevented this increase. Tissue edema, evidenced by extracellular expansion and increased water content, was detected in LR- but not PR-resuscitated muscle. **Conclusions:** Systemic hypotension and hindlimb ischemia-reperfusion with LR-resuscitation imposed pro-oxidative and pro-inflammatory stress and initiated apoptotic mechanisms in muscle. PR blunted oxidative and inflammatory stress and suppressed pro-apoptotic signaling in the resuscitated hindlimb.

INTRODUCTION

Hemorrhage is the leading cause of death associated with combat-related casualties in modern warfare.[8] During Operation Iraqi Freedom III, advances in Kevlar body armor worn by soldiers in the battlefield sharply lowered the incidence and severity of head and torso trauma, but the extremities were unprotected and vulnerable to blast injuries that often resulted in severe blood loss[41] Although tourniquet application is an effective battlefield technique to stanch bleeding from wounded limbs, prolonged application imposes ischemia, which in turn damages distal musculature.[16,20,26,41]

Tourniquet removal reintroduces oxygenated blood, causing explosive formation of reactive oxygen derivatives including hydrogen peroxide (H_2O_2), hydroxyl radical (OH•) and superoxide ($•O_2^-$) within the reperfused muscle.[37] Although pharmacological antioxidants have been advocated for resuscitation, antioxidant-enhanced resuscitative fluids are not currently used in clinical and military settings.[38] Serum nitric oxide (•NO) concentrations have been found to be depressed in hemorrhagic animal models resuscitated with lactate Ringer's, concordant with increased $•O_2^-$ condensation with •NO to produce peroxynitrite ($ONOO^-$).[39] Fluid resuscitation with pyruvate would introduce a potent, membrane-permeable antioxidant[28,29,30] to the reperfused limb, capable of neutralizing reactive oxygen and nitrogen species.[15]

Ischemia-reperfusion induced skeletal muscle apoptosis is initiated through uncoupling of the electron transport chain and opening of mitochondrial permeability transition pores.[14] Crystalloid resuscitation with lactate Ringer's potentiates apoptosis of skeletal myocytes by upregulating the pro-apoptotic protein Bax and restricting phosphorylation of the anti-apoptotic protein Bad.[10,19] Pyruvate, through preservation of

cellular energetics and cellular redox potentials, may promote inner mitochondrial membrane integrity and, thus, suppress cellular apoptosis.[21]

This study tested, in domestic goats subjected to severe hemorrhage and hindlimb ischemia-reperfusion, the hypothesis that controlled volume expansion with pyruvate-fortified Ringer's solution attenuated the formation of reactive oxygen and nitrogen species and local inflammation, preserved anti-apoptotic mediators, and decreased expression of pro-apoptotic mediators in reperfused hindlimb muscle. These potentially cytoprotective mechanisms were examined in gastrocnemius 4 h after hemorrhagic shock and unilateral occlusion-reperfusion of the femoral vessels, combined with fluid resuscitation with lactate- or pyruvate-fortified Ringer's crystalloid solution.

MATERIALS AND METHODS

Animal experimentation was approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center, and was conducted in accordance with the *Guide to the Care and Use of Laboratory Animals* (NIH publication 85-23, revised 1996). Studies were conducted in 17 male goats weighing 20.3 ± 0.5 kg. Goats subjected to hemorrhage were resuscitated with Ringer's solutions containing 130 mM Na^+, 109 mM Cl^-, 4 mM K^+, 3 mM Ca^{2+} and either 28 mM lactate (LR group; n=6) or 28 mM pyruvate (PR group; n=7). Sham animals (n=4) were anesthetized and surgically instrumented, but were subjected to neither controlled hemorrhage nor resuscitation. All goats were fasted for 24 h before experimentation.

Surgical Preparation

Goats were sedated with diazepam (0.25 mg/kg) and ketamine (5 mg/kg), intubated and mechanically ventilated with room air supplemented with 100% O_2. Anesthesia was maintained with 1-2% isoflurane. Body temperature was kept at 38-39.5°C by placing the animal on an electric heating pad.[1]

The left internal jugular vein was accessed through an 8 cm craniocaudal neck incision and catheterized, and then heparin (500U/kg) was administered to maintain catheter patency. The left common carotid artery was catheterized and connected to an in-line, CDXress 3cc arterial pressure transducer (Maxxim Medical) to monitor blood pressure and heart rate (Hewlett Packard model 78354A vital sign monitor). The right femoral vessels were exposed just inferior to the inguinal ligament.

Experimental Protocol

Controlled withdrawal of blood (20 ml/min) from the jugular vein was performed to lower mean arterial pressure (MAP) to 47.7 ± 1.0 mmHg (total withdrawn volume 233 ± 13 ml). Once MAP had stabilized for 5 min an atraumatic vascular clamp was applied to the femoral artery and vein just inferior to the inguinal ligament to produce hindlimb ischemia. After 30 min of ischemia, fluid resuscitation was initiated by continuous roller pump infusion of lactate- or pyruvate-fortified Ringer's solution into the jugular vein at a rate sufficient to deliver 3 times the hemorrhage volume over 90 min. After 90 min of hindlimb ischemia and 60 min resuscitation the vascular clamp was released, and after another 30 min, fluid resuscitation was stopped. The goat was then allowed to recover for 3.5 h. Arterial blood was sampled periodically for measuring PO_2, PCO_2 and pH,

which were kept within normal limits by adjusting supplemental O_2 and altering ventilatory frequency and tidal volumes. After sedimentation of formed elements by centrifugation, plasma was flash-frozen in liquid N_2 and stored at -80°C.

Gastrocnemius metabolites and enzymes

At 3.5 h recovery, post-ischemic gastrocnemius was sampled for analyses of enzyme activities, metabolites and protein content. Tissues were snap-frozen *in situ* with Wollenberger tongs pre-cooled in liquid N_2. Additional gastrocnemius biopsies were excised and fixed in 95% ethanol formalin. After fixation, tissues were sectioned and washed with distilled H_2O, embedded in paraffin, sectioned to 5 µm and stained with hematoxylin and eosin. Microscopic images were captured with an Olympus U-PS 4X70 camera. Proteins were extracted from snap-frozen and pulverized muscle and concentrations determined colorimetrically with a Coomassie Plus Kit (Pierce, Rockford, IL). Enzyme activities were measured by spectrophotometric assays[5] in a Shimadzu Instruments model UV-1600 spectrophotometer, and expressed as U/mg protein.[17,30,44]

Immunoblot analyses of pro- and antiapoptotic proteins

Muscle proteins were extracted for immunoblot analyses of Bcl-xL and PARP-2.[22,42] Total protein concentrations in extracts were measured by the method of Bradford.[6] Proteins (50 µg/lane) were separated by electrophoresis (100V for 90 min) on 10% polyacrylamide gels, and then electrophoretically transferred (350 amp current for 210 min) onto nitrocellulose membranes. Membranes were then incubated in 5% electrophoresis grade, nonfat milk (Bio-Rad) for 2 h to block nonspecific binding. After

one 5 min wash with TBS and two 10 min washes with TTBS, membranes were incubated with primary antibodies for 2 h at room temperature with gentle shaking. After another TBS-TTBS wash, membranes were exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h. Primary antibodies were mouse monoclonal anti-poly(ADP-ribose) (Millipore, Temecula, CA) and rabbit polyclonal anti-Bcl-2L1 (Abcam, Cambridge, MA). Anti-rabbit and anti-mouse secondary antibodies were from Kirkegaard and Perry (Gaithersburg, MD). Membranes were washed for 60 min in TBS-TTBS, and then immune complexes were detected by employing enhanced chemiluminescence (Amersham Bioscience). Images were captured with a Canon digital camera. All membranes were stripped and exposed to an anti-actin antibody (Stressgen, Victoria, BC); analyte band densities were normalized to densities of actin bands. Target protein and actin band densities were analyzed with an AlphaEase FC 4.0 densitometer program (AlphaInnotech, San Leandro, CA).

Nitrotyrosine

Muscle protein extracts were analyzed for nitrotyrosine content using an enzyme-linked immunosorbence assay (ELISA) kit (Northwest Life Science Specialties, Vancouver, WA). A Power Wave XS plate reader was employed for analysis of ELISA at 450 nm (Bio-Tek, Winooski, VT).

Statistical analysis

Values are means ± SEM. Intra-group comparisons of different time points were accomplished by one-way ANOVA for repeated measures. When ANOVA detected statistical significance, a Student-Newman-Keul *post hoc* test was performed to detect

specific differences. Comparisons of the 3 groups (sham, LR, PR) also employed one-way ANOVA with *post hoc* Student-Newman-Keul testing. Single comparisons of unpaired values employed Student's T-test. P values <0.05 were taken to indicate statistically significant differences. Statistical analyses were performed with Sigma Stat software version 3.1 (Aspire Software Int., Ashburn, VA).

RESULTS

Muscle metabolite contents

Lactate and pyruvate contents in gastrocnemius were assayed at 4 h reperfusion, i.e. 3.5 h after completing LR or PR resuscitation. There were no significant differences in lactate content among the groups (Figure 1A). PR resuscitation increased pyruvate content by 67 and 66% *vs.* LR-treated and sham muscle, respectively ($p<0.05$; Figure 1B). The ratio of lactate/pyruvate content, a measure of NADH/NAD^+ redox state, was increased by 122% in LR-treated muscle *vs.* sham ($p<0.05$; Figure 1C). Thus, LR and PR resuscitation produced effects on the redox state of hindlimb muscle which persisted for 3.5 h post-resuscitation.

Muscle enzyme activities

Activities of glycolytic (LDH), energy shuttling (CK), antioxidative (glutathione reductase, glutathione peroxidase) and pro-oxidant (NADPH oxidase) enzymes were measured in sham, LR and PR gastrocnemius at 4 h reperfusion. LDH activity (Figure 2A) fell 35% in the LR group *vs.* sham ($p<0.05$) but was preserved in the PR group. CK activity (Figure 2B) also fell, by 20%, in the LR group *vs.* sham ($p<0.05$); PR

resuscitation did not appreciably protect CK activity. Activity of glutathione reductase, which utilizes NADPH reducing power to maintain glutathione antioxidant reserve, increased by 69% in the LR group and by 120% in the PR group *vs.* the sham value (Figure 2C). Glutathione peroxidase did not differ among the groups. PR resuscitation suppressed activity of the pro-oxidant enzyme NADPH oxidase, a major source of superoxide, by 54% *vs.* LR-resuscitated muscle (Figure 2E).

Pro- and anti-apoptotic factors

Within the poly(ADP-ribose) polymerase (PARP) family the isotypes PARP-1 and PARP-2 are specifically activated in response to DNA strand breaks associated with apoptosis.[2,4] The effects of LR *vs.* PR resuscitation on cleavage and activation of PARP-2 were examined (Figure 3). The ratio of cleaved to uncleaved PARP-2, a measure of PARP-2 activation, doubled in LR-resuscitated *vs.* non-ischemic sham muscle ($p<0.01$). Resuscitation with PR appreciably lowered PARP-2 cleavage ($p<0.05$ *vs.* LR). In addition, cellular content of the anti-apoptotic protein Bcl-xL fell by 18% in the LR resuscitated *vs.* sham muscle, but was fully maintained in the PR-resuscitated muscle (Figure 4). Collectively, these findings demonstrate initiation of pro-apoptotic signaling cascades in the LR-resuscitated hindlimb muscle, and dampening of these pro-apoptotic mechanisms following fluid resuscitation with PR.

Tyrosine nitration and tissue edema

Nitration of tyrosine residues, a consequence of chemical attack by reactive nitrogen species, is a footprint of nitrosative stress in muscle.[24,45] 3.5 h after completing

LR resuscitation, muscle content of nitrotyrosine increased by 114% *vs.* sham muscle ($p<0.05$; Figure 5). No increase in nitrotyrosine was evident following PR resuscitation. Muscle water content, a measure of tissue edema, was markedly increased in LR-resuscitated *vs.* sham muscle (81.4 ± 2.2 *vs.* 74.7 ± 1.2 ml/100g; $p<0.01$; Figure 6). This increase in water content represents a 26% decrease in dry mass per g tissue. PR attenuated the increase in tissue water content. Histological examination confirmed expansion of the extracellular compartment in the LR-resuscitated *vs.* sham muscle (Figure 7). The extracellular space in the PR-resuscitated muscle was not expanded, and resembled that of the sham control muscle.

DISCUSSION

This study tested the hypothesis that fluid resuscitation with pyruvate-fortified Ringer's protects hindlimb muscle more effectively than conventional lactate Ringer's solution in goats subjected to hemorrhagic shock and femoral occlusion-reperfusion. Relative to muscle of non-hemorrhaged sham goats, muscle of LR-resuscitated goats exhibited increased measures of oxidative (NADPH oxidase activity) and nitrosative (nitrotyrosine content) stress, tissue edema and activation of pro-apoptotic signaling (PARP-2 cleavage and Bcl-xL depletion). PR-resuscitation minimized or prevented these measures of injury in post-ischemic hindlimb muscle.

Anti-apoptotic effects of pyruvate

The use of tourniquets to achieve hemostasis imposes severe ischemia on the hindlimb musculature. Subsequent volume expansion with reperfusion of ischemic muscle elicits formation of harmful free radicals,[37,38,48] inflammation and eventual cell

death.[9,25,46] Apoptosis, i.e. programmed cell death, involves cellular shrinkage, chromatin condensation, membrane blebbing and internucleosomal DNA fragmentation, culminating in energy-dependent cellular fragmentation into membrane-bound apoptotic bodies.[13] Studies in rat skeletal muscle revealed that pro-apoptotic cascades initiated by ischemia-reperfusion can begin as early as 2 – 6 h after the onset of ischemia, and progress to cell death in stages over several hours - days.[16,48] Moreover, the use of lactate Ringer's for volume expansion may actually promote end-organ apoptosis.[10,11,18,19] In the current study, substituting pyruvate for lactate in the Ringer's solution dampened pro-apoptotic mediators, thus substantiating previous studies demonstrating pyruvate's preservation of cellular integrity in animal models of hemorrhagic shock.[18,33,34]

Pyruvate infusion after controlled hemorrhage has been found to reduce apoptotic indicators in liver, lung and human endothelial cells.[18,19,25,27,34,43] Ischemia-induced apoptosis is initiated by the opening of mitochondrial permeability transition (MPT) pores.[14] Kerr *et al.* demonstrated pyruvate's effective stabilization and resealing of MPT pores in an isolated heart model of ischemia and reperfusion.[21] Pyruvate Ringer's has been shown to enhance cellular contents of the anti-apoptotic proteins Bcl-2 and Bcl-xL.[18,25,27,34,43] Bcl-xL, a natural suppressor of MPT opening, cytochrome c release and apoptosis,[34] was preserved in PR-treated muscle, while LR-treated muscle exhibited diminished Bcl-xL content. Preventing MPT pore opening will retain cytochrome c in the mitochondrial inter-membrane space, thus limiting caspase activation and maintaining electron flux through the respiratory chain. The increased nitrotyrosine content of LR-resuscitated muscle indicates appreciable nitrosative stress, which may have contributed to the observed reduction in Bcl-xL content, and, thus, precipitated MPT opening and

progression of apoptosis. Conversely, suppression by PR of free radical formation, as indicated by the dampening of tyrosine nitration, could have stabilized the mitochondrial membrane.[27,43]

Activity of the cytosolic enzyme lactate dehydrogenase provided additional information on tissue injury. Lactate dehydrogenase activity fell in LR-resuscitated *vs.* sham muscle, but not in PR-resuscitated muscle, suggesting that PR ameliorated cellular injury and leakage of soluble cytosolic enzymes. Collectively, these results indicate that the combination of hemorrhage and hindlimb ischemia-reperfusion induced oxidative stress sufficient to injure the muscle and initiate the apoptotic cascade, and that resuscitation with PR was more effective than LR at mitigating cellular injury and apoptosis.[9,12,16]

Although the exact molecular mechanism through which pyruvate suppresses apoptosis is unknown, it is well established that pyruvate enhances cellular energy states by bolstering oxidative metabolism.[23,28,30,47] Lactate-induced conversion of NAD^+ to NADH is linked to increased formation of reactive oxygen species in myocardium.[3] On the other hand, enhancement by PR of tissue pyruvate content is linked to increased cytosolic NAD^+/NADH ratio via the lactate dehydrogenase equilibrium, which may relieve the restraint of glycolysis imposed by excessive cytosolic NADH.[7,35] Moreover, pyruvate augments GSH redox state, thereby enhancing cellular defenses against oxidative stress,[23,34,36,40] and functions as an antioxidant, detoxifying peroxides and peroxynitrite in direct, nonenzymatic reactions.[30,31] The marked increase in glutathione reductase activity after PR treatment may afford another mechanism to maintain the glutathione redox state in the face of ischemia-reperfusion-induced oxidative stress.

Poly(ADP-ribose) polymerase-2 (PARP-2) is activated in response to the fragmentation of chromatin DNA that occurs with oxidative stress.[2,4,10] Activated PARP-2 consumes NAD^+ as substrate for poly(ADP-ribose) synthesis, depleting cells of an essential cofactor for energy production.[2,33] NAD^+-depleted cells must consume ATP to regenerate the cofactor. Further reductions in cellular ATP content could induce synthesis and activation of pro-apoptotic proteins and propagation of programmed cell death cascades.[12] Marked PARP-2 cleavage and over-activation was evident in post-ischemic muscle of the LR group, but PR dampened PARP-2 cleavage.

Anti-oxidative and –inflammatory effect of pyruvate

Prolonged ischemia causes inflammation in skeletal muscle.[9] In LR-resuscitated muscle, increased nitrosative stress and NADPH oxidase activity, a major source of superoxide, favors development of inflammation in the reperfused tissue.[32] PR, through its antioxidative properties, suppresses these inflammatory mechanisms. PR resuscitation appears to dampen the effects of circulating inflammatory mediators at the end-organ as well as locally derived inflammatory cascades.

Muscle H_2O content was markedly increased in the LR-treated *vs.* non-ischemic sham gastrocnemius, an indication of tissue edema. H&E stains confirmed the presence of tissue edema in the LR group. The interstitial compartment was markedly expanded in LR-treated *vs.* sham and PR-treated muscle. Pyruvate's anti-inflammatory properties,[15] specifically its suppression of PARP-2 cleavage, may help reduce post-ischemic interstitial edema. With diminished PARP-2 activation, pyruvate may reduce neutrophil infiltration and subsequent local inflammation.[45,46]

Limitations

Limitations of this study must be acknowledged. First, while the duration of ischemia, reperfusion and recovery in our model appears to be sufficient to evoke early apoptotic events in skeletal muscle, longer ischemic insults and protracted reperfusion may be necessary to demonstrate the complete apoptotic spectrum. Indeed, Cowled *et al.* reported that no cellular damage could be detected in rat skeletal muscle subjected to 2 h ischemia and 2 h reperfusion.[9] Secondly, the optimal dosage of pyruvate for maximum benefit remains undefined. A pyruvate concentration of 28 mEq/L in Ringer's was used in this study, representing equilmolar replacement of lactate in conventional LR. At the infusion rate of 10 ml Ringer's solution $\bullet min^{-1}$, i.e. 0.4-0.5 $ml \bullet kg^{-1} \bullet min^{-1}$, plasma pyruvate increased to approximately 0.5-0.6 mM, which is below the concentration range of *c.* 3-5 mM found to afford maximal protection to isolated[7] and *in situ*[23,42] hearts subjected to ischemia-reperfusion. The goat was chosen as the animal model due to its substantial hindlimb musculature, but the goat model itself poses a significant limitation, due to the limited availability of antibodies specific for caprine proteins.

Conclusions

In summary, resuscitation with pyruvate-enriched Ringer's diminished tissue damage and suppressed early events of the apoptotic cascade in hindlimb muscle subjected to ischemia-reperfusion in the setting of severe hemorrhage. Pyruvate's antiapoptotic properties may be directly related to its anti-oxidative and anti–inflammatory effects within the ischemic tissue. To our knowledge, this is the first study examining the effects of pyruvate-enriched resuscitation on apoptosis in post-ischemic

skeletal muscle. Future work will aim to establish the optimal pyruvate concentration to maximize its salutary effects.

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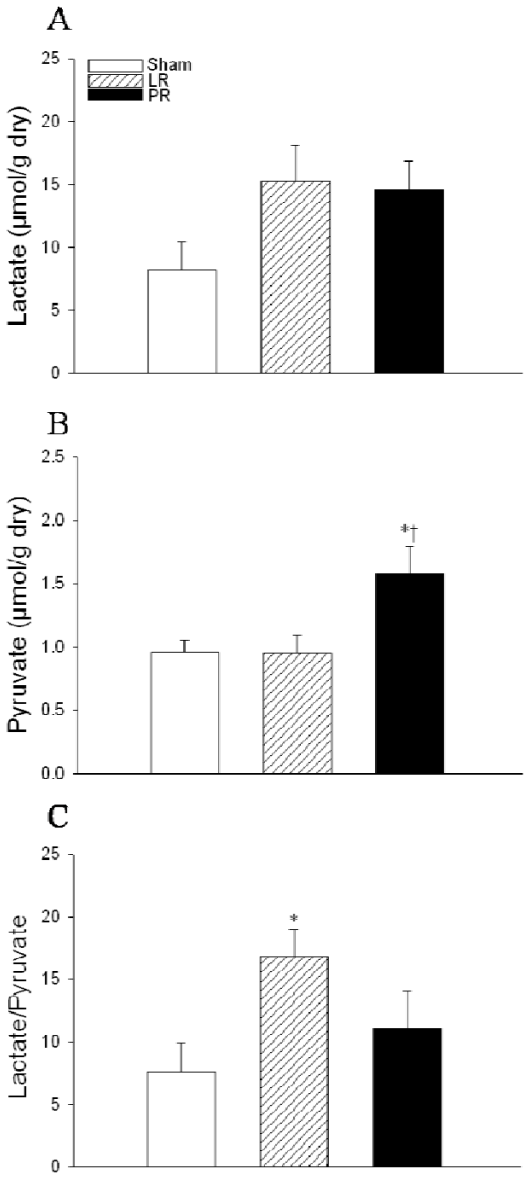

Figure 1. *Lactate and pyruvate content in post-ischemic hindlimb muscle.* Lactate (panel A) and pyruvate (panel B) contents in gastrocnemius sampled 4 h after hindlimb reperfusion in goats resuscitated with LR (hatched bars, n=6) or PR (solid bars, n=7), and in non-ischemic sham muscle (open bars, n=4). Panel C: lactate/pyruvate content ratio. Values in this and the other figures are means ± SEM. * $p<0.05$ *vs.* sham; † $p<0.05$ *vs.* LR.

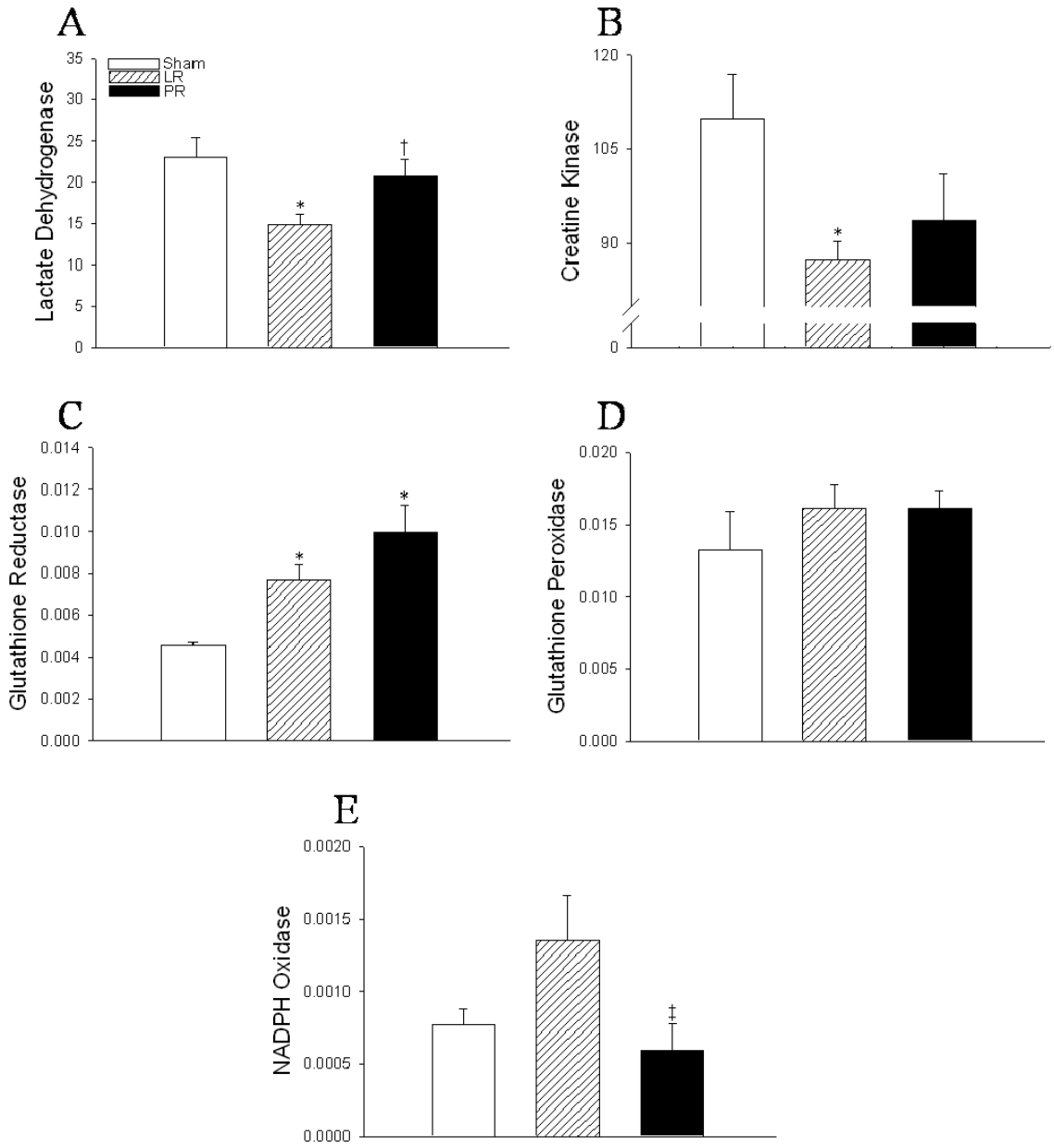

Figure 2. *Muscle enzymes.* Activities (U/mg protein) of (A) lactate dehydrogenase, (B) creatine kinase, (C) glutathione reductase, (D) glutathione peroxidase and (E) NADPH oxidase were measured in ischemic gastrocnemius protein extracts from sham (open bars), LR-resuscitated (hatched bars) and PR-resuscitated (solid bars) muscle. * $p<0.05$ *vs.* sham; † $p<0.05$ *vs.* LR; ‡ $p = 0.056$.

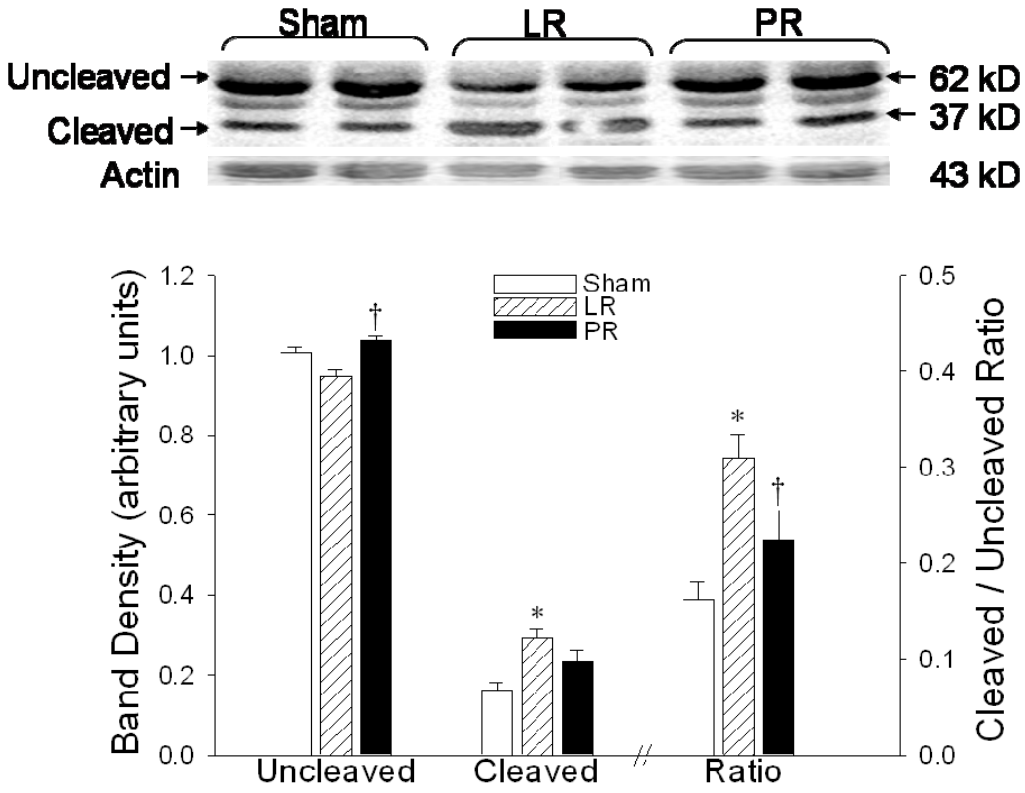

Figure 3. *Poly(ADP-ribose) polymerase-2 (PARP-2) cleavage.* Top: representative immunoblot of uncleaved and cleaved PARP-2. Bottom: actin-normalized band densities of uncleaved and cleaved PARP-2, and the uncleaved/cleaved PARP-2 ratio in sham (open bars), LR-resuscitated (hatched bars) and PR-resuscitated (solid bars) muscle. * $p<0.05$ *vs.* sham; † $p<0.05$ *vs.* LR.

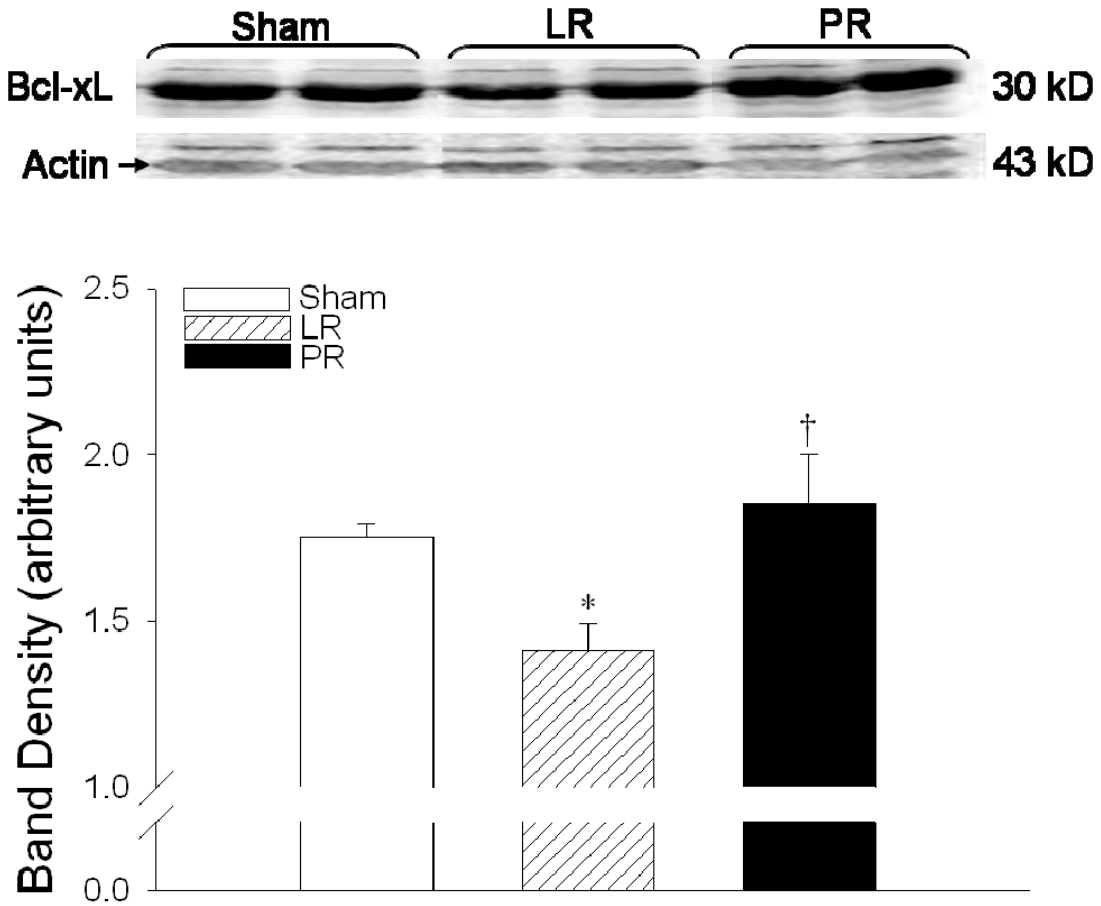

Figure 4. *Bcl-xL content.* Top: representative immunoblot of Bcl-xL. Bottom: actin-normalized Bcl-xL band densities in sham (open bars), LR-resuscitated (hatched bars) and PR-resuscitated (solid bars) muscle. * $p<0.05$ *vs.* sham; † $p<0.05$ *vs.* LR.

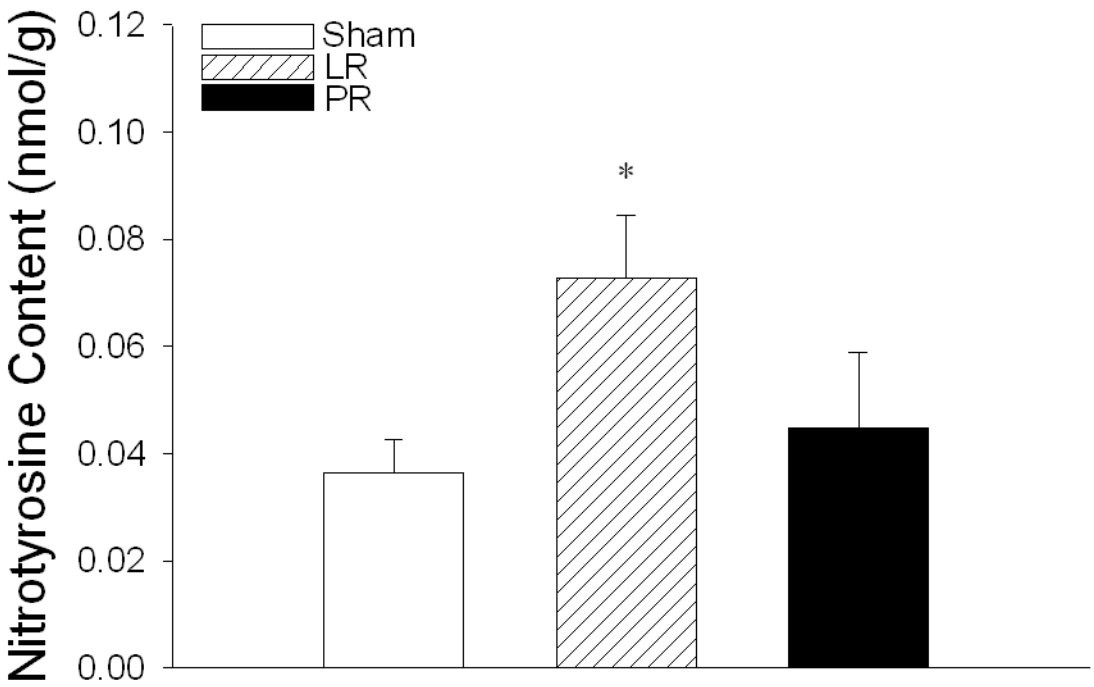

Figure 5. *Nitrotyrosine content.* Nitrotyrosine content in sham (open bars), LR-resuscitated (hatched bars) and PR-resuscitated (solid bars) muscle. * $p<0.05$ *vs.* sham.

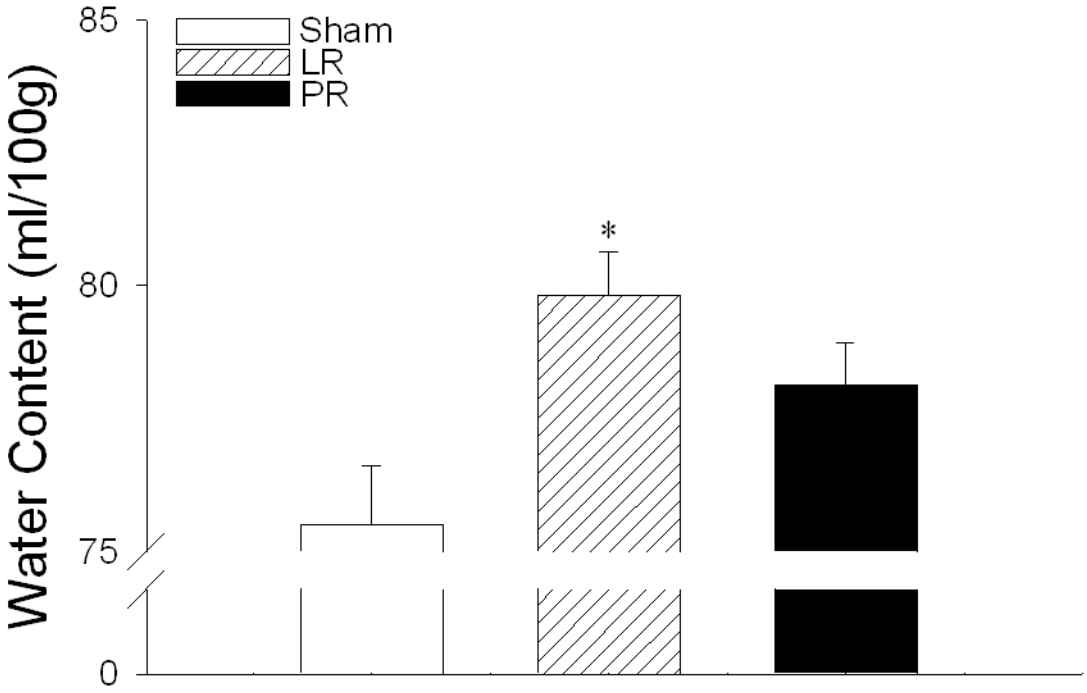

Figure 6. *Muscle water content.* Water content of sham (open bars), LR-resuscitated (hatched bars) and PR-resuscitated (solid bars) muscle. Values equal to means ± SEM. * $p<0.05$ *vs.* sham.

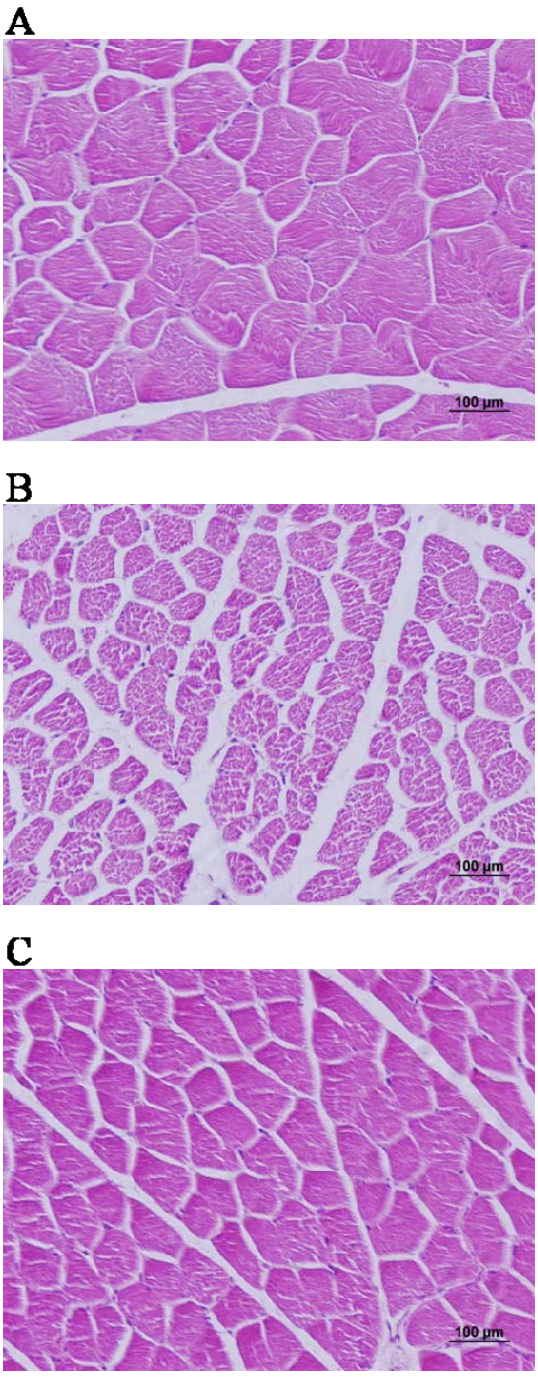

Figure 7. *Histological examination of gastrocnemius at 4 h recovery.* 200X magnification of hematoxylin and eosin stained sections. Panel A: Sham muscle; Panel B: LR-resuscitated muscle; Panel C: PR-resuscitated muscle. Bars in each panel indicate 100 µm.

CHAPTER IV

The goat as an animal model for acute surgical research: an overview

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ABSTRACT

This article reviews the use of goats as animal models for biomedical research. Appropriate methods for induction of anesthetics, intubation and surgical maintenance of the goat during acute experimentation are described. A current hemorrhagic shock protocol exemplifying the use of goats for cardiovascular research is presented, and challenges encountered in the development and refinement of this preparation are discussed. The article also addresses the risks imposed by the Q fever pathogen Coxiella burnetii, and measures which have proven effective in minimizing zoonotic transmission of this pathogen to laboratory personnel. Finally, the article reviews current costs of the goat, and discusses the availability of antibodies and primers specific to goat proteins and mRNA transcripts. With appropriate knowledge of its capabilities and limitations, the goat is a suitable animal model for a variety of biomedical research applications.

CURRENT RESEARCH EMPLOYING THE GOAT AS AN ANIMAL MODEL

The goat (*Capra hircus*) is a large animal model used in scientific research, teaching and testing. Although goats still comprise a small percentage of the total animals used in research, their use as an accepted experimental animal model is increasing steadily. Exemplifying this trend is the number of articles listed in the Pubmed database containing the words "goat" or "caprine", which in the last ten years is double the number published in the 1970's (Table 1).[15] The steady rise in goat research can be directly attributed to the more moderate costs of goats as compared to the popular but increasingly expensive canine and swine models, better understanding of handling and an increased breadth of experimental studies in which a goat model can be ideally employed.

The increased role of the goat in biomedical research is due in part to an overall increase in the breadth of scientific research in general. The anatomy and availability of the goat make it ideal for investigations ranging from surgical research to bacteriology. Research utilizing the goat as the primary animal model includes but is not limited to orthopedic, reproductive, infectious disease, chemotherapeutic, obstetric, gastrointestinal, cardiovascular, veterinary, agricultural, genetic and endocrine related areas of investigation.[7,28] Figure 1 illustrates the distribution of research involving goats identified by an advanced Pubmed search of 1,124 titles containing the word "goat" published from January 1, 2000 to January 18, 2009. This literature search reveals that the most popular research fields utilizing goats are investigations involving reproductive / embryologic studies (20.6%) and infectious disease research (17.7%). The goat is particularly facile for research in these areas due to its longer gestation time of 143-154

days *vs.* dogs and swine, and its susceptibility to a large number of infectious agents that are also infectious in humans.[2]

The United States Department of Defense uses goats for a variety of military research applications and training exercises.[3] Although much of this work is classified, goats are used in the field to train medics in the proper application of triage skills, stabilization techniques and patient transport.[3] Ruminants are also utilized in blast injury studies involving the testing of ballistic vests, as well as in training surgeons to repair acute traumatic injuries to the extremities.[8,21]

The goat is especially suitable for studies requiring large amounts of hindlimb muscle tissue because it possesses well-developed and easily discernable gastrocnemius and soleus muscle, and the lack of extensive inguinal adipose tissue facilitates exposure and catheterization of the femoral vessels. The goat's long, easily extendable neck and minimal adipose tissue affords an excellent surgical field for studies requiring isolation of the major neck vessels.

HANDLING, SEDATION, INTUBATION AND INTRAOPERATIVE MANAGEMENT

The authors have implemented a goat model for investigating injury to hindlimb musculature and internal organs challenged by hemorrhagic shock. Approval for the use of goats in these studies was obtained from the University of North Texas Health Science Center's (UNTHSC) Institutional Animal Care and Use Committee, and all experimentation was performed in accordance with the *Guide to Care and Use of Laboratory Animals* (NIH publication 85-23, revised 1996).[5,13] Goats were delivered to

the UNTHSC's Department of Laboratory Animal Medicine the morning of experimentation in order to minimize contact with personnel and potential exposure to other laboratory animals housed in the UNTHSC vivarium. Goats were fasted at least 24 h before experimentation, and water was withheld 6 h before any procedure in order to minimize the possibility of aspiration.[2] A minimum one-hour adaptation period elapsed before administering induction anesthetics. During this time goats were housed in a holding area, in accordance with National Research Council guidelines, with the room maintained at slightly subatmospheric pressures to prevent dissemination of pathogens.[20] All laboratory personnel coming into direct contact with the goat were required to wear appropriate personal protective equipment, consisting of a gown made of spunbond fabric, latex gloves, shoe covers, surgical cap and an N92 Respirator.

Induction of general anesthesia

The goat was next prepared for induction of anesthesia under the guidance of a licensed veterinary assistant.[5] A relatively docile species, goats are generally easy to handle. Although male goats are more prone to resist handling, this behavior is readily controlled by cornering the animal before attempting any positioning maneuvers.[2] Proper handling of the goat was accomplished by hooking an arm around either the neck or torso, or by grabbing the horns, thus allowing laboratory personnel to hold the goat in a vertical position resting on its haunches (Figure 2A).[25] Once positioned in this manner, goats typically assume a calm demeanor. The neck of the goat then was shaved with conventional clippers from the superior border of the clavicle to the mandibular angle (Figure 2B). Shaving ensured proper visualization of right and left external jugular veins

as well as ready access to the trachea should an emergent tracheostomy be necessary. The epigastric surface of the goat's abdomen was also shaved in anticipation of the gastrostomy performed for stomach decompression.

Sedation of goats in our studies was accomplished through a controlled percutaneous injection of the induction anesthetics diazepam (0.25 mg/kg) and ketamine (5 mg/kg) into the left external jugular vein.[2] This specific drug combination eliminates many of ketamine's untoward side-effects such as increased resting muscle tone, trembling, and increases in intraocular pressure, mean arterial pressure and body temperature.[1] A minimum of 30 minutes should elapse before taking baseline measurements to allow the systemic effects of the single dose of ketamine to abate.[1,2] An 18-gauge needle was introduced bevel-side up into the left external jugular vein, and the diazepam/ketamine was injected after ensuring proper needle placement within the vessel through gentle withdrawal of venous blood (Figure 2C). The goat should be noticeably comatose within 10 s after completing the injection.

In our study there were three instances of acute pulmonary edema arising 60 – 90 min after administration of induction anesthesia. These goats eventually succumbed to the effects of hypoxia as a result of the rapid onset and progression of the edema. Intravenous diazepam/ketamine administration has been reported to cause this type of acute pulmonary insufficiency.[4] It is proposed that propylene glycol, a component of diazepam, may cause increased vasoactivity in the lungs of anesthetized animals, leading to an increase in fluid accumulation in the pulmonary interstitium.[27]

Intubation technique

Intubation is achieved by either placing the goat in a sternal recumbent position or maintaining the positioning used for diazepam/ketamine injection. The authors prefer the latter positioning technique as it allows for easy extension of the neck to facilitate insertion of the endotracheal tube (Figure 2D). Laboratory personnel performing the intubation should face the goat and use a straight-blade laryngoscope held upside down allowing for proper placement at the back of the tongue. With the tip of the blade at the glottis the tongue is compressed and the vocal cords exposed.[2] Spontaneous respiration moves the epiglottis in an anterior direction, which facilitates visualization of the vocal cords. An endotracheal tube, 6-8 mm in diameter, is then introduced between the vocal cords by guiding it down the blade of the laryngoscope over a rigid wire stylus (Figure 2D). Once proper placement of the tube has been confirmed through detection of air passing through the tube, the cuff is inflated and the tube secured with a gauze tie passed behind the lower incisors and around the lower jaw.[2] Securing the endotracheal tube in this manner permits nasal drainage of oral secretions throughout the experiment, thereby obviating the risk of aspiration around the inflated cuff.

Once intubated, the goat is carefully placed on the operating table in a supine position, connected to the ventilator, and all limbs secured with conventional rope ties. The ventilator used during our experimentation was a MDS Matrix model 3000 Veterinary Anesthesia Ventilator, and the vaporizer was a VAS 2000 #00191OF, both manufactured by Vet Equip, Inc. (Pleasanton, CA). Ventilatory settings for goats weighing between 20 and 25 kg are presented in Table 3. These settings should be adjusted after baseline blood gases are obtained to maintain arterial pH between 7.3 and

7.4.[2] Tidal volume should approximate 12.9 ml/kg.[2] For optimal ventilation, the goat should be positioned in a left lateral decubitus position during experimentation. Placement in a right lateral decubitus position could lead to caval compression, impeding venous return to the right atrium. Proper visualization during isolation of the major blood vessels in the neck can be obtained by temporarily placing the goat in a supine position, and then repositioning it into a left lateral decubitus position after completing the isolation.

Maintenance anesthesia and intraoperative assessment of the anesthetic plane

Isoflurane was selected as the gaseous anesthetic agent for this investigation. Isoflurane does not produce as severe a cardiorespiratory depression and sensitization as other inhaled anesthetics and is administered at concentrations 1 – 1.5%,[2,7] thereby achieving an alveolar concentration sufficient to suppress all movement.[26] Other accepted gaseous anesthetic agents suitable for goats include halothane, methoxyflurane and nitrous oxide.[2] Consideration of protocol and experimental end-points should guide the attending veterinarian's choice of appropriate anesthetic agent.[7]

Accurate monitoring of the anesthetic plane can be accomplished through serial monitoring of jaw tension, hemodynamic changes, pedal and palpebral reflexes, eye position and papillary light reflex.[2,26] It is important to note that these signs can only be accurately assessed after the systemic effects of ketamine have subsided, generally 15-30 min after administration.[2] The animal will experience pain if the anesthesia plane is too low. Intraoperative assessment of pain relies on physiologic responses. An abrupt increase in mean arterial pressure, heart rate or respiratory rate (if the goat is allowed

ventilating spontaneously) may indicate the animal is perceiving pain. Spontaneous mastication also indicates a suboptimal anesthetic plane.[7] Sweating, tearing and movement also indicate a low anesthetic plane, but are associated with a more definitive awareness of pain and should not be used as end-points.[26] Also, the use of anticholinergic agents will confound many of these intraoperative signs.

Anatomy and physiology of the ruminant stomach

Intraoperative maintenance of the goat requires careful attention. Intravenous fluid administration is achieved through a calculated drip rate of 4 ml/kg/hr.[7] Body temperature should be closely monitored through serial rectal temperature readings. The normal range of rectal temperatures in the goat is 38.0 to 39.5°C (100.4 to 103.1°F). A heating pad should be placed on the operating table prior to restraining the goat to ensure maintenance of body temperature during the operation.

Goats are classified as true small ruminant laboratory animals. This designation refers to the complex anatomy of their gastrointestinal tract. The four-part ruminant stomach is divided into the forestomach, composed of the reticulum, the rumen and the omasum, and the abomasums or "true" stomach (Figure 3). Food is processed and digested in these compartments, producing volatile gases that are voided through eructation.[2] A sedated and intubated goat may experience abdominal buildup of these gases during the course of a surgical protocol, leading to the appropriately termed ruminant tympany or "bloat".[7] Bloating may lead to severe abdominal distention, which eventually could impede the descent of the diaphragm, thus contributing to ventilatory insufficiency and a diminished blood oxygen saturation.[7] Decompressing the stomach

via gastrostomy is a simple approach to relieve the buildup of gastrointestinal gases, especially in goats fasted less than 24 h. Preferably, the rumen or the reticulum of the forestomach is vented, as these compartments comprise approximately 80% of the total stomach capacity in post-weaning goats.[29] An alternative approach involves passing a stomach tube during intubation.[7] Finally, it is important to avoid placing the goat in a right lateral decubitus position during experimentation, as this position increases intraabdominal pressure and contributes to the accumulation of "bloat" in the rumen.[2]

Management of intraoperative oral secretions

Goats produce copious amounts of oral secretions during experimentation. Care must be taken to monitor the integrity of the endotracheal tube's cuff throughout the experiment as aspiration of these secretions could complicate post-operative recovery in chronic investigations. Further, as noted above the endotracheal tube should be secured to the lower jaw to permit drainage of secretions from the nasal cavities. Studies that are not concerned with hemodynamic end points may employ 0.1-1.0 mg/kg of atropine to minimize intraoperative production of oral secretions.[6] Atropine diminishes oral secretions through its anticholinergic properties. Introduction of atropine into the systemic circulation can obscure the aforementioned indicators used to assess the plane of anesthesia, and this factor should be taken into account when monitoring the animal's level of sedation. For hemodynamic studies, glycopyrolate is a good alternative, as this anticholinergic agent does not increase the heart rate and thus cardiac output of the goat to nearly the extent seen with atropine.

Ventilatory medicine

When formulating the ventilatory management strategy for our goat experimentation, we initially elected to not use positive pressure ventilation. Rather, we reasoned that permissive hypercapnea would effectively drive the goat's apneustic center. This assumption proved incorrect as heroic efforts to prevent diminished O_2 saturation and dangerously low blood pH levels were required. Accordingly, subsequent experiments utilized active positive pressure ventilation. Figure 4A compares arterial pCO_2 in goats without ventilatory support (93 ± 9.2 mmHg) vs. those that were actively ventilated (37 ± 3.1 mmHg, respectively, $p<0.001$). The hypercapnea in the unventilated group produced a marked respiratory acidosis that contrasted with the near-normal pH found in actively ventilated goats (7.15 ± 0.09 vs. 7.47 ± 0.02; $p<0.001$) (Figure 4B). Gross uncorrected hypercapnea and the resultant acidemia may lead to life-threatening metabolic impairment and dampening of contractile force in skeletal and cardiac muscles especially during long experimental protocols.[7,19]

SURGICAL AND EXPERIMENTAL PROTOCOL

The following is a description of the procedures we conducted during experimentation on goats to investigate controlled hemorrhagic shock with hindlimb ischemia-reperfusion. This protocol exemplifies the surgical and experimental procedures that can be accomplished in goats.

Surgical preparation

After diazepam/ketamine induction, intubation and establishment of an acceptable plane of isoflurane anesthesia, central venous access was obtained through an 8 cm

anterior neck incision in order to expose the left internal jugular vein and left common carotid artery. The jugular vein was catheterized with 3 mm o.d. polyethylene tubing to permit controlled blood withdrawal (10cc/min) to mimic hemorrhage, and to enable controlled fluid resuscitation (3 * hemorrhage volume over 90 min) by roller pump infusion of crystalloid solution. Heparin was administered at a dose of 500U/kg to maintain catheter patency. Care should be taken when administering heparin, as an overdose may result in hemolysis, which can taint plasma samples.[14] A right femoral dissection was performed and the femoral vasculature exposed just inferior to the inguinal ligament so that ischemia and reperfusion of the hindlimb could be imposed. The femoral vein was catheterized with a 2 mm o.d. polyethylene tube inserted distal to the most proximal tributary vessel allowing collateral drainage of the hindlimb musculature (Figure 5).

Hemodynamic measurements

The left common carotid artery was isolated, catheterized with 3 mm polyethylene tubing and connected to an inline, CDXress 3cc arterial pressure transducer (Maxxim Medical). Systolic, diastolic and mean arterial blood pressures and heart rate were measured with a Hewlett Packard vital sign monitor, model 78354A (Table 2).

Whole blood chemistry

Baseline arterial blood samples were taken after catheterization of the left common carotid artery, and additional samples were taken at defined stages of the protocol. Arterial whole blood pH, pO_2, pCO_2, K^+, hemoglobin, hematocrit, HCO_3^-, base

excess, glucose, and lactate concentrations were measured in an Instrumentation Laboratory model 1730 blood gas analyzer and model 682 Co-Oximeter. Pyruvate was extracted from centrifuged and deproteinated baseline plasma samples and spectrophotometrically assayed.[18] Arterial pH was adjusted through ventilatory control of pCO_2. Hematocrit values were consistently found to be at the lower end of the accepted range of 22-38% expected in goats (21.8 ± 0.7%).[2]

Complete blood count (CBC) with differential

Whole blood (2 ml) was collected from the carotid artery before initiating controlled hemorrhage, transferred to a purple-topped collection tube containing ethylenediaminetetraacetic acid (EDTA) and stored at room temperature. Next, a peripheral smear of this sample was prepared on a glass slide. White blood cell and platelet counts were obtained manually using a hemocytometer and automatically using a Coulter Counter. The white cell differential was determined manually by Wright's staining. Neutrophils were the most abundant leukocytes at prehemorrhage baseline, and lymphocytes also were prominent (Table 2). The values are within the normal ranges reported in goat.[2]

Expermental protocol

A controlled hemorrhage was accomplished by withdrawing blood from the left internal jugular vein at a rate of 10ml/min. Once mean arterial pressure had fallen to the target 50mmHg and stabilized at this level for five min, a nontraumatic vascular clamp was applied simultaneously to the femoral artery and vein just inferior to the inguinal

ligament (Figure 5). Withdrawal of approximately 15% of blood volume was required to achieve the target blood pressure, and elicited an appropriate baroreceptor-mediated physiologic increase in heart rate (Figure 6). 30 min after hemorrhage heart rate was 129 ± 4, a 25% increase ($p<0.001$) over the pre-hemorrhage rate of 103 ± 5 (Figure 6). 30 min after placement of the femoral vasoclamp, fluid resuscitation with Ringer's solution was initiated by continuous infusion into the internal jugular vein. The resuscitative fluid was delivered by roller pump and adjusted to accomplish a 3:1 volume ratio of resuscitative fluid:hemorrhaged blood over 90 min of infusion. Conventional mixed racemic D/L-lactate Ringer's (B. Braun Medical Inc, Bethlehem, PA) or pyruvate Ringer's prepared by equimolar substitution of 28 mM sodium pyruvate for sodium lactate (Sigma Chemical Co, St. Louis, MO) constituted the control and experimental infusates, respectively. After 90 min of hindlimb ischemia the vascular clamp was released to initiate femoral reperfusion. Thirty minutes later, i.e. at 90 min infusion, resuscitation with the Ringer's solution was stopped. The goat was then allowed to recovery for 3.5 h.

TRANSMITTABLE INFECTIOUS DISEASES

The goat is host to a number of infectious agents and, thus, a vector for disease. Diseases caused by pathogens potentially harbored in goats include infectious abortion, scrapie, goat pox, foot and mouth disease, caprine arthritis, blue tongue, contagious ecthyma, ulcerative dermatosis, anthrax, chlamydiosis, malignant edema, blackleg, black disease, enterotoxemias, leptospirosis, tuberculosis, salmonellosis, shipping fever, tularemia, listeriosis, paratuberculosis (Johne's Disease), caseous lymphadenitis,

coxiellosis, coccidiomycosis, ovine babesiasis, brucellosis, melioidosis, tuberculosis, mycoplasmosis, staphylococcal mastitis, toxoplasmosis, coccidiosis, sarcosporidiosis and udder warts.[17,25]

Many of the diseases that goats may carry are zoonotic, i.e. they pose the risk of transmission to laboratory personnel. Of particular concern in ruminants is *Coxiella burnetii*. *C. burnetii*, an obligate intracellular bacterium, is the smallest pathogenic organism morphologically similar to the bacterial genus rickettsiae.[24] This highly infectious agent can cause Q fever in humans after the transfer of just a single organism.[9] The etymology of this disease derives from the word "query", referring to its variable presentation in animals and humans.[22] The goat is a known host of *C. burnetii*, chronically harboring the bacteria for up to 2 kidding seasons after initial infection.[11,16,24] Placental materials from aborting domestic ruminants and unpasteurized milk are the two most common sources of Q fever in farm animals, and the most common route of transmission to humans is inhalation of aerosolized bacterium.[24,30]

Since the 1960's reports of Q fever outbreaks involving research laboratory personnel have been documented, the most recent being in 2009 when >70% of laboratory personnel working with infected pregnant sheep had positive titers for *C. burnetii*.[9,12,23] Most studies during which infections occurred involved exposure to placental fluids. Diagnosis was confirmed through serologic testing. Fortunately, none of these cases proved fatal.[9,23] These experiences have prompted many institutions to require that personnel not only adhere to occupational health programs mandated by the National Institute of Health, but also receive a baseline physical examination to determine immune status, wear personal protective equipment including respiratory

protection when working with goats and that goat experimentation be conducted at slightly subatmospheric pressure to control airflow and thereby minimize dispersal of *C. burnetii*.[10]

Q fever infection requires intensive diagnostic and treatment modalities in only 2% of contaminated patients. The natural history of the acute form of Q fever after exposure to *C. burnetii* includes an asymptomatic 14-39 d incubation period, followed by development of an unexplained fever lasting more than 7 days, and "flu-like" symptoms including headache, myalgias and night sweats. As the acute phase progresses, cough and fatigue may accompany the development of pneumonia, hepatitis and, less commonly, a rash.[9,16,22,30] Serology study with immunofluorescent assay provides definitive diagnosis. Treatment of acute Q fever consists of a two-week course of doxycycline. Approximately 0.2-0.5% of infected patients develop the chronic form of Q fever which may lead to immunocompromise, valvular lesions and/or vascular aneurysms.[22] Importantly, pregnant women should avoid all contact with any animal that may harbor *C. burnetii*, as Q fever may compromise a pregnancy during any of the three trimesters.[22]

Other considerations and limitations

Domestic goats for biomedical research are generally less costly than dogs and swine, two comparable large mammalian species. Table 4 presents the mean prices of canines, swine and goats from 3 vendors of farm-raised animals from 2006 – 2009. Dogs have been consistently more expensive than the other species throughout this period. Small and very large goats (0 – 34 kg and 57+ kg) remain less costly than swine. In addition to the purchase price of these animals, there are costs for vivarium care,

especially for chronic studies, as well as the costs of personal protective equipment, professional mask fittings, vaccinations and education of laboratory personnel.

Molecular studies performed with goat tissue are challenged by the limited availability of species-specific antibodies and primer sequences. An advanced Pubmed search for the number of primer sequences specific to the goat reveals 5,005 nucleotide sequences. Further searching within the Pubmed database disclosed 1,361 proteins specific for *Capra hircus*. The same search revealed 4,250 proteins specific for *C. burnetii,* indicating that Q fever continues to be an active area of research.

Conclusion

The goat is a docile and affordable large animal that has seen an increasing trend in usage since the 1970's. Its size and prominent hindlimb musculature make the goat especially suitable for studies of trauma, hemorrhagic shock and fluid resuscitation. Educating the veterinary and biomedical research communities about the goat and the potential pitfalls that may be encountered during experimentation will further enhance the effective utilization of this docile ruminant in biomedical research.

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TABLES

Table 1. *Results of Pubmed literature search using the terms "goat", "caprine" and "ruminant" from 1970 - present*

	1970-1979	1980-1989	1990-1999	2000-present	Species total
Goat	652	851	996	1,124	3,623
Caprine	71	262	329	346	1,008
Ruminant	207	163	243	294	907
Total	930	1,276	1,568	1,764	5,538

Values are the number of article titles in the PubMed database associated with each index term.

Table 2. *Positive pressure ventilatory settings for goats*

	V_T (ml)	Respiratory frequency (min^{-1})	PEEP (cm H_2O)	% O_2
20-25 kg Goat	258-323	10-30	5-10	100

Accepted ventilatory ranges for positive pressure ventilation in the goat as described by Allen *et al*. Tidal volumes represent 12.9 ml/kg body mass.[2] Respiratory rate and PEEP are titrated according to desired pH range. V_T: tidal volume; PEEP: positive end expiratory pressure.

Table 3. *Vital signs, blood chemistry, blood metabolites and complete blood count with differential in the goat*

Vital Signs			
Temperature (˚C)	Heart Rate (min^{-1})	MAP (mmHg)	Pulse Pressure (mmHg)
38.5 ± 0.2	97 ± 3.9	90 ± 3.9	21.0 ± 1.4

Blood Chemistry			
Na^+ (mEq/L)	K^+ (mEq/L)	HCO_3^- (mEq/L)	Base Excess
141.5 ± 0.7	4.3 ± 0.2	27.1 ± 0.8	+ 3.0 ± 0.7

Blood Metabolites		
Glucose (mg/dL)	Lactate (mEq/L)	Pyruvate (mEq/L)
66.6 ± 4.7	1.6 ± 0.2	0.3 ± 0.0

Complete Blood Count			
Total WBC's	Hgb (mg/dL)	Hct (%)	Plt ($10^5/mm^3$)
8,200 ± 800	9.1 ± 0.4	21.8 ± 0.7	9.1 ± 1.6

Differential				
Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes
59.6 ± 3.0%	0.5 ± 0.2%	0.2 ± 0.1%	37.0 ± 3.1%	2.2 ± 0.3%

Mean values ± SE from 21 goats. MAP: mean arterial pressure; WBC: white blood cells; Hgb: hemoglobin; Hct: hematocrit; Plt: platelets.

Table 4. *North Texas prices of experimental swine, caprines and canines*

Species	2006	2007	2008	2009
Swine (> 3 weeks - 34 kg)	$175.00 (1)	$155.00 (2)	$172.00 (3)	$163.00 (2)
Swine (35-68 kg)	$202.00 (1)	$205.00 (2)	$229.00 (3)	$225.00 (2)
Swine (68+ kg)	$275.00 (1)	$275.00 (2)	$287.00 (3)	$288.00 (2)
Caprine (0-34 kg)	$173.00 (1)	$171.00 (2)	$167.00 (3)	$150.00 (2)
Caprine (35-57 kg)	$220.00 (1)	$225.00 (2)	$239.00 (3)	$225.00 (2)
Caprine (58+ kg)	$275.00 (1)	$255.00 (2)	$253.00 (3)	$238.00 (2)
Canine (23+ kg)	$450.00 (2)	$550.00 (2)	$550.00 (2)	$550.00 (2)

Mean pricing for farm-raised swine, caprine and canine experimental animal models listed by local vendors supplying the North Texas region from 2006 – 2009. Number in parentheses = number of vendor prices averaged to obtain value.

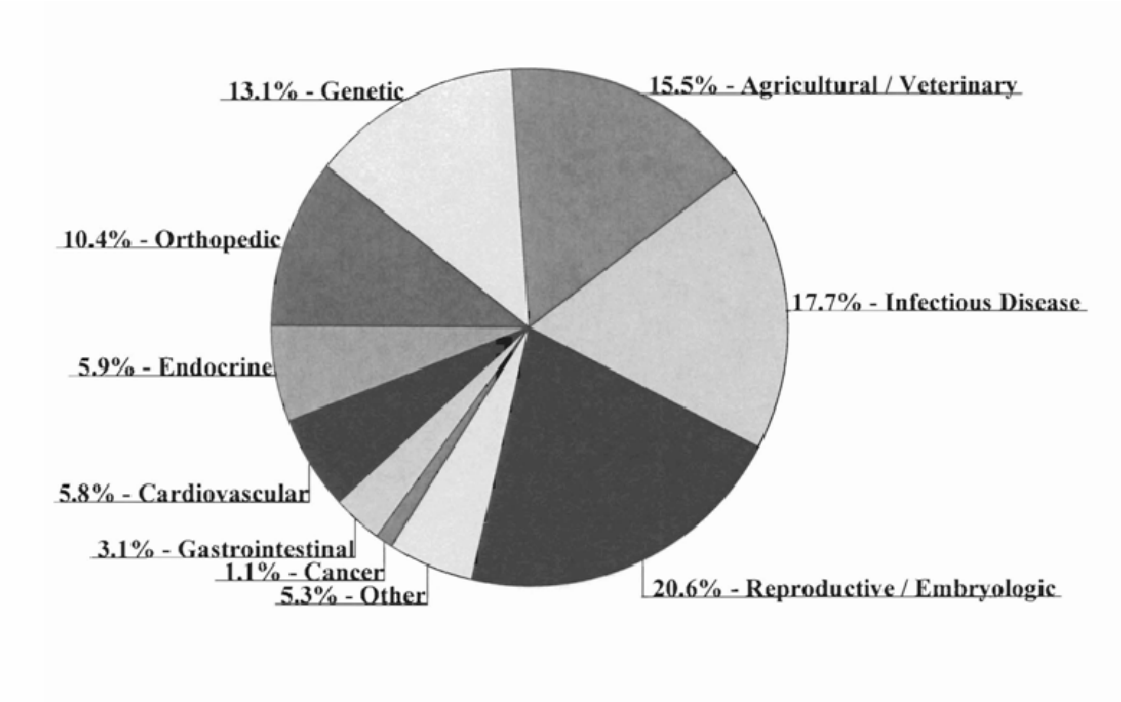

Figure 1. *Research involving the goat*. Review of all Pubmed titles containing the word "goat" published from January 1, 2000 to January 1, 2009. Percentages were computed from a total of 1,124 titles.

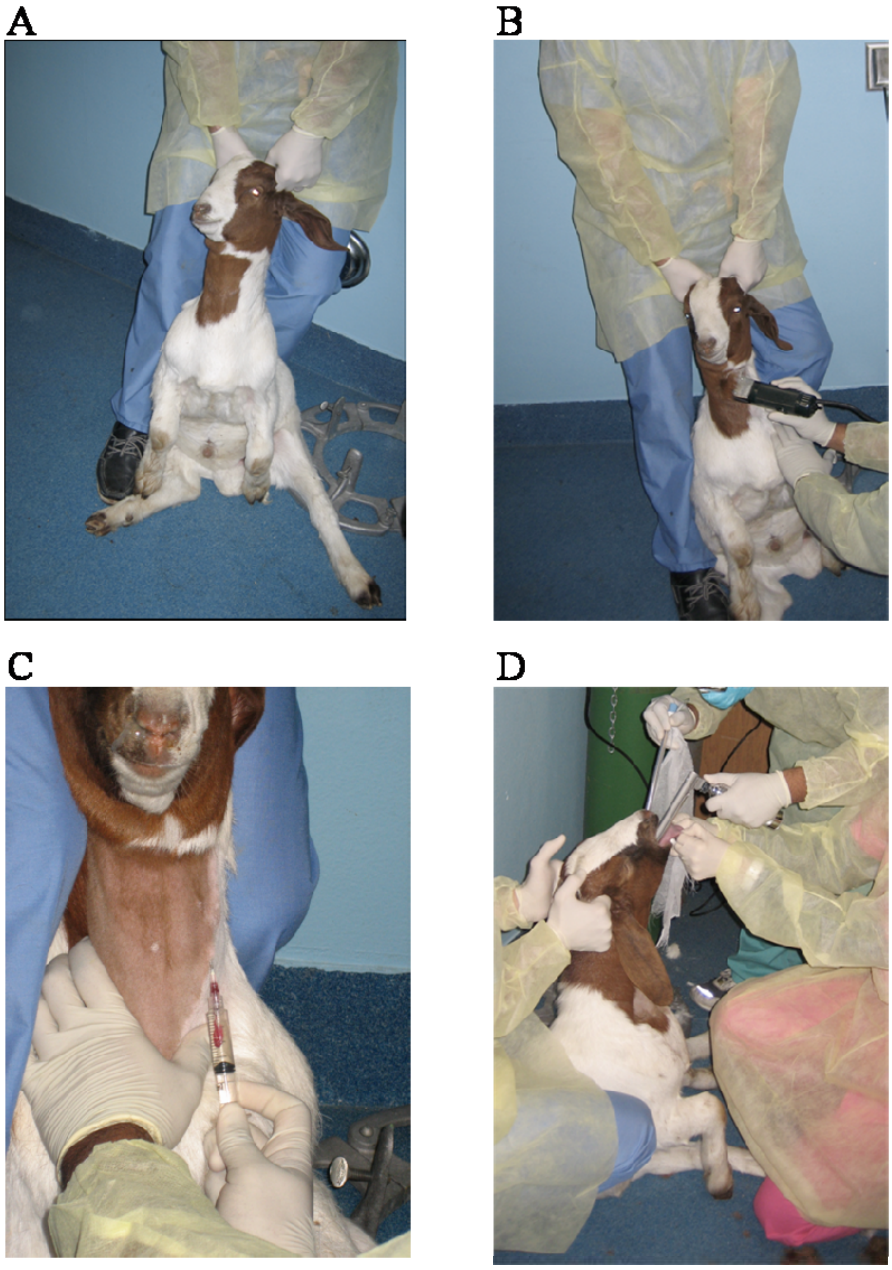

Figure 2. *Positioning, induction of anesthesia and intubation of the goat.* Panel A: The goat is positioned vertically on its haunches and straddled by laboratory personnel. Panel B: Clippers are used to shave the hair covering the neck vessels as well as the trachea. Panel C: The external jugular is pierced, aspirated and injected with the induction anesthetic. Panel D: The obtunded goat's head is extended and an endotracheal tube is placed after visualization of the vocal cords.

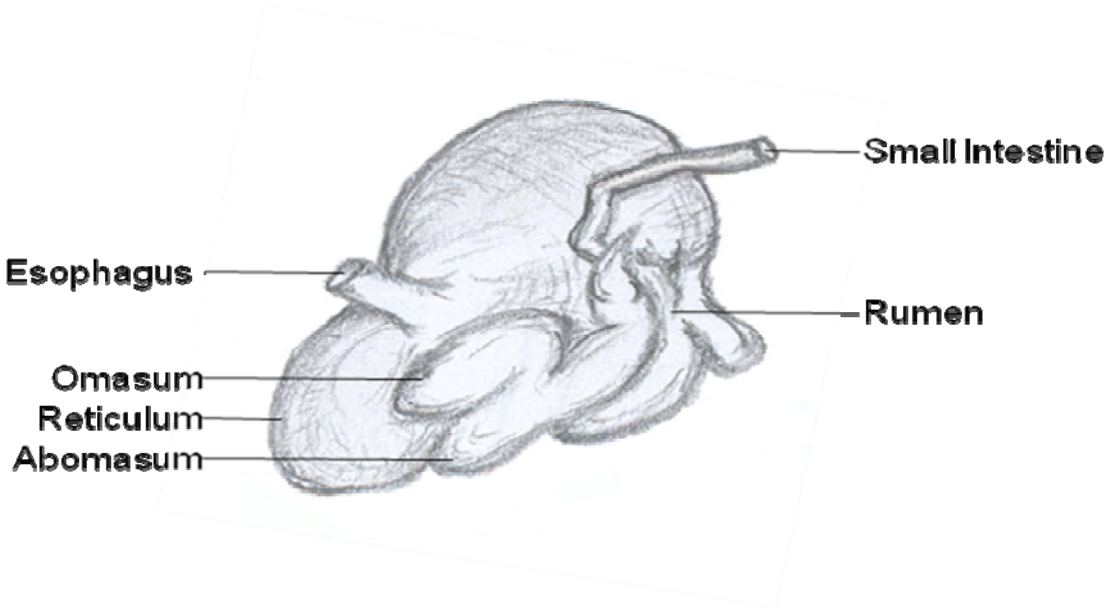

Figure 3. *Stomach anatomy of the goat.* Anatomic depiction of the stomach and its four divisions (original work by the author).

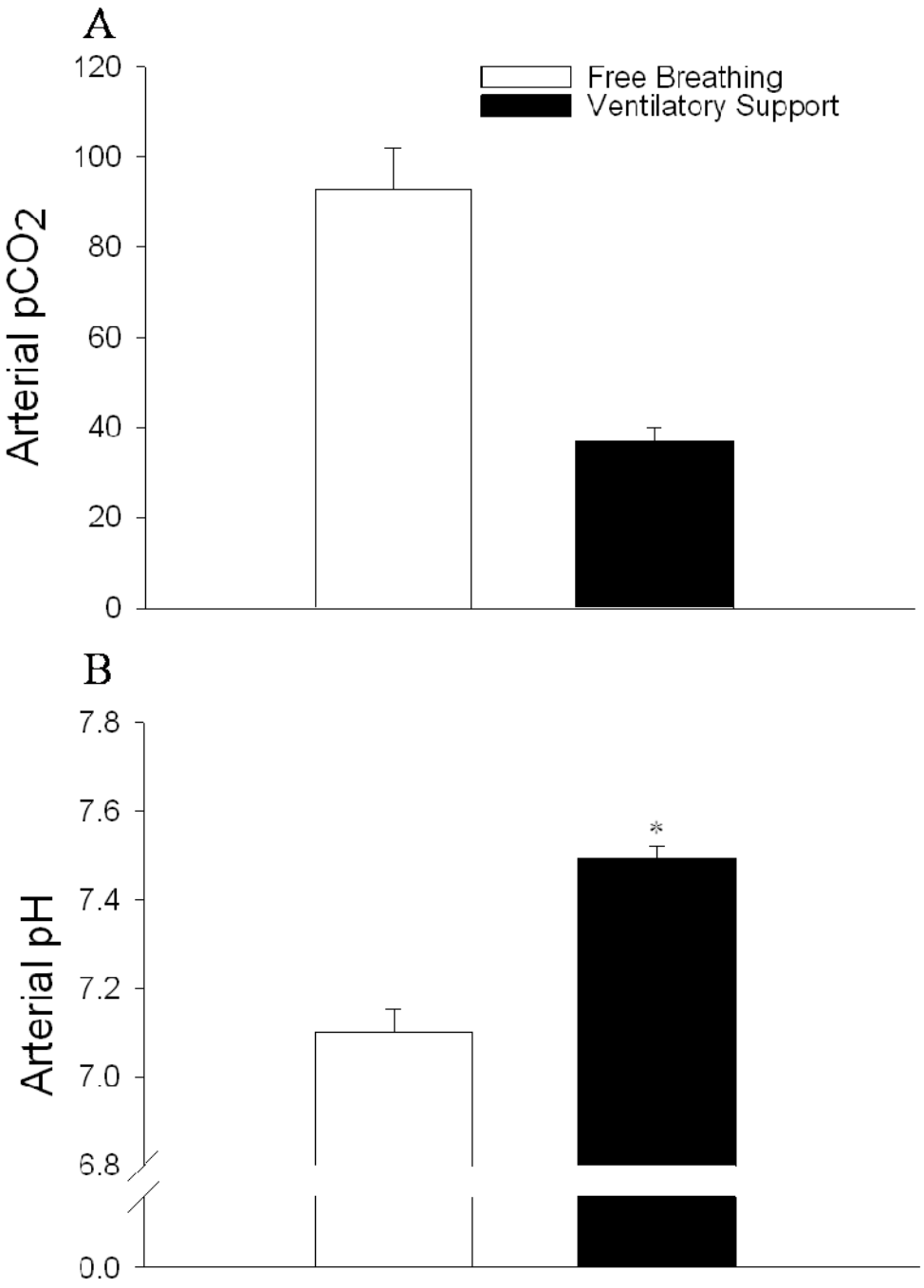

Figure 4. *Arterial pCO_2 (A) and pH (B) values in free-breathing vs. actively ventilated goats.* Whole blood arterial pCO_2 and pH values from 5 goats allowed to self-ventilate on room air were compared to the respective values in 16 goats actively ventilated with 100% O_2. Values are means ± SEM. * $p<0.05$ vs. free-breathing values.

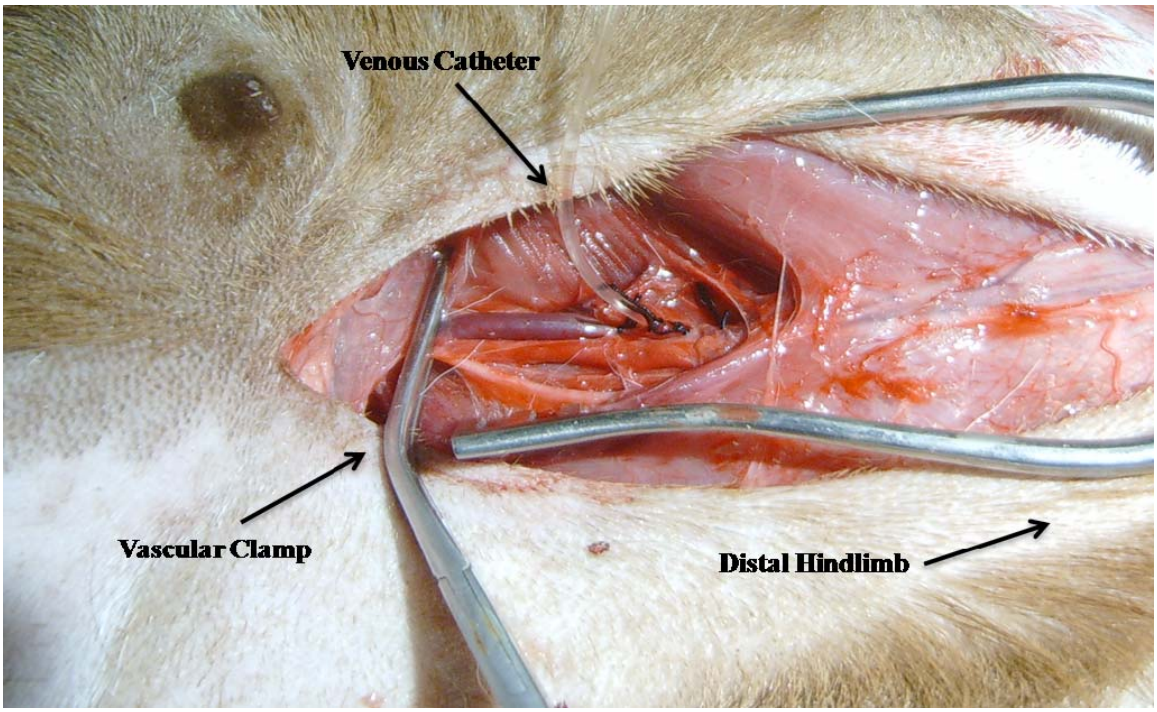

Figure 5. *Hindlimb anatomy of the goat.* Gross visualization of the hindlimb femoral vessels just distal to the inguinal ligament and exposed with a Weitlaner retractor. The femoral vein is catheterized and both vessels occluded with an atraumatic vascular clamp.

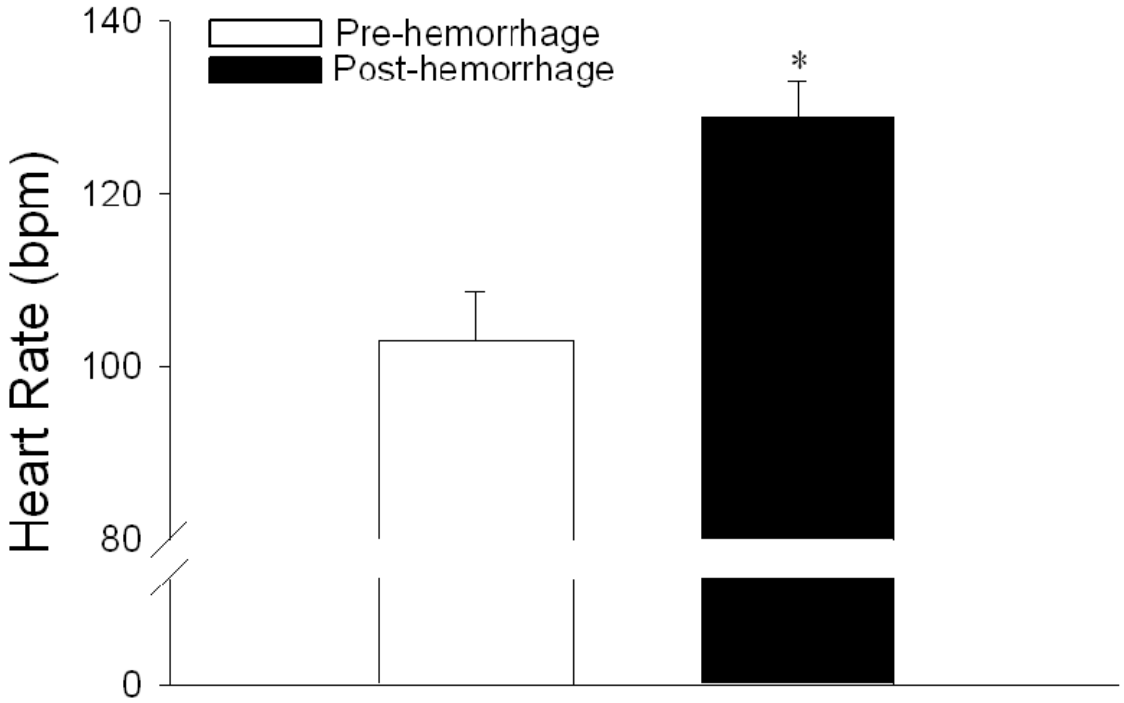

Figure 6. *Heart rate before and after hemorrhage.* Heart rates were measured in 21 experiments at pre-hemorrhage baseline and 30 min after a controlled hemorrhage to a mean arterial pressure of *c.* 50 mmHg. Values are means ± SEM. * $p<0.05$ vs. baseline heart rate.

CHAPTER V

CONCLUSIONS

The first purpose of this investigation was to determine the ability of pyruvate Ringer's infusion to bolster hemodynamics and combat oxidative and inflammatory changes in the setting of controlled hemorrhagic shock with resuscitation. We found that by fortifying conventional Ringer's solution with 28 mM pyruvate, resuscitative efforts stabilized post-shock arterial pressure and suppressed circulating inflammation and myocardial oxidative stress. Second, we sought to determine the effect pyruvate-fortified Ringer's resuscitation had on hindlimb muscle after vasoclamp-mediated ischemia-reperfusion. Pyruvate treatment attenuated hindlimb damage by providing an optimal cytosolic redox environment, thus effectively abrogating oxidative stress and the initiation stages of apoptosis. Importantly, no detrimental outcomes were associated with pyruvate administration. From these results, we conclude that:

1. Systemic hypotension and hindlimb ischemia-reperfusion with conventional LR treatment imposed pro-oxidative and pro-inflammatory stress both systemically and locally, thus preventing stabilization of MAP during recovery and initiating apoptotic mechanisms within the hindlimb musculature.

2. Pyruvate-fortified Ringer's effectively stabilizes hemodynamics and dampens systemic inflammation after hemorrhagic shock with resuscitation and hindlimb ischemia-reperfusion.

3. Pyruvate-fortified resuscitation blunted oxidative and inflammatory stress within the ischemic hindlimb and suppressed pro-apoptotic signaling.

4. These results suggest that pyruvate-fortified Ringer's resuscitation after hemorrhagic shock with hindlimb ischemia improves overall systemic stabilization and affords cytoprotection to tissue experiencing ischemia-reperfusion by suppressing oxidative and inflammatory stress.

CHAPTER VI

PROPOSAL OF FUTURE STUDIES

This investigation, for the first time, provides evidence concerning the beneficial effects of pyruvate-fortified Ringer's (PR) solution in the setting of hemorrhagic shock with resuscitation and hindlimb ischemia. The studies provide evidence of pyruvate's anti-oxidative and –inflammatory effects within previously ischemic skeletal muscle as well as within the circulation. Despite this compelling evidence, more studies should be undertaken to fully grasp the extent of protection PR may afford a patient experiencing hemorrhagic shock and tourniquet application. The following experiments are proposed to continue this area of investigation:

1. Determine the effect pyruvate-fortified Ringer's has on the progression of apoptosis in a setting of prolonged recovery after hemorrhagic shock with ischemia-reperfusion after tourniquet application-release.

2. Determine the effect pyruvate-fortified Ringer's has on vasculature subjected to ischemia-reperfusion *vs.* other conventional resuscitative vehicles.

3. Determine the extent to which PR dampens the systemic inflammatory response syndrome (SIRS) through investigations of circulating inflammatory mediators.

4. Determine the concentration of circulating pyruvate during resuscitative measures that confers maximal protection to end organs.

Hindlimb ischemia induces apoptosis

Sufficient cellular stress will disrupt mitochondrial membranes allowing cytochrome c release from the inner-mitochondrial space. With cytochrome c activation of the caspase cascade, apoptosis progresses toward ultimate nuclear degradation and cell death.[5,6] We demonstrated pyruvate's ability to dampen the initiation of apoptosis in skeletal muscle exposed to oxidative stress. How pyruvate affects the apoptotic mediators responsible for progression of cell death has yet to be determined in our current model. Sharma *et al.* demonstrated, in canine hippocampal tissue subjected to cardiac arrest with resuscitation, a blunting of caspase-3 cleavage with pyruvate infusion.[14] Apoptotic studies utilizing pyruvate administration have demonstrated diminished caspase-3 activation in hepatic,[11] lens epithelial,[14] endothelial[9] and neurologic tissue.[17,18] Apoptosis is a known time-dependent event. The timing of apoptotic events within ischemic skeletal muscle is variable and has yet to be completely substantiated.[4,7] Indeed, in our goat hindlimb muscle, caspase-3 cleavage was equivocal suggesting an inadequate amount of time had elapsed for progression of apoptosis.

Pyruvate's effect on K_{ATP} channels within the vasculature

Preserving systemic vasoconstriction after hemorrhagic shock with resuscitation affords the trauma patient compensatory mechanisms that serve to improve mean arterial pressures.[13] Mongan *et al.* reported pyruvate delays the onset of vascular failure in a pig model of hypovolemic shock.[12] This group hypothesized that pyruvate's reduction of metabolic acidosis

and stabilization of cytoplasmic phosphorylation potentials allows vascular smooth muscle sarcolemmal K_{ATP} channels to remain closed, thereby preventing a decrease in intracellular Ca^{++} and preserving contractile function.[1,12] Further studies have determined that the opening of K_{ATP} channels causes increased free radical production within vascular smooth muscle.[8] Thus, preventing K_{ATP} channel opening would preserve vascular contractility and potentiate pyruvate's already established antioxidative effects.[19] Finally, preservation of the vascular endothelium dampens downstream ischemia-reperfusion injury in skeletal muscle, identifying another way pyruvate may confer protection through its effects on the vasculature.[20]

Hypovolemic shock invokes SIRS during and after infusion of resuscitative crystalloids

Systemic inflammatory response syndrome is a known sequelae of hemorrhagic shock.[13] Resuscitation with lactate Ringer's has demonstrated increased neutrophil activation in animal models that have been subjected to hemorrhage as well as shams that were not hemorrhaged.[15] Increased circulating neutrophils were found in the plasma of the LR resuscitated group in our study. These studies highlight the need for a resuscitative vehicle that dampens SIRS after hemorrhagic shock.[13]

Sodium pyruvate in our hemorrhagic shock study attenuated systemic increases in circulating neutrophils. Hemorrhagic shock studies utilizing ethyl pyruvate have demonstrated a diminished expression of IL-6 mRNA after resuscitation.[21] A study by Cai *et al.* provides further evidence of ethyl pyruvate's anti-inflammatory effects by demonstrating diminished circulating TNF-α levels and improved survival in a rodent model of awake hemorrhage.[3] The mechanisms through which sodium pyruvate blunts the systemic inflammatory response have yet to be elucidated.

Optimal circulating pyruvate concentration

The optimal concentration of circulating pyruvate in the hemorrhagic shock setting has yet to be determined. Bünger *et al.*, working with various pyruvate concentrations within guinea-pig myocardium, demonstrated maximal ventricular work output and myocardial phosphorylation potential at a pyruvate concentration of 5 mM.[2] Further, in a model of hemorrhagic shock, Mongan *et al.* demonstrated a significant and sustained stabilization of hemodynamics with a pyruvate infusion of greater than 5 mM.[12] Increasing the circulating glutathione (GSH) / glutathione disulfide (GSSG) ratio would provide further evidence of pyruvate robust antioxidative effect during recovery form hemorrhagic shock.[10,13] The current study witnessed a promising upward trend in the GSH/GSSG ratio during resuscitation. Optimized circulating pyruvate concentrations are needed to fully appreciate this effect as well as others.

Based on these principles, we hypothesize that pyruvate-fortified Ringer's solution does diminish the systemic inflammatory response associated with hemorrhagic shock, while significantly dampening the progression stages of apoptosis in end-organs. That stated, the following specific aims have been identified for future investigation:

1. Determine the effect pyruvate Ringer's has on tissue caspases in a model of hemorrhagic shock with resuscitation and hindlimb ischemia after prolonged recovery.

2. Determine the effect pyruvate Ringer's has on K_{ATP} channels within vessels exposed to ischemia-reperfusion.

3. Determine the optimal concentration of circulating pyruvate that maximizes its salutary effects within the circulation and end-organs previously exposed to hemorrhagic shock

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