

May 2013

Mechanism of Ascorbate Protection Against Sepsis-Induced Capillary Blood Flow Impairment

Dan Secor

The University of Western Ontario

Supervisor

Dr. Karel Tymł

The University of Western Ontario

Graduate Program in Pharmacology and Toxicology

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

© Dan Secor 2013

Follow this and additional works at: <https://ir.lib.uwo.ca/etd>

 Part of the [Pharmacology Commons](#)

Recommended Citation

Secor, Dan, "Mechanism of Ascorbate Protection Against Sepsis-Induced Capillary Blood Flow Impairment" (2013). *Electronic Thesis and Dissertation Repository*. 1270.
<https://ir.lib.uwo.ca/etd/1270>

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact tadam@uwo.ca, wlsadmin@uwo.ca.

**MECHANISM OF ASCORBATE PROTECTION AGAINST SEPSIS-INDUCED
CAPILLARY BLOOD FLOW IMPAIRMENT**

(Thesis format: Integrated Article)

by

Dan Secor

Graduate Program in Pharmacology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

© Dan Secor 2013

Abstract

Sepsis is a systemic inflammatory response to an infection. The overwhelming inflammation has many deleterious effects, including cessation of capillary blood flow. This cessation may lead to organ failure and subsequent death, but the cause of cessation during sepsis is not fully understood. Ascorbate (reduced vitamin C) has been shown to restore capillary blood flow by an unknown mechanism. I hypothesized that activation of both platelets and the coagulation pathway in sepsis contributes to the cessation of capillary blood flow and that ascorbate protects against cessation by reducing platelet activation.

Using intravital microscopy in the mouse hindlimb skeletal muscle *in vivo*, I observed that sepsis impairs capillary blood flow and increases both adhesion of platelets/platelet aggregates to the capillary wall and deposition of fibrin plaques in the same capillaries. Platelet depletion, blocking of P-selectin (a key adhesion molecule), antithrombin, and eptifibatide (anti-aggregatory agent) all reduced the capillary blood flow impairment and platelet adhesion. Intravenous bolus injection of ascorbate reduced platelet adhesion in capillaries, via the endothelial nitric oxide synthase (eNOS) system.

To study any direct effects of ascorbate on platelet function, I used an *ex vivo* model (isolated mouse platelets) examining platelet aggregation under septic conditions. Here, thrombin, ADP, and thromboxane (agents released into the blood during sepsis), but not lipopolysaccharide (LPS), tumor necrosis factor (TNF α) or septic plasma, increased platelet aggregation and surface P-selectin protein expression. Ascorbate inhibited the increased aggregation and P-selectin expression.

Next, an *in vitro* mouse microvascular endothelial cells model was used to study the effect of ascorbate on platelet-endothelial cell adhesion. LPS and TNF α increased platelet adhesion and P-selectin mRNA expression in endothelial cells. LPS also increased P-selectin-containing endothelial granule secretion. Ascorbate prevented the increased adhesion and granule secretion but did not affect mRNA expression.

Thus, I conclude that impairment of blood flow in the septic microvasculature requires platelets and is reduced by anti-coagulant/anti-aggregatory agents. Ascorbate prevents platelet-endothelial adhesion and platelet aggregation, partly through reducing

P-selectin protein expression at the platelet/endothelial cell surface. Thus ascorbate reduces adhering platelets in septic capillaries leading to restoration of blood flow.

Keywords: Ascorbate, sepsis, capillary blood flow, platelet aggregation/adhesion, P-selectin

Co-Authorship

In the investigation entitled:

Impaired microvascular perfusion in sepsis requires activated coagulation and P-selectin mediated platelet adhesion in capillaries (Chapter 2),

Ms. F. Li assisted in preparation of septic mice including intravenous injections, video recordings, and analysis of blood flow stoppage in capillaries. Drs. M. Sharpe, C. Ellis, P. Gross, and J. Wilson participated in manuscript writing.

Ascorbate reduces mouse platelet aggregation and surface P-selectin expression in an ex vivo model of sepsis (Chapter 3),

Drs. M. Sharpe, C. Ellis, and Mr. S. Swarbreck participated in manuscript writing.

Ascorbate reduces P-selectin mediated platelet-endothelial adhesion in an in vitro model of sepsis via reduced endothelial granule secretion (Chapter 4),

Mr. S. Swarbreck assisted in the preparation of septic mice and mRNA collection and analysis. Ms. S. Seghal measured, in blinded fashion, the platelet-endothelial adhesion to verify my measurements of this adhesion.

In all of the above investigations, all work not specified above was performed by myself. All manuscripts were written principally by myself. Dr. K. Tymbal assisted in the experimental design, helpful discussions, and preparation of the final manuscripts and helped in the preparation of this thesis.

In this thesis (Chapters 2-4), I use “we” to retain the original version of the published manuscript. The usage of “we” reflects the co-authorships of the paper.

Acknowledgments

The research contained in this thesis has been supported by a grant from the Heart and Stroke Foundation on Ontario (NA 5941, awarded to K. Tyml) and by salary awards of D. Secor (Ontario Graduate Scholarship in Science and Technology 2010 – 2011, Queen Elizabeth II Scholarship in Science and Technology 2011-2012)

First and foremost, I would like to thank my supervisor, Dr. Karel Tyml, for his support and guidance throughout my completion of this thesis. I have learned a great deal from him in terms of experimental design, research skills, writing and revising manuscripts, and analyzing of data. He has allowed me to investigate my own ideas and use my own approach to science. For this, he has my gratitude.

I am grateful for my advisory committee members, Drs. Doug Jones, Michael Sharpe, Christopher Ellis, Ravi Taneja, and Gediminas Cepinskas, for their guidance and advice during my PhD program and for their review of this thesis.

I would like to thank Ms. Fuyan Li for all her technical help and her valuable teachings. I thank Mr. Scott Swarbreck, a fellow PhD student in Dr. Tyml's lab, for his technical assistance and support over the last five years as well as for his friendship. I also thank Dr. David Freeman for the use of his platelet aggregometer.

Finally, I would like to thank my wife, Christina, for all her support during my studies and my daughter, Brooklyn, for providing inspiration.

Table of Contents

<i>Abstract</i>	ii
<i>Co-authorship</i>	iv
<i>Acknowledgements</i>	v
<i>Table of Contents</i>	vi
<i>List of Tables</i>	xi
<i>List of Figures</i>	xii
<i>List of Appendices</i>	xiv
<i>List of Abbreviations</i>	xv
Chapter 1: Introduction	1
1.1 Sepsis	1
1.1.1 Clinical significance	1
1.1.2 Pathophysiology of sepsis	2
1.1.3 Capillary blood flow impairment	3
1.1.4 ROS and sepsis	6
1.1.4.1 Reactive oxygen species in sepsis	7
1.1.5 NO and sepsis	7
1.1.5.1 Nitric oxide effects during sepsis	8
1.2 Coagulation	9
1.2.1 Overview	9
1.2.2 Platelets	11
1.2.3 Fibrin	12
1.2.4 Endothelial cells	13
1.2.5 Platelet adhesion, aggregation, and thrombi formation	13
1.3 Ascorbate	15
1.3.1 Ascorbate treatment	15
1.3.2 Mechanism of protection by ascorbate	16

1.4	Rationale, Hypothesis, and Objectives _____	20
1.4.1	Rationale _____	20
1.4.2	Hypotheses _____	21
1.4.3	Objectives _____	21
1.5	References _____	22
Chapter 2:	<i>Impaired microvascular perfusion in sepsis requires activated coagulation and P-selectin mediated platelet adhesion in capillaries</i> ____	41
2.1	<i>Introduction</i> _____	42
2.2	<i>Methods</i> _____	43
2.2.1	Animal preparation _____	43
2.2.2	Intravital microscopy _____	43
2.2.3	Biochemical and blood platelet count analyses _____	45
2.2.4	Experimental design: (a) Role of platelet and coagulation functions in blood flow stoppage _____	45
2.2.5	Experimental design: (b) Roles ROS and NO in platelet adhesion in the septic capillary bed _____	46
2.2.6	Statistics _____	47
2.3	<i>Results</i> _____	47
2.3.1	Role of platelet and coagulation functions in blood flow stoppage _____	47
2.3.2	Role of ROS and NO _____	52
2.4	<i>Discussion</i> _____	56
2.4.1	Role of platelet and coagulation functions in blood flow stoppage _____	56
2.4.2	Role of ROS and NO in septic capillaries _____	57
2.4.3	Methodological limitations _____	60
2.5	<i>References</i> _____	61

Chapter 3:	<i>Ascorbate reduces mouse platelet aggregation and surface P-selectin expression in an ex vivo model of sepsis</i>	66
3.1	<i>Introduction</i>	67
3.2	<i>Methods</i>	69
3.2.1	Reagents used	69
3.2.2	Experimental design	69
3.2.3	Platelet collection and isolation, and plasma collection	70
3.2.4	Platelet aggregation	70
3.2.5	Flow cytometry	71
3.2.6	Statistical analysis	72
3.3	<i>Results</i>	74
3.3.1	LPS and TNF α do not initiate platelet aggregation nor increase P-selectin surface expression	74
3.3.2	Ascorbate reduces platelet aggregation to thrombin, ADP, and U46619	77
3.3.3	Ascorbate reduces the sensitivity of P-selectin surface expression to thrombin	77
3.3.4	Ascorbate reduces thrombin-induced platelet aggregation independent of NOS	78
3.4	<i>Discussion</i>	85
3.5	<i>References</i>	90
Chapter 4:	<i>Ascorbate reduces P-selectin mediated platelet-endothelial adhesion in an in vitro model of sepsis via reduced endothelial granule secretion</i>	100
4.1	<i>Introduction</i>	101
4.2	<i>Methods</i>	103

4.2.1	Reagents used _____	103
4.2.2	Endothelial cell harvest and culture _____	103
4.2.3	Platelet collection and isolation _____	104
4.2.4	<i>In vitro</i> adhesion model to mimic the platelet-endothelial cell interaction _____	104
4.2.5	Septic mouse model _____	105
4.2.6	P-selectin and PSGL1 mRNA expression in cultured endothelial cells and skeletal muscle homogenates _____	105
4.2.7	Von Willebrand factor (vWF) ELISA _____	106
4.2.8	Western blots _____	107
4.2.9	Statistical analysis _____	107
4.3	<i>Results</i> _____	108
4.3.1	Ascorbate prevents increased platelet-endothelial cell adhesion in an <i>in vitro</i> model of sepsis _____	108
4.3.2	LPS and TNF α increase P-selectin mRNA expression but not PSGL1 mRNA _____	108
4.3.3	Ascorbate reduces vWF release in LPS treated endothelial cells _____	113
4.3.4	Sepsis increases expression of P-selectin mRNA but not protein in muscle homogenates _____	113
4.4	<i>Discussion</i> _____	117
4.4.1	Methodological limitations _____	119
4.5	<i>References</i> _____	122
Chapter 5: Summary, Discussion, and Future Directions _____		129
5.1	<i>Thesis Summary</i> _____	129
5.2	<i>General Discussion</i> _____	134
5.2.1	Comparing the <i>in vivo</i> , <i>ex vivo</i> , and <i>in vitro</i> models of sepsis _____	134

5.2.1.1	Mouse models of sepsis _____	134
5.2.1.2	Methodological limitations to the models _____	136
5.2.2	Mechanism of capillary blood flow impairment during sepsis _____	138
5.2.3	Capillary plugging in septic skeletal muscle _____	140
5.2.3.1	Microthrombi formation _____	141
5.2.3.2	RBC adhesion _____	143
5.2.4	Ascorbate and capillary blood flow impairment during sepsis _____	143
5.2.5	The protective effect of ascorbate and eNOS _____	145
5.2.6	Ascorbate as a treatment for sepsis _____	146
5.2.7	Overall implications of this research _____	148
5.3	<i>Future Directions</i> _____	149
5.3.1	Effect of eNOS inhibition on platelet adhesion, <i>in vitro</i> _____	149
5.3.2	Mechanism of ascorbate inhibition of TNF α -induced platelet adhesion, <i>in vitro</i> _____	150
5.4	<i>References</i> _____	152
	<i>Appendices</i> _____	163
	<i>Vita</i> _____	167

List of Tables

Table 1.1.	Possible underlying processes/events involved in the sepsis-induced capillary blood flow impairment, and possible protective effect of ascorbate treatment (intravenous bolus injection) _____	18
------------	--	----

List of Figures

Figure 1.1	A simplified scheme representing the mechanism of LPS-induced sepsis _____	4
Figure 1.2	Mechanism of restored nitric oxide (NO) production by ascorbate during sepsis _____	19
Figure 2.1	Sepsis increases blood flow stoppage, platelet adhesion and fibrin deposition in capillaries in mouse skeletal muscle _____	48
Figure 2.2	Effect of P-selectin blocking antibody, eptifibatide, and antithrombin on platelet adhesion and blood flow stoppage _____	50
Figure 2.3	Sepsis increases thrombosis in capillaries _____	51
Figure 2.4	Effect of ascorbate and gp91phox knockout on platelet adhesion and blood flow stoppage _____	53
Figure 2.5	Effect of ascorbate on platelet adhesion and blood flow stoppage in wild type and eNOS knockout mice _____	54
Figure 2.6	Effects of tetrahydrobiopterin (BH ₄) and NO donor SNAP on platelet adhesion and blood flow stoppage in capillaries of wild type and eNOS ^{-/-} mice (top), and effect of iNOS knockout on adhesion/stoppage (bottom) _____	55
Figure 2.7	Platelet adhesion in septic capillaries predicts capillary blood flow impairment _____	58
Figure 3.1	Dose response to thrombin on mouse platelet aggregation <i>ex vivo</i> _____	73
Figure 3.2	LPS and TNF α have no effect on mouse platelet aggregation <i>ex vivo</i> _____	75
Figure 3.3	Effect of LPS, TNF α , and thrombin on P-selectin surface expression on isolated platelets _____	76
Figure 3.4	Ascorbate reduces thrombin-, ADP-, and U46619-induced platelet aggregation _____	79
Figure 3.5	Dose response to thrombin and to thrombin plus ascorbate on P-selectin surface expression on isolated platelets _____	81
Figure 3.6	Effect of ascorbate on thrombin-induced platelet aggregation is independent of platelet NOS _____	82

Figure 3.7	Effect of ascorbate on thrombin-induced P-selectin surface expression on isolated platelets is independent of platelet NOS _____	84
Figure 4.1	Effect of P-selectin block on LPS or TNF α treated endothelial cell-platelet adhesion _____	109
Figure 4.2	Effect of ascorbate on LPS or TNF α treated endothelial cell-platelet adhesion _____	110
Figure 4.3	Effect of ascorbate on P-selectin mRNA expression in LPS or TNF α treated endothelial cells _____	111
Figure 4.4	Effect of ascorbate on P-selectin glycoprotein ligand-1 (PSGL1) mRNA expression in LPS or TNF α treated endothelial cells _____	112
Figure 4.5	Effect of ascorbate on endothelial cell granule secretion _____	114
Figure 4.6	Effect of ascorbate on P-selectin mRNA expression in septic mice _____	115
Figure 4.7	Effect of ascorbate on P-selectin protein expression in septic mice _____	116
Figure 4.8	P-selectin protein expression in the endothelial cell model _____	121
Figure 5.1	The effect of ascorbate on platelet-endothelial function in septic capillaries _____	133
Figure 5.2	Image of fibrin plaques in septic capillaries _____	142

List of Appendices

Appendix 1	Copyright approval for previously published work _____	164
Appendix 2	Ethics approval for animal use _____	166

List of Abbreviations

5HT	5-hydroxytryptamine (serotonin)
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BH ₂	Dihydrobiopterin
BH ₄	Tetrahydrobiopterin
cGMP	Cyclic guanosine monophosphate
CLP	Cecal ligation and perforation
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
DNAME	N-Nitro-D-arginine methyl ester
EC	Endothelial cell
EDL	Extensor digitorum longus
eNOS	Endothelial nitric oxide synthase
FeCl ₃	Ferric chloride
FIP	Feces injection into peritoneum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPIIb/IIIa	Glycoprotein IIb/IIIa
HRP	Horse radish peroxidase
ICU	Intensive care unit
IFN γ	Interferon gamma
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase

LDL	Low-density lipoprotein
LNAME	N-Nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
M ϕ	Macrophages
MOF	Multiple organ failure
mRNA	Messenger Ribonucleic acid
NADPH	β -Nicotinamide adenine dinucleotide phosphate
NF κ B	Nuclear factor kappa B
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NSF	N-ethylmaleimide sensitive factor
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered solution
PE	Phycoerythrin
PECAM	Platelet endothelial cell adhesion molecule-1
PKC	Protein kinase C
PMN	Polymorphonuclear neutrophils
PS	Phosphatidylserine
PSGL1	P-selectin glycoprotein receptor-1
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cell
ROS	Reactive oxygen species
SNAP	S-nitroso-N-acetylpenicillamine
SVCT2	Sodium-dependent vitamin C transporter 2

TNF α	Tumor necrosis factor alpha
TF	Tissue factor
tPA	Tissue plasminogen activator
TRITC	Tetramethylrhodamine isothiocyanate
TXA	Thromboxane A ₂
uPA	Urokinase plasminogen activator
US	United States of America
vWF	von Willebrand factor
WBC	White blood cell

Chapter 1

INTRODUCTION

This thesis focuses on the capillary blood flow impairment during sepsis, the coagulation system, a potential key component of this impairment, and the protective effect of ascorbate (a reduced form of vitamin C) against this impairment. The introduction will provide background information on: i) sepsis, including factors that contribute to the capillary blood flow impairment, ii) coagulation, including formation of micro-thrombi, and iii) ascorbate and its possible use as a treatment for sepsis.

1.1 SEPSIS

1.1.1 Clinical significance

Sepsis is the tenth leading cause of mortality in the United States of America (57) and is the leading cause of mortality in non-coronary intensive care units in North America (101), with a mortality rate near 40% (75). Despite extensive research, the mortality rate for sepsis increased between 1995 and 2005 (81). In low-income countries, the mortality rate is likely higher as treatment is dependent on available intensive care unit services. In some countries, there has been no improvement in treating sepsis in the last 10 years (11, 69).

Sepsis is a systemic inflammatory response syndrome due to an infection (15, 27).

Patients are considered septic when they have 2 or more of the following features: core

temperature $\geq 38^{\circ}\text{C}$ or $\leq 36^{\circ}\text{C}$, heart rate ≥ 90 beats/min, respiratory rate > 20 breaths/min or partial pressure of carbon dioxide < 32 mmHg or mechanical ventilation, and white blood cell (WBC) counts $> 12,000/\text{mm}^3$ or $< 4,000/\text{mm}^3$. 'Severe sepsis' is when there is dysfunction in 2 or more organs and 'septic shock' is when hypotension is present (15).

Current treatment focuses on treating the infection and the features (i.e., maintaining blood pressure), while treatments for the inflammatory response are ineffective or only have marginal benefits (27). Despite extensive research and numerous clinical trials, only one pharmacological treatment reached clinical approval for treatment of sepsis, activated protein C, and has since been withdrawn from the market for lack of efficacy (74, 94). The lack of progress in determining effective treatments represents our limited understanding of the mechanisms involved in the pathophysiology of sepsis.

1.1.2 Pathophysiology of sepsis

On the cellular level, the inflammatory response in sepsis is a result of overwhelming production of cytokines by the host to defend against an invading pathogen. In the case of a gram-negative bacterial infection, sepsis is initiated by the endotoxin lipopolysacchride (LPS), a component of gram negative bacteria. LPS results in the release of cytokines such as tumour necrosis factor α (TNF α), interleukin-6 (IL-6), and interferon γ (IFN γ) (2, 97) from macrophages and lymphocytes. This process is mediated through toll-like receptor 4 (90) and proteins of the complement system (125). These cytokines, along with LPS, further stimulate macrophages, neutrophils, platelets, and endothelial cells to express adhesion proteins and chemokines as well as produce reactive oxygen species (ROS) and nitric oxide (NO) (90). The end result of these processes includes many

serious physiological alterations including a multitude of circulatory dysfunctions such as hypotension, decreased systemic vascular resistance, impaired oxygen utilization, maldistribution of blood flow in the microcirculation, impaired capillary blood flow, and decreased cellular communication along the vascular wall (15, 27, 60, 109, 110, 112). These dysfunctions eventually progress towards tissue injury and multiple organ failure (49) (Figure 1.1).

1.1.3 Capillary blood flow impairment

As mentioned, one consequence of systemic inflammation is an impairment of capillary blood flow. This impairment has been observed and defined as a decrease in perfused capillaries and an increase in non-perfused capillaries in rat and mouse skeletal muscle (3, 60, 107, 108). In other words, there is an increase in the percentage of stopped-flow capillaries during sepsis. A similar impairment of capillary blood flow is evident in other organs and in septic patients (10, 14). The microvascular dysfunction leads to poor oxygen transport and tissue hypoxia due to increased oxygen diffusion distances from red blood cells in capillaries to the mitochondria of surrounding cells (30, 40). Tissue hypoxia due to this impairment may account for one third of patients who die from organ failure even when shock is prevented (98, 99). The underlining mechanism of the capillary blood flow impairment is not yet known.

The impairment of flow is heterogeneous in nature meaning that capillaries in a capillary unit supplied by a common arteriole and drained by a common venule include both perfused and stopped-flow capillaries (107). This indicates that the impairment is

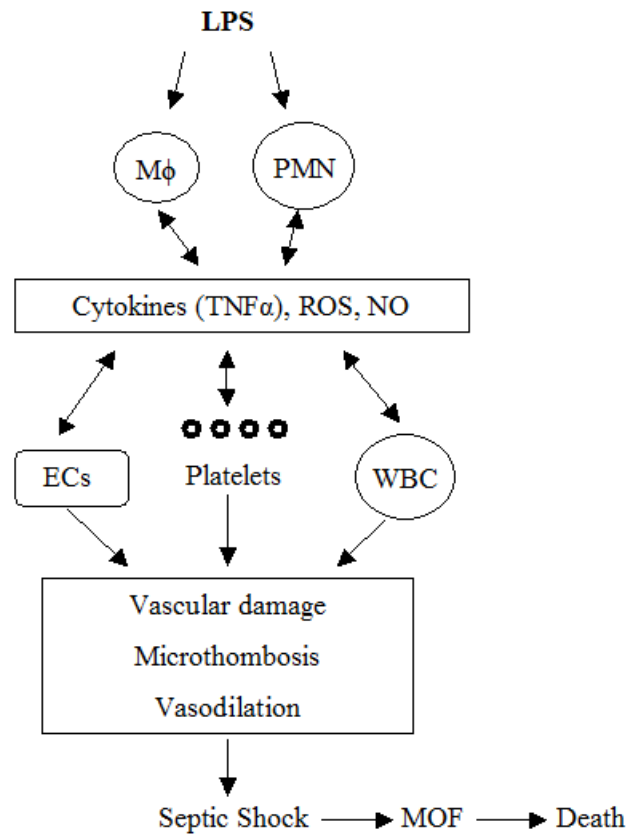


Figure 1.1. A simplified scheme representing the mechanism of LPS-induced sepsis.

LPS from gram negative bacteria enters circulation and stimulates macrophages (M ϕ) and neutrophils (PMN) to release cytokines such as TNF α . These cytokines (along with LPS) activate endothelial cells (ECs), platelets, and white blood cells (WBC) to further produce cytokines as well as ROS and NO. These mediators, together with the ECs, platelets, and WBC, cause vascular damage, microthrombosis, and vasodilation potentially leading to septic shock, multiple organ failure (MOF), and/or death.

not due to dysfunction of larger blood vessels. Since the microcirculation in skeletal muscle does not contain precapillary sphincters (31) (i.e., smooth muscle cells encircling the entrance segment of the capillary) there is no obvious physiological process (e.g., constriction and relaxation of sphincters) that could explain this heterogeneous capillary blood flow. Thus, the stoppage of flow in capillaries seen during sepsis is most likely an outcome of pathological processes initiated by sepsis.

There are various microvascular dysfunctions in sepsis that could contribute to the capillary blood flow impairment (Table 1.1, columns 1 and 2, on page 18), including the plugging of the capillary lumen by adhering leukocytes, stiffened erythrocytes and/or micro-thrombi containing platelets and fibrin plaques. Leukocytes are known to have increased adhesion to the endothelium during sepsis (25, 79) and could possibly plug capillaries. However, leukocytes are located primarily in the lung and liver during sepsis (80, 82). Accordingly, it was concluded that leukocytes are not responsible for the impairment in septic rat skeletal muscle (91). Based on this report we predicted that, in our mouse skeletal muscle model of sepsis, leukocytes may have a minor/negligible role in the impairment. Erythrocytes have been shown to have decreased deformity during sepsis (9), and therefore they may contribute to capillary plugging. Platelets become activated during sepsis, leading to their increased aggregation and adhesion to the capillary wall (66). Combined with increase fibrin formation (detailed in section 1.2.2), this would progress to the formation of microthrombi that could block the flow in the capillary. Since little is known about the mechanism of capillary plugging, this thesis will focus on investigating the role of platelets and microthrombi in the capillary blood flow impairment

Clinically, a lowered platelet count in systemic blood correlates with increased severity of sepsis (77). One possibility is that platelets are 'trapped' in the capillaries due to the formation of microthrombi. It was observed that the blood flow in a single capillary has intermittent stoppage periods until it ultimately becomes permanently stopped. This could be explained by the process of ongoing formation and breakdown (thrombolysis) of thrombi in capillaries during the initial stage of sepsis. The progressing severity of sepsis eventually tilts the equilibrium towards microthrombi formation leading to the plugging of capillaries (35).

1.1.4 ROS and sepsis

ROS are highly reactive molecules due to an unpaired electron in their outer orbit. The main biological radicals include superoxide, hydroxyl and peroxynitrite radicals, and hydrogen peroxide (non-radical) (41). Superoxide and hydrogen peroxide react with other molecules until they are eventually converted to water by superoxide, catalase, and the glutathione peroxidase enzyme system (28). However, when NO levels are high, superoxide reacts with the NO to form peroxynitrite (96). Peroxynitrite is known to have toxic effects on cells by interacting with lipids, protein, and DNA (41).

The major vascular source of ROS is NADPH oxidase found in neutrophils, macrophages, and endothelial cells (43). The superoxide producing NADPH oxidase is composed of 6 subunits (p22phox, gp91phox, p47phox, p67phox, rac, and p40phox) and translocates to the plasma membrane upon stimulation to form a catalytically active oxidase (54). LPS and inflammatory cytokines are known to stimulate NADPH oxidase, and thus NADPH oxidase has an important role in the pathophysiology of sepsis (67, 83).

1.1.4.1 ROS in sepsis

Septic patients often show signs of oxidative stress and decreased levels of the antioxidant ascorbate, which correlate with lower levels of survival (22, 38). During sepsis, ROS has been shown to cause endothelial damage and dysfunction (6), increase the expression of adhesion proteins (124), enhance platelet adhesion and activation, and promote coagulation (21, 47, 87). Sepsis was shown to increase ROS production in skeletal muscle in part by NADPH oxidase (123). In particular, LPS+INF γ treatment has been shown to increase superoxide production in endothelial cells (120). It is important to note that increased ROS in the septic capillary will cause the conversion of tetrahydrobiopterin (BH₄) to dihydrobiopterin (BH₂) (59), limiting the available BH₄ needed for proper vascular homeostasis (see section 1.1.5.1).

ROS has been implicated as a potential cause for the sepsis-induced capillary blood flow impairment. Removal of a major source of ROS by gp91phox (a subunit of NADPH oxidase) knockout, reduced sepsis-induced capillary blood flow impairment (107). Furthermore, treatment of septic rodents with the anti-oxidant ascorbate prevented and reversed the capillary blood flow impairment in septic models (3, 107, 108).

1.1.5 NO and sepsis

Although NO is primarily known as a vasodilator, it is a very important signalling molecule for vascular homeostasis with effects including inhibition of platelet adhesion and aggregation (44). Endogenous NO is produced from 3 distinct nitric oxide synthases

(NOS): neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3).

NO is synthesized by these enzymes in a 2 step oxidation reaction resulting in the conversion of L-arginine to L-citrulline. This reaction requires oxygen and NADPH as co-substrates, the calcium-dependent enzyme calmodulin and the co-factors flavin adenine dinucleotide, flavin mononucleotide, heme, and BH₄. During this reaction, electrons are passed from the NADPH to the heme in the oxygenase domain with assistance from the co-factors. This flow of electrons needs to be tightly regulated and if interrupted it will lead to the production and release of superoxide. This is referred to as the 'uncoupled' state of NOS. One such disruption to the flow is when the availability of BH₄ becomes limited (33).

1.1.5.1 NO effects during sepsis

Sepsis is associated with the excessive production of NO as plasma levels of nitrite and nitrate are elevated (5) in septic patients. This elevation occurs due to increased expression of iNOS by LPS and inflammatory mediators such as TNF α , IL-6, and IFN γ (53). The increased NO is responsible for the impaired vascular reactivity and vasodilation leading to hypotension during sepsis (89).

The sepsis-induced capillary blood flow impairment is not affected by genetic deletion of each of the 3 NOS isoforms (eNOS, nNOS, or iNOS)(107). This suggests that the impairment is not due to the overexpression of iNOS or the subsequent changes in vascular reactivity. As mentioned previously, ascorbate treatment rescued the capillary blood flow in septic mice. Interestingly, this reversal was not seen in the eNOS^{-/-} mice

(107). Located in endothelial cells and platelets (93), eNOS generates the majority of vascular NO under normal conditions (33) and contributes to vascular homeostasis by regulating vasomotor tone (88). NO also inhibits vascular smooth muscle cell proliferation (39), and platelet aggregation and adhesion (1). Since, during sepsis, BH₄ may be limited (due to conversion to BH₂ by ROS), eNOS can become uncoupled from BH₄, leading to reduced NO synthesis and thus a reduction of its local effects. Furthermore, the uncoupled eNOS produces superoxide, further contributing to the effects of ROS in the capillary in particular, increasing the formation of peroxynitrite and subsequent damage caused by this highly reactive molecule. Impaired eNOS function, combined with other factors, contributes to the impairment of the capillary blood flow (107).

1.2 COAGULATION

1.2.1 Overview

Coagulation is the process by which thrombi are formed to produce and maintain vascular homeostasis. It contains both a cellular component (platelets; see section 1.2.1) and a protein component (fibrin; see section 1.2.2). Thrombus formation is in constant equilibrium with the breakdown of the clot by fibrinolytic factors (35). Both excessive (hypercoagulation) and insufficient (hypocoagulation) coagulation will lead to disease states (26, 35, 35). Thus any alteration to the equilibrium can lead to a pathological state.

The coagulation cascade has 2 separate pathways that ultimately lead to platelet activation and fibrin formation. The intrinsic or contact pathway begins with exposed

tissue collagen (normally separated from the blood by endothelial cells forming the wall of blood vessels) which will directly activate the platelets and provide a site for the formation of a complex that will eventually convert prothrombin into thrombin. The extrinsic or tissue factor (TF) pathway begins when the protein, tissue factor, comes in contact with the blood. TF is normally exposed on cells that are not normally in contact with the blood such as fibroblast and smooth muscle cells. However endothelial cells and WBCs can express TF on their surface following stimulation by inflammatory mediators (63). Tissue factor will bind and activate factor VII to form the tissue factor-factor VIIa complex. This complex will activate factor X (to factor Xa) that subsequently cleaves prothrombin into its active form thrombin (95). Thrombin then cleaves fibrinogen to fibrin as well as activating platelets and endothelial cells(24). Thrombin is one of the strongest platelet activators *in vivo* (63).

Septic patients commonly manifest coagulation disorders such as thrombocytopenia (reduced platelet counts) and disseminated intravascular coagulation (DIC) (63). This is due to cross-talk between the inflammatory and coagulation pathways. For example, $\text{TNF}\alpha$ can upregulate TF (12). Furthermore, direct stimulation by LPS can also increase TF on endothelial cells (19). Besides affecting thrombin generation by TF, inflammation can alter coagulation by reducing the function of the anticoagulation system. This system includes protein C (degrades coagulation factors Va and VIIIa), antithrombin (inhibits thrombin), and tissue factor pathway inhibitor (inhibits TF-factor VIIa complex) (63). Finally, inflammation can disrupt the coagulation system by impairing the fibrinolytic system (34).

1.2.2 Platelets

Platelets are fully differentiated anuclear cells formed from megakaryocytes in the bone marrow. They are approximately 1 – 3 microns in diameter and have a lifespan of 7 days. They circulate in the blood and contribute to vascular homeostasis. When activated, they form and strengthen thrombi at the site of vascular damage. Pathological disorders can occur when platelet function is altered. High counts or overactive platelets will lead to enhance clotting disorders (e.g., stroke), whereas low counts or inactive platelets will lead to bleeding complications (50).

Platelets are activated by a variety of agents such as thrombin, adenosine diphosphate (ADP), thromboxane A₂ (TXA), collagen, and serotonin (5HT) (32, 68). Three major events accompany platelet activation: shape change, granule secretion, and cell surface integrin activation. Platelets change to a more spherical shape with pseudopods on its surface. This new shape favours platelet rolling thus increasing adhesion (58). Platelets secrete 2 types of granules: alpha and dense. Dense granules contain small molecules (ADP, adenosine triphosphate (ATP), serotonin and calcium) whereas alpha granules contain pro-coagulatory proteins (P-selectin, fibrinogen, thrombospondin, von Willebrand factor and others). The release of molecules from these granules acts as a positive feedback for further platelet activation (32, 68). Activation of cell surface integrins such as glycoprotein IIb/IIIa (GPIIb/IIIa) contributes to platelet adhesion and aggregation (85).

Reduced platelet counts (thrombocytopenia) is a common feature during sepsis (64). However, the cause of this reduction is unclear. Due to cross-talk between the inflammatory and coagulation pathways, in particular the increased levels of thrombin,

ADP, TXA, and 5HT (20, 51, 63, 71), platelets can become activated during sepsis leading to increased aggregation and adhesion to the vascular wall (64, 65, 102). Platelets can be activated by both endotoxin (LPS) and inflammatory cytokines (126, 127). Activated platelets can in turn increase the expression of tissue factor on monocytes (104). Furthermore, ROS will potentiate platelet aggregation (46, 56, 100, 113) and activated platelets will release ROS (another positive feedback mechanism) (8, 113).

1.2.3 Fibrin

Fibrin is an insoluble fibrous protein that forms 3-dimensional matrix structures that participate in the formation of a clot. Besides physically supporting the clot, fibrin triggers signalling events that mediate cellular functions (117). Fibrin matrices form when thrombin cleaves the insoluble fibrinogen into fibrin monomers that polymerise and branch to form the fibrin network. The architecture of the fibrin network determines the structural integrity of the clot (114).

The breakdown of fibrin (fibrinolysis) is also very important for homeostasis. Fibrin is lysed by the protease plasmin, which is formed by cleavage of the circulating plasminogen. There are 2 plasminogen activators that facilitate this process: tissue plasminogen activator (tPA), and urokinase plasminogen activator (uPA). Both of these enzymes are inhibited by plasminogen activator inhibitor-1 (PAI-1) (42).

Fibrin deposition is a common consequence of sepsis. Inflammatory mediators activate mononuclear cells and endothelial cells to express tissue factor (63). Moreover, septic patients have elevated PAI-1 levels (103). Although, levels of tPA are also

increased, the net effect is antifibrinolytic (103). These 2 points suggest that sepsis produces the state of fibrin clot formation and stabilization.

1.2.4 Endothelial cells

Endothelial cells line the blood vessel walls, providing a barrier between the vascular lumen and the surrounding cells. Besides the barrier function of the cells, they also participate in both the inflammation and coagulation process. Endothelial cells express various adhesion proteins which participate in the inflammatory response in the microvasculature (111). Specifically, endothelial cells contain pro-coagulatory factors such as von Willebrand factor (vWF) and P-selectin in Weibel-Palade bodies that are exposed to the surface when the cells become 'activated'. Endothelial cells can be activated by LPS, inflammatory mediators such as $\text{TNF}\alpha$, and thrombin (37, 52, 73). Also, it is well established that there is endothelial damage and dysfunction during sepsis resulting in a loss of barrier function including microvascular leak and exposure of the subendothelial matrix (or basement membrane) (62).

1.2.5 Platelet adhesion, aggregation, and thrombi formation

On the molecular level, there are many proteins that facilitate the adhesion of platelets to the endothelium, to fibrin, and to other platelets to form micro-thrombi. These include P-selectin (CD62P), platelet endothelial cell adhesion molecule-1 (PECAM), vWF, and glycoprotein IIb/IIIa (GPIIb/IIIa or integrin $\alpha_{\text{IIb}}\beta_3$) (85).

P-selectin (CD62P) is a 140 kDa glycoprotein that is found in the alpha granules in platelets and Weibel-Palade bodies in ECs. Upon stimulation of either cell type, P-

selectin is exposed on the surface of the cell where it can interact with its counter receptor P-selectin glycoprotein receptor-1 (PSGL1)(13). PSGL1 is found on both platelets (36) and ECs (23). Thus, the P-selectin-PSGL1 interaction could occur between platelet-endothelium, endothelium-platelet, and platelet-platelet. Once bound, the interaction between the proteins leads to platelet rolling, adhesion, and aggregation. Furthermore, the binding can signal other effects such as platelet activation (13, 17).

Adhesion of platelets to the endothelium by PECAM, although possible, does not occur under physiological conditions and is not the primary function of this protein (118).

vWF participates in platelet adhesion and aggregation by acting as a binding partner, linking proteins together to cause adhesion. Like P-selectin, vWF is stored in alpha granules and Weibel-Palade bodies and is released upon stimulation. vWF will interact with GPIIb/IIIa as well as the platelet integrin GPIb, thus linking 2 platelets together and initiating platelet aggregation. Furthermore, vWF will bind collagen (a part of the sub endothelial matrix). Therefore vWF will link platelets to the sub-endothelial matrix, causing platelet adhesion to the vessel wall (72).

GPIIb/IIIa binds to vWF, fibrinogen or fibrin, fibronectin, and vitronectin. Like many integrins, GPIIb/IIIa can exist in a low active or high active state depending on its conformation (85, 92). Found constitutively expressed on the surface of platelets, GPIIb/IIIa will change conformation to a high active state when activation of the platelet occurs (68, 92). When in this state, GPIIb/IIIa binds to vWF (causing platelet aggregation and adhesion), fibrinogen or fibrin (linking the platelet to the clot), and to fibronectin and vitronectin (i.e., proteins found in the sub-endothelial matrix) to participate in platelet adhesion.

All of these processes come together during sepsis, which increases (i) coagulation, leading to increased platelet activation, (ii) endothelial cell activation and (iii) fibrin formation (which increases P-selectin expression, vWF release, and GPIIb/IIIa activation). The resulting binding interactions between platelets, ECs, the subendothelial matrix, and the fibrin network lead to the formation of microthrombi.

1.3 ASCORBATE

1.3.1 Ascorbate treatment

Ascorbate (reduced vitamin C) is the most abundant water soluble antioxidant. It will react with most ROS including superoxide and peroxynitrite. After reacting, the ascorbate radical has a low reactivity due to resonance stabilization of the unpaired electron and readily dismutates to ascorbate and dehydroascorbic acid (18). Ascorbate can also recycle other antioxidants, such as α -tocopherol (vitamin E) and glutathione from the radical species (78).

Circulating ascorbate levels are significantly lowered in septic patients and animal models of sepsis (3, 38). In these models, this is likely due to impairment of cellular mechanisms of ascorbate production during sepsis (55). In critically ill patients, this depletion is due to increased requirement for ascorbate (7, 70) caused by increased ROS levels (115, 116). Reduced ascorbate levels correlate with increased severity and mortality of septic patients (16, 38, 41).

Clinically, ascorbate, in combination with vitamin E, has been shown to reduce mortality in critically ill patients in a randomized, double-blind, placebo controlled trial

(22). Furthermore, ascorbate and vitamin E reduced multiple organ failure and shortened the ICU stay in patients post trauma or major surgery (84). Finally, an extremely high dose of ascorbate decreased mortality of severely burned patients (105).

In experimental animal models of sepsis, ascorbate bolus given intravenously has been shown to improve arteriolar responsiveness, arterial blood pressure, liver function, capillary blood flow, and survival (3, 107, 108, 121, 122). In particular, ascorbate bolus prevented the sepsis-induced capillary blood flow impairment when administered at the onset of sepsis (3). Further, when administered late into sepsis (i.e. when the capillary blood flow impairment was already present), ascorbate improved capillary blood flow (107, 108). Finally, ascorbate has been shown to improve survival in a mouse CLP (cecal ligation and perforation) model when given early (121) and in a mouse FIP (feces injection into peritoneum) model when given late (107). Table 1.1 (columns 3 and 4) indicates the protective effects of ascorbate and their potential role in the capillary blood flow impairment during sepsis.

1.3.2 Mechanism of protection by ascorbate

We have recently discovered that the protective effect of ascorbate in sepsis is eNOS-dependent. Furthermore, application of exogenous NO, or the eNOS cofactor BH₄ directly on the impaired capillaries also reversed the impairment (107). Application of BH₄ was ineffective in eNOS^{-/-} mice indicating that its protective effect is also eNOS-dependent (107). As stated previously, BH₄ is known to be oxidized by ROS and, when in this state, is no longer a cofactor for eNOS and thus will ‘uncouple’ from eNOS (59, 61). In this uncoupled state, eNOS produces superoxide instead of NO, effectively

reducing NO production to the local environment (61) and further increasing ROS due to the formation of peroxynitrite. Therefore, we propose that ascorbate is readily absorbed into the endothelial cells (115, 116), where it prevents and reverses the oxidization of BH₄, effectively 're-coupling' to eNOS and thus restoring the local NO production. This local NO then provides beneficial effects alongside any other effects of ascorbate (Figure 1.2).

NO is known to inhibit platelet aggregation and adhesion thus contributing to the maintenance of blood flow in the microvasculature. Primarily known to prevent platelet activation through a cGMP pathway, NO has also been shown prevent this activation by other pathways. Specifically, NO has been shown to reduce P-selectin expression on ECs (4) and on platelets (44) thus reducing platelet-endothelium adhesion (44, 45, 76, 86). Thus ascorbate, through NO, may modulate P-selectin function. Since NO has anti-platelet properties, and platelets are believed to involve in septic capillary plugging, the restored local NO production will lead to a reduction in the capillary blood flow impairment.

Table 1.1. Possible underlying processes/events involved in the sepsis-induced capillary blood flow impairment, and possible protective effect of ascorbate treatment (intravenous bolus injection).

Pathophysiological process/ event during sepsis	Involvement of process/event in capillary blood flow impairment	Effect of ascorbate treatment on process/event	Protection by ascorbate ?
Decreased blood pressure (3)	Unlikely	Prevents decrease (3)	None
Increased drainage pressure (49)	Unlikely	No effect	None
Increased WBC adhesion (80)	Likely*	Reduced <i>in vitro</i> (25)	None
Increased RBC stiffening, adhesion (10,29)	Likely	Unknown	Possible
Increased platelet adhesion (67)	Likely	Unknown	Possible
Increased microthrombi formation (64)	Very likely	Unknown	Possible
Increased EC swelling (47)	Possible	Possibly reduced	Possible
Increased edema (63)	Possible	Unknown	None

WBC – white blood cell, RBC – red blood cell, EC – endothelial cell, *unlikely in skeletal muscle

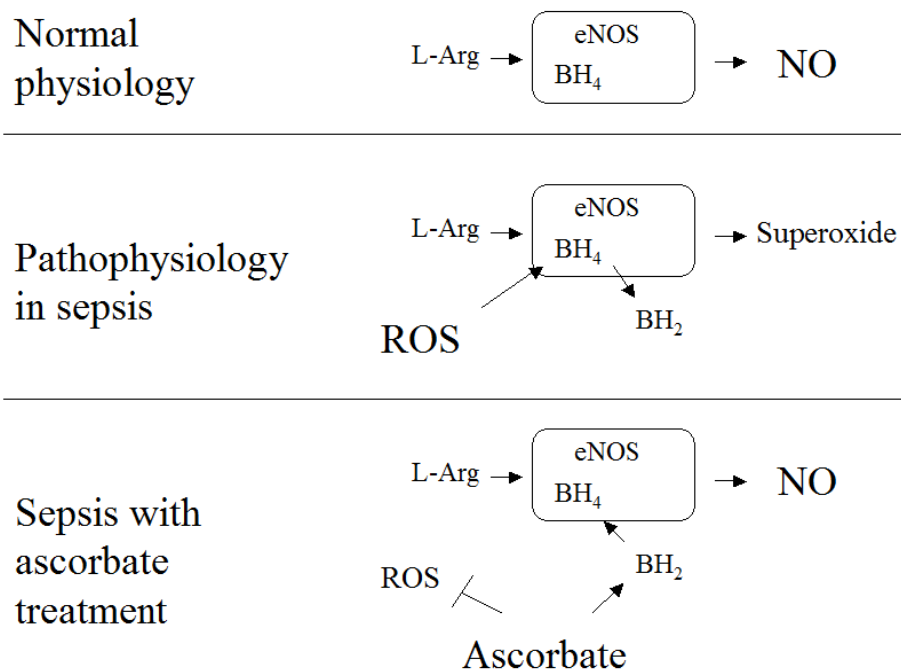


Figure 1.2. Mechanism of restored nitric oxide (NO) production by ascorbate during sepsis. Under normal physiological conditions, local NO is produced from L-arginine (L-arg) through the enzyme endothelial nitric oxide synthase (eNOS) with the aid of the cofactor tetrahydrobiopterin (BH₄). During sepsis, increased levels of reactive oxygen species (ROS), causes the conversion of BH₄ to dihydrobiopterin (BH₂). BH₄ becomes limited, effectively ‘uncoupling’ from eNOS, which produces superoxide instead of NO. Treatment with ascorbate will decrease the ROS as well as convert BH₂ back to BH₄ restoring eNOS function and NO production.

1.4 RATIONALE, HYPOTHESIS, and OBJECTIVES

1.4.1 Rationale

Despite extensive research, there is no pharmacological therapy for the treatment of sepsis. This represents a lack of understanding of the complex cellular and molecular mechanisms that govern the disease. The sepsis-induced capillary blood flow impairment has been associated with poor prognosis of sepsis and is a possible mechanism for the multiple organ failure seen in severe sepsis (99). However, the precise mechanisms of this impairment remain unclear. The impairment is a physical hindrance of blood flow in the capillary and one of the most likely candidates to cause this hindrance is the formation of micro-thrombi. To this extent, the roles of platelets and fibrin with regards to the capillary blood flow impairment have not been investigated.

Over 10 years ago, Armour and coworkers (3) first demonstrated that treatment with ascorbate had beneficial effects on capillary blood flow during sepsis. Since then, there have been numerous studies confirming the beneficial effect of ascorbate in sepsis (3, 106-108, 119, 121, 122). The mechanism of ascorbate's effect on the capillary blood flow impairment is unresolved. It is known that the effect of ascorbate is eNOS dependent, presumably due to the restoration of local NO. NO plays an important role in the homeostasis of coagulation, and thus any contribution to the capillary blood flow impairment by the coagulation system (i.e., microthrombi formation), will be altered by the increased local NO. Further information regarding this will (i) further the

understanding of the molecular interactions occurring during sepsis, and (ii) encourage the use of ascorbate as a therapy for septic patients.

1.4.2 Hypotheses

1) Increased platelet adhesion, aggregation and microthrombi formation during sepsis lead to plugging of capillaries, resulting in capillary blood flow impairment.

2) Ascorbate lowers platelet aggregation and adhesion by decreasing P-selectin expression, thus improving capillary blood flow during sepsis.

1.4.3 Objectives

1) Determine the effect of sepsis on capillary blood flow and on platelet adhesion in capillaries of mouse skeletal muscle, and examine the roles of the coagulation system, ROS and NO in sepsis-induced impairment of capillary blood flow and platelet adhesion. This objective will be addressed in Chapter 2.

2) Use an *ex vivo* model to examine i) the effect of sepsis on platelet aggregation and P-selectin expression and ii) the protection by ascorbate against sepsis-induced aggregation and P-selectin expression. This objective will be addressed in Chapter 3.

3) Use an *in vitro* model of microvascular endothelial cells to examine i) the effect of sepsis on platelet-endothelial cell adhesion and P-selectin expression in endothelial cells and ii) the protection by ascorbate against sepsis-induced platelet-endothelial adhesion and P-selectin expression in endothelial cells. This objective will be addressed in Chapter 4.

1.5 REFERENCES

1. Alheid U, Frolich JC, Forstermann U. Endothelium-derived relaxing factor from cultured human endothelial cells inhibits aggregation of human platelets. *Thromb Res* 47(5): 561-571, 1987.
2. Andreasen AS, Krabbe KS, Krogh-Madsen R, Taudorf S, Pedersen BK, Moller K. Human endotoxemia as a model of systemic inflammation. *Curr Med Chem* 15(17): 1697-1705, 2008.
3. Armour J, Tynl K, Lidington D, Wilson JX. Ascorbate prevents microvascular dysfunction in the skeletal muscle of the septic rat. *J Appl Physiol* 90(3): 795-803, 2001.
4. Armstead VE, Minchenko AG, Schuhl RA, Hayward R, Nossuli TO, Lefer AM. Regulation of P-selectin expression in human endothelial cells by nitric oxide. *Am J Physiol* 273(2 Pt 2): H740-6, 1997.
5. Arnalich F, Hernanz A, Jimenez M, Lopez J, Tato E, Vazquez JJ, Montiel C. Relationship between circulating levels of calcitonin gene-related peptide, nitric oxide metabolites and hemodynamic changes in human septic shock. *Regul Pept* 65(2): 115-121, 1996.
6. Azevedo LC, Janiszewski M, Soriano FG, Laurindo FR. Redox mechanisms of vascular cell dysfunction in sepsis. *Endocr Metab Immune Disord Drug Targets* 6(2): 159-164, 2006.

7. Baines M, Shenkin A. Lack of effectiveness of short-term intravenous micronutrient nutrition in restoring plasma antioxidant status after surgery. *Clin Nutr* 21(2): 145-150, 2002.
8. Bakdash N, Williams MS. Spatially distinct production of reactive oxygen species regulates platelet activation. *Free Radic Biol Med* 45(2): 158-166, 2008.
9. Bateman RM, Jagger JE, Sharpe MD, Ellsworth ML, Mehta S, Ellis CG. Erythrocyte deformability is a nitric oxide-mediated factor in decreased capillary density during sepsis. *Am J Physiol Heart Circ Physiol* 280(6): H2848-56, 2001.
10. Bateman RM, Sharpe MD, Ellis CG. Bench-to-bedside review: microvascular dysfunction in sepsis--hemodynamics, oxygen transport, and nitric oxide. *Crit Care* 7(5): 359-373, 2003.
11. Becker JU, Theodosis C, Jacob ST, Wira CR, Groce NE. Surviving sepsis in low-income and middle-income countries: new directions for care and research. *Lancet Infect Dis* 9(9): 577-582, 2009.
12. Bevilacqua MP, Pober JS, Majeau GR, Fiers W, Cotran RS, Gimbrone MA, Jr. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. *Proc Natl Acad Sci U S A* 83(12): 4533-4537, 1986.
13. Blann AD, Nadar SK, Lip GY. The adhesion molecule P-selectin and cardiovascular disease. *Eur Heart J* 24(24): 2166-2179, 2003.

14. Boerma EC, van der Voort PH, Spronk PE, Ince C. Relationship between sublingual and intestinal microcirculatory perfusion in patients with abdominal sepsis. *Crit Care Med* 35(4): 1055-1060, 2007.
15. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* 101(6): 1644-1655, 1992.
16. Borrelli E, Roux-Lombard P, Grau GE, Girardin E, Ricou B, Dayer J, Suter PM. Plasma concentrations of cytokines, their soluble receptors, and antioxidant vitamins can predict the development of multiple organ failure in patients at risk. *Crit Care Med* 24(3): 392-397, 1996.
17. Carlow DA, Gossens K, Naus S, Veerman KM, Seo W, Ziltener HJ. PSGL-1 function in immunity and steady state homeostasis. *Immunol Rev* 230(1): 75-96, 2009.
18. Carr A, Frei B. Does vitamin C act as a pro-oxidant under physiological conditions?. *FASEB J* 13(9): 1007-1024, 1999.
19. Colucci M, Balconi G, Lorenzet R, Pietra A, Locati D, Donati MB, Semeraro N. Cultured human endothelial cells generate tissue factor in response to endotoxin. *J Clin Invest* 71(6): 1893-1896, 1983.

20. Cook JA, Wise WC, Halushka PV. Elevated thromboxane levels in the rat during endotoxic shock: protective effects of imidazole, 13-azaprostanic acid, or essential fatty acid deficiency. *J Clin Invest* 65(1): 227-230, 1980.
21. Cooper D, Stokes KY, Tailor A, Granger DN. Oxidative stress promotes blood cell-endothelial cell interactions in the microcirculation. *Cardiovasc Toxicol* 2(3): 165-180, 2002.
22. Crimi E, Liguori A, Condorelli M, Cioffi M, Astuto M, Bontempo P, Pignalosa O, Vietri MT, Molinari AM, Sica V, Della Corte F, Napoli C. The beneficial effects of antioxidant supplementation in enteral feeding in critically ill patients: a prospective, randomized, double-blind, placebo-controlled trial. *Anesth Analg* 99(3): 857-63, table of contents, 2004.
23. da Costa Martins P, Garcia-Vallejo JJ, van Thienen JV, Fernandez-Borja M, van Gils JM, Beckers C, Horrevoets AJ, Hordijk PL, Zwaginga JJ. P-selectin glycoprotein ligand-1 is expressed on endothelial cells and mediates monocyte adhesion to activated endothelium. *Arterioscler Thromb Vasc Biol* 27(5): 1023-1029, 2007.
24. De Caterina R, Sicari R. Cellular effects of thrombin: pharmacology of the receptor(s) in various cell types and possible development of receptor antagonists. *Pharmacol Res* 27(1): 1-19, 1993.
25. De la Fuente M, Victor VM. Ascorbic acid and N-acetylcysteine improve in vitro the function of lymphocytes from mice with endotoxin-induced oxidative stress. *Free Radic Res* 35(1): 73-84, 2001.

26. de Laforcade A. Diseases associated with thrombosis. *Top Companion Anim Med* 27(2): 59-64, 2012.
27. Dellinger RP, Levy MM, Carlet JM, Bion J, Parker MM, Jaeschke R, Reinhart K, Angus DC, Brun-Buisson C, Beale R, Calandra T, Dhainaut JF, Gerlach H, Harvey M, Marini JJ, Marshall J, Ranieri M, Ramsay G, Sevransky J, Thompson BT, Townsend S, Vender JS, Zimmerman JL, Vincent JL, International Surviving Sepsis Campaign Guidelines Committee, American Association of Critical-Care Nurses, American College of Chest Physicians, American College of Emergency Physicians, Canadian Critical Care Society, European Society of Clinical Microbiology and Infectious Diseases, European Society of Intensive Care Medicine, European Respiratory Society, International Sepsis Forum, Japanese Association for Acute Medicine, Japanese Society of Intensive Care Medicine, Society of Critical Care Medicine, Society of Hospital Medicine, Surgical Infection Society, World Federation of Societies of Intensive and Critical Care Medicine. Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2008. *Crit Care Med* 36(1): 296-327, 2008.
28. Eaton S. The biochemical basis of antioxidant therapy in critical illness. *Proc Nutr Soc* 65(3): 242-249, 2006.
29. Eichelbronner O, Sibbald WJ, Chin-Yee IH. Intermittent flow increases endotoxin-induced adhesion of human erythrocytes to vascular endothelial cells. *Intensive Care Med* 29(5): 709-714, 2003.

30. Ellis CG, Bateman RM, Sharpe MD, Sibbald WJ, Gill R. Effect of a maldistribution of microvascular blood flow on capillary O₂ extraction in sepsis. *Am J Physiol Heart Circ Physiol* 282(1): H156-64, 2002.
31. Eriksson E, Germann G, Mathur A. Microcirculation in muscle. *Ann Plast Surg* 17(1): 13-16, 1986.
32. Flaumenhaft R. Molecular basis of platelet granule secretion. *Arterioscler Thromb Vasc Biol* 23(7): 1152-1160, 2003.
33. Forstermann U. Janus-faced role of endothelial NO synthase in vascular disease: uncoupling of oxygen reduction from NO synthesis and its pharmacological reversal. *Biol Chem* 387(12): 1521-1533, 2006.
34. Fourrier F. Severe sepsis, coagulation, and fibrinolysis: dead end or one way?. *Crit Care Med* 40(9): 2704-2708, 2012.
35. Franchini M, Mannucci PM. The hemostatic balance revisited through the lessons of mankind evolution. *Intern Emerg Med* 3(1): 3-8, 2008.
36. Frenette PS, Denis CV, Weiss L, Jurk K, Subbarao S, Kehrel B, Hartwig JH, Vestweber D, Wagner DD. P-Selectin glycoprotein ligand 1 (PSGL-1) is expressed on platelets and can mediate platelet-endothelial interactions in vivo. *J Exp Med* 191(8): 1413-1422, 2000.
37. Frenette PS, Moyna C, Hartwell DW, Lowe JB, Hynes RO, Wagner DD. Platelet-endothelial interactions in inflamed mesenteric venules. *Blood* 91(4): 1318-1324, 1998.

38. Galley HF, Davies MJ, Webster NR. Ascorbyl radical formation in patients with sepsis: effect of ascorbate loading. *Free Radic Biol Med* 20(1): 139-143, 1996.
39. Garg UC, Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest* 83(5): 1774-1777, 1989.
40. Goldman D, Bateman RM, Ellis CG. Effect of sepsis on skeletal muscle oxygen consumption and tissue oxygenation: interpreting capillary oxygen transport data using a mathematical model. *Am J Physiol Heart Circ Physiol* 287(6): H2535-44, 2004.
41. Goode HF, Webster NR. Free radicals and antioxidants in sepsis. *Crit Care Med* 21(11): 1770-1776, 1993.
42. Gramling MW, Church FC. Plasminogen activator inhibitor-1 is an aggregate response factor with pleiotropic effects on cell signaling in vascular disease and the tumor microenvironment. *Thromb Res* 125(5): 377-381, 2010.
43. Griendling KK, Sorescu D, Lassegue B, Ushio-Fukai M. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arterioscler Thromb Vasc Biol* 20(10): 2175-2183, 2000.
44. Gries A, Bode C, Peter K, Herr A, Bohrer H, Motsch J, Martin E. Inhaled nitric oxide inhibits human platelet aggregation, P-selectin expression, and fibrinogen binding in vitro and in vivo. *Circulation* 97(15): 1481-1487, 1998.

45. Gries A, Herr A, Motsch J, Holzmann A, Weimann J, Taut F, Erbe N, Bode C, Martin E. Randomized, placebo-controlled, blinded and cross-matched study on the antiplatelet effect of inhaled nitric oxide in healthy volunteers. *Thromb Haemost* 83(2): 309-315, 2000.
46. Handin RI, Karabin R, Boxer GJ. Enhancement of platelet function by superoxide anion. *J Clin Invest* 59(5): 959-965, 1977.
47. Herkert O, Djordjevic T, BelAiba RS, Gorlach A. Insights into the redox control of blood coagulation: role of vascular NADPH oxidase-derived reactive oxygen species in the thrombogenic cycle. *Antioxid Redox Signal* 6(4): 765-776, 2004.
48. Hinshaw LB. Sepsis/septic shock: participation of the microcirculation: an abbreviated review. *Crit Care Med* 24(6): 1072-1078, 1996.
49. Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. *N Engl J Med* 348(2): 138-150, 2003.
50. Huebsch LB, Harker LA. Disorders of platelet function: mechanisms, diagnosis and management. *West J Med* 134(2): 109-127, 1981.
51. Jabs CM, Ferrell WJ, Robb HJ. Plasma changes in endotoxin and anaphylactic shock (ATP, ADP and Creatine phosphorus). *Ann Clin Lab Sci* 9(2): 121-132, 1979.
52. Kameda H, Morita I, Handa M, Kaburaki J, Yoshida T, Mimori T, Murota S, Ikeda Y. Re-expression of functional P-selectin molecules on the endothelial cell surface by repeated stimulation with thrombin. *Br J Haematol* 97(2): 348-355, 1997.

53. Kirkeboen KA, Strand OA. The role of nitric oxide in sepsis--an overview. *Acta Anaesthesiol Scand* 43(3): 275-288, 1999.
54. Kleniewska P, Piechota A, Skibska B, Goraca A. The NADPH oxidase family and its inhibitors. *Arch Immunol Ther Exp (Warsz)* 60(4): 277-294, 2012.
55. Korcok J, Wu F, Tymi K, Hammond RR, Wilson JX. Sepsis inhibits reduction of dehydroascorbic acid and accumulation of ascorbate in astroglial cultures: intracellular ascorbate depletion increases nitric oxide synthase induction and glutamate uptake inhibition. *J Neurochem* 81(1): 185-193, 2002.
56. Krotz F, Sohn HY, Gloe T, Zahler S, Riexinger T, Schiele TM, Becker BF, Theisen K, Klauss V, Pohl U. NAD(P)H oxidase-dependent platelet superoxide anion release increases platelet recruitment. *Blood* 100(3): 917-924, 2002.
57. Kung HC, Hoyert DL, Xu J, Murphy SL. Deaths: final data for 2005. *Natl Vital Stat Rep* 56(10): 1-120, 2008.
58. Kuwahara M, Sugimoto M, Tsuji S, Matsui H, Mizuno T, Miyata S, Yoshioka A. Platelet shape changes and adhesion under high shear flow. *Arterioscler Thromb Vasc Biol* 22(2): 329-334, 2002.
59. Kuzkaya N, Weissmann N, Harrison DG, Dikalov S. Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. *J Biol Chem* 278(25): 22546-22554, 2003.

60. Lam C, Tyml K, Martin C, Sibbald W. Microvascular perfusion is impaired in a rat model of normotensive sepsis. *J Clin Invest* 94(5): 2077-2083, 1994.
61. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, Harrison DG. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* 111(8): 1201-1209, 2003.
62. Lee WL, Liles WC. Endothelial activation, dysfunction and permeability during severe infections. *Curr Opin Hematol* 18(3): 191-196, 2011.
63. Levi M. The coagulant response in sepsis and inflammation. *Hamostaseologie* 30(1): 10-2, 14-6, 2010.
64. Levi M. Platelets in sepsis. *Hematology* 10 Suppl 1: 129-131, 2005.
65. Levi M, Ten Cate H. Disseminated intravascular coagulation. *N Engl J Med* 341(8): 586-592, 1999.
66. Levi M, van der Poll T, Buller HR. Bidirectional relation between inflammation and coagulation. *Circulation* 109(22): 2698-2704, 2004.
67. Li JM, Shah AM. Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. *Am J Physiol Regul Integr Comp Physiol* 287(5): R1014-30, 2004.
68. Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. *Arterioscler Thromb Vasc Biol* 30(12): 2341-2349, 2010.

69. Linde-Zwirble WT, Angus DC. Severe sepsis epidemiology: sampling, selection, and society. *Crit Care* 8(4): 222-226, 2004.
70. Long CL, Maull KI, Krishnan RS, Laws HL, Geiger JW, Borghesi L, Franks W, Lawson TC, Sauberlich HE. Ascorbic acid dynamics in the seriously ill and injured. *J Surg Res* 109(2): 144-148, 2003.
71. Luna A, Villanueva E, Hernandez-Cueto C, Morales I. Study of 5-hydroxytryptamine (serotonin) in pericardial fluid in different causes of death (II.). Experimental study of 5-HT levels in two types of shocks (hemorrhagic and septic) in dogs. *Z Rechtsmed* 89(4): 227-236, 1983.
72. Luo GP, Ni B, Yang X, Wu YZ. von Willebrand factor: more than a regulator of hemostasis and thrombosis. *Acta Haematol* 128(3): 158-169, 2012.
73. Lush CW, Cepinskas G, Sibbald WJ, Kvietys PR. Endothelial E- and P-selectin expression in iNOS- deficient mice exposed to polymicrobial sepsis. *Am J Physiol Gastrointest Liver Physiol* 280(2): G291-7, 2001.
74. Marti-Carvajal AJ, Sola I, Gluud C, Lathyris D, Cardona AF. Human recombinant protein C for severe sepsis and septic shock in adult and paediatric patients. *Cochrane Database Syst Rev* 12: CD004388, 2012.
75. Martin CM, Priestap F, Fisher H, Fowler RA, Heyland DK, Keenan SP, Longo CJ, Morrison T, Bentley D, Antman N, STAR Registry Investigators. A prospective,

observational registry of patients with severe sepsis: the Canadian Sepsis Treatment and Response Registry. *Crit Care Med* 37(1): 81-88, 2009.

76. Matsushita K, Morrell CN, Cambien B, Yang SX, Yamakuchi M, Bao C, Hara MR, Quick RA, Cao W, O'Rourke B, Lowenstein JM, Pevsner J, Wagner DD, Lowenstein CJ. Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. *Cell* 115(2): 139-150, 2003.

77. Mavrommatis AC, Theodoridis T, Orfanidou A, Roussos C, Christopoulou-Kokkinou V, Zakyntinos S. Coagulation system and platelets are fully activated in uncomplicated sepsis. *Crit Care Med* 28(2): 451-457, 2000.

78. May JM. How does ascorbic acid prevent endothelial dysfunction?. *Free Radic Biol Med* 28(9): 1421-1429, 2000.

79. McCuskey RS, Urbaschek R, Urbaschek B. The microcirculation during endotoxemia. *Cardiovasc Res* 32(4): 752-763, 1996.

80. McDonald B, Kubes P. Neutrophils and intravascular immunity in the liver during infection and sterile inflammation. *Toxicol Pathol* 40(2): 157-165, 2012.

81. Melamed A, Sorvillo FJ. The burden of sepsis-associated mortality in the United States from 1999 to 2005: an analysis of multiple-cause-of-death data. *Crit Care* 13(1): R28, 2009.

82. Mizgerd JP. Molecular mechanisms of neutrophil recruitment elicited by bacteria in the lungs. *Semin Immunol* 14(2): 123-132, 2002.

83. Muzaffar S, Shukla N, Angelini G, Jeremy JY. Nitroaspirins and morpholinonydnonimine but not aspirin inhibit the formation of superoxide and the expression of gp91phox induced by endotoxin and cytokines in pig pulmonary artery vascular smooth muscle cells and endothelial cells. *Circulation* 110(9): 1140-1147, 2004.
84. Nathens AB, Neff MJ, Jurkovich GJ, Klotz P, Farver K, Ruzinski JT, Radella F, Garcia I, Maier RV. Randomized, prospective trial of antioxidant supplementation in critically ill surgical patients. *Ann Surg* 236(6): 814-822, 2002.
85. Nurden AT, Nurden P. A review of the role of platelet membrane glycoproteins in the platelet-vessel wall interaction. *Baillieres Clin Haematol* 6(3): 653-690, 1993.
86. Omi H, Okayama N, Shimizu M, Fukutomi T, Imaeda K, Okouchi M, Itoh M. Statins inhibit high glucose-mediated neutrophil-endothelial cell adhesion through decreasing surface expression of endothelial adhesion molecules by stimulating production of endothelial nitric oxide. *Microvasc Res* 65(2): 118-124, 2003.
87. Ovechkin AV, Lominadze D, Sedoris KC, Robinson TW, Tyagi SC, Roberts AM. Lung ischemia-reperfusion injury: implications of oxidative stress and platelet-arteriolar wall interactions. *Arch Physiol Biochem* 113(1): 1-12, 2007.
88. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327(6122): 524-526, 1987.
89. Parratt JR. Nitric oxide in sepsis and endotoxaemia. *J Antimicrob Chemother* 41 Suppl A: 31-39, 1998.

90. Peters K, Unger RE, Brunner J, Kirkpatrick CJ. Molecular basis of endothelial dysfunction in sepsis. *Cardiovasc Res* 60(1): 49-57, 2003.
91. Piper RD, Pitt-Hyde ML, Anderson LA, Sibbald WJ, Potter RF. Leukocyte activation and flow behavior in rat skeletal muscle in sepsis. *Am J Respir Crit Care Med* 157(1): 129-134, 1998.
92. Quinn MJ, Byzova TV, Qin J, Topol EJ, Plow EF. Integrin α IIb β 3 and its antagonism. *Arterioscler Thromb Vasc Biol* 23(6): 945-952, 2003.
93. Randriamboavonjy V, Fleming I. Endothelial nitric oxide synthase (eNOS) in platelets: how is it regulated and what is it doing there?. *Pharmacol Rep* 57 Suppl: 59-65, 2005.
94. Ranieri VM, Thompson BT, Barie PS, Dhainaut JF, Douglas IS, Finfer S, Gardlund B, Marshall JC, Rhodes A, Artigas A, Payen D, Tenhunen J, Al-Khalidi HR, Thompson V, Janes J, Macias WL, Vangerow B, Williams MD, PROWESS-SHOCK Study Group. Drotrecogin alfa (activated) in adults with septic shock. *N Engl J Med* 366(22): 2055-2064, 2012.
95. Rapaport SI. Regulation of the tissue factor pathway. *Ann N Y Acad Sci* 614: 51-62, 1991.
96. Ray R, Shah AM. NADPH oxidase and endothelial cell function. *Clin Sci (Lond)* 109(3): 217-226, 2005.

97. Rittirsch D, Flierl MA, Ward PA. Harmful molecular mechanisms in sepsis. *Nat Rev Immunol* 8(10): 776-787, 2008.
98. Rivers E, Nguyen B, Havstad S, Ressler J, Muzzin A, Knoblich B, Peterson E, Tomlanovich M, Early Goal-Directed Therapy Collaborative Group. Early goal-directed therapy in the treatment of severe sepsis and septic shock. *N Engl J Med* 345(19): 1368-1377, 2001.
99. Sakr Y, Dubois MJ, De Backer D, Creteur J, Vincent JL. Persistent microcirculatory alterations are associated with organ failure and death in patients with septic shock. *Crit Care Med* 32(9): 1825-1831, 2004.
100. Salvemini D, de Nucci G, Sneddon JM, Vane JR. Superoxide anions enhance platelet adhesion and aggregation. *Br J Pharmacol* 97(4): 1145-1150, 1989.
101. Sands KE, Bates DW, Lanken PN, Graman PS, Hibberd PL, Kahn KL, Parsonnet J, Panzer R, Orav EJ, Snyderman DR, Black E, Schwartz JS, Moore R, Johnson BL, Jr, Platt R, Academic Medical Center Consortium Sepsis Project Working Group. Epidemiology of sepsis syndrome in 8 academic medical centers. *JAMA* 278(3): 234-240, 1997.
102. Schouten M, Wiersinga WJ, Levi M, van der Poll T. Inflammation, endothelium, and coagulation in sepsis. *J Leukoc Biol* 83(3): 536-545, 2008.
103. Semeraro N, Ammollo CT, Semeraro F, Colucci M. Sepsis, thrombosis and organ dysfunction. *Thromb Res* 129(3): 290-295, 2012.

104. Shebuski RJ, Kilgore KS. Role of inflammatory mediators in thrombogenesis. *J Pharmacol Exp Ther* 300(3): 729-735, 2002.
105. Tanaka H, Matsuda T, Miyagantani Y, Yukioka T, Matsuda H, Shimazaki S. Reduction of resuscitation fluid volumes in severely burned patients using ascorbic acid administration: a randomized, prospective study. *Arch Surg* 135(3): 326-331, 2000.
106. Tynml K. Critical role for oxidative stress, platelets, and coagulation in capillary blood flow impairment in sepsis. *Microcirculation* 18(2): 152-162, 2011.
107. Tynml K, Li F, Wilson JX. Septic impairment of capillary blood flow requires nicotinamide adenine dinucleotide phosphate oxidase but not nitric oxide synthase and is rapidly reversed by ascorbate through an endothelial nitric oxide synthase-dependent mechanism. *Crit Care Med* 36(8): 2355-2362, 2008.
108. Tynml K, Li F, Wilson JX. Delayed ascorbate bolus protects against maldistribution of microvascular blood flow in septic rat skeletal muscle. *Crit Care Med* 33(8): 1823-1828, 2005.
109. Tynml K, Wang X, Lidington D, Ouellette Y. Lipopolysaccharide reduces intercellular coupling in vitro and arteriolar conducted response in vivo. *Am J Physiol Heart Circ Physiol* 281(3): H1397-406, 2001.
110. Tynml K, Yu J, McCormack DG. Capillary and arteriolar responses to local vasodilators are impaired in a rat model of sepsis. *J Appl Physiol* 84(3): 837-844, 1998.

111. Vallet B, Wiel E. Endothelial cell dysfunction and coagulation. *Crit Care Med* 29(7 Suppl): S36-41, 2001.
112. Vincent JL. Clinical sepsis and septic shock--definition, diagnosis and management principles. *Langenbecks Arch Surg* 393(6): 817-824, 2008.
113. Wachowicz B, Olas B, Zbikowska HM, Buczynski A. Generation of reactive oxygen species in blood platelets. *Platelets* 13(3): 175-182, 2002.
114. Whittaker P, Przyklenk K. Fibrin architecture in clots: a quantitative polarized light microscopy analysis. *Blood Cells Mol Dis* 42(1): 51-56, 2009.
115. Wilson JX. Mechanism of action of vitamin C in sepsis: ascorbate modulates redox signaling in endothelium. *Biofactors* 35(1): 5-13, 2009.
116. Wilson JX, Dixon SJ, Yu J, Nees S, Tyml K. Ascorbate uptake by microvascular endothelial cells of rat skeletal muscle. *Microcirculation* 3(2): 211-221, 1996.
117. Wolberg AS. Determinants of fibrin formation, structure, and function. *Curr Opin Hematol* 19(5): 349-356, 2012.
118. Woodfin A, Voisin MB, Nourshargh S. PECAM-1: a multi-functional molecule in inflammation and vascular biology. *Arterioscler Thromb Vasc Biol* 27(12): 2514-2523, 2007.

119. Wu F, Schuster DP, Tyml K, Wilson JX. Ascorbate inhibits NADPH oxidase subunit p47phox expression in microvascular endothelial cells. *Free Radic Biol Med* 42(1): 124-131, 2007.
120. Wu F, Tyml K, Wilson JX. iNOS expression requires NADPH oxidase-dependent redox signaling in microvascular endothelial cells. *J Cell Physiol* 217(1): 207-214, 2008.
121. Wu F, Wilson JX, Tyml K. Ascorbate protects against impaired arteriolar constriction in sepsis by inhibiting inducible nitric oxide synthase expression. *Free Radic Biol Med* 37(8): 1282-1289, 2004.
122. Wu F, Wilson JX, Tyml K. Ascorbate inhibits iNOS expression and preserves vasoconstrictor responsiveness in skeletal muscle of septic mice. *Am J Physiol Regul Integr Comp Physiol* 285(1): R50-6, 2003.
123. Yu G, Peng T, Feng Q, Tyml K. Abrupt reoxygenation of microvascular endothelial cells after hypoxia activates ERK1/2 and JNK1, leading to NADPH oxidase-dependent oxidant production. *Microcirculation* 14(2): 125-136, 2007.
124. Yung LM, Leung FP, Yao X, Chen ZY, Huang Y. Reactive oxygen species in vascular wall. *Cardiovasc Hematol Disord Drug Targets* 6(1): 1-19, 2006.
125. Zhang X, Kimura Y, Fang C, Zhou L, Sfyroera G, Lambris JD, Wetsel RA, Miwa T, Song WC. Regulation of Toll-like receptor-mediated inflammatory response by complement in vivo. *Blood* 110(1): 228-236, 2007.

126. Zielinski T, Wachowicz B, Saluk-Juszczak J, Kaca W. Polysaccharide part of *Proteus mirabilis* lipopolysaccharide may be responsible for the stimulation of platelet adhesion to collagen. *Platelets* 13(7): 419-424, 2002.
127. Zimmerman GA, McIntyre TM, Prescott SM, Stafforini DM. The platelet-activating factor signaling system and its regulators in syndromes of inflammation and thrombosis. *Crit Care Med* 30(5 Suppl): S294-301, 2002.

Chapter 2

Impaired microvascular perfusion in sepsis requires activated coagulation and P-selectin mediated platelet adhesion in capillaries

A version of this chapter has been published previously

Secor D, Li F, Ellis CG, Sharpe MD, Gross PL, Wilson JX and Tysl K. Impaired microvascular perfusion in sepsis requires activated coagulation and P-selectin-mediated platelet adhesion in capillaries. *Intensive Care Med.* 36: 11: 1928-1934, 2010.

2.1 INTRODUCTION

Severe sepsis is a systemic inflammatory response in which impaired microvascular perfusion precipitates organ failure and death (31). Impaired perfusion is seen as increased number of stopped-flow capillaries and decreased number of perfused capillaries (13). The impairment increases the diffusion distance for oxygen to parenchymal cells, leading to tissue hypoxia and organ failure (3, 9). Septic impairment of capillary blood flow has been visualized in animal organs by intravital microscopy (6, 13) and in human tissues by orthogonal polarization spectral imaging and sidestream dark-field imaging (8, 26). Despite the prevalence of capillary flow impairment and its importance in determining the clinical outcome of sepsis (26), the mechanisms of this impairment are unknown (19).

Intravascular coagulation in sepsis (2, 7) could be a contributory mechanism. Inflammatory and coagulation pathways cross-activate (15), including initiation of coagulation and activation of platelets by lipopolysaccharide (LPS) and inflammatory cytokines (15, 16), as well as augmentation of inflammation by activated platelets (1, 11, 17, 23, 32). Therefore, sepsis-induced platelet adhesion to capillary endothelium, platelet aggregates and/or subsequently formed microthrombi could plug capillaries. To our knowledge, there is no report addressing this possible mechanism. The overall aim of the present study was to use high-resolution intravital microscopy to examine in “real time” platelet adhesion and microthrombi formation in septic capillaries. The study had 2 specific objectives. First, we used treatments known to affect platelet and coagulation functions to establish the role of these functions in blood flow stoppage in septic capillaries. Second, we

manipulated the production of reactive oxygen species (ROS) and nitric oxide (NO) in the microvasculature to determine if the role of platelets in septic blood flow stoppage is oxidant- and NO-dependent. We discovered that platelet adhesion co-localized with fibrin deposition in septic capillaries contributes critically to blood flow stoppage.

2.2 METHODS

2.2.1 Animal preparation

We used a fluid resuscitated model of polymicrobial sepsis in mice as described previously (28, 29). Briefly, male wild type, eNOS^{-/-}, iNOS^{-/-} and gp91phox^{-/-} mice (C57BL/6, 18 - 27 g body weight, Jackson Laboratory, Bar Harbor, ME) were injected with feces into the peritoneum (FIP; 50 ml/kg of mixture of saline and feces at 75 mg/ml). For controls, we used naive or sham mice (saline-injected intraperitoneally, 50 ml/kg). Immediately after FIP, mice were injected subcutaneously with 1 ml saline containing the analgesic buprenorphine (4 µg/ml). Mice were studied by intravital microscopy at 6-7 h post-FIP because capillary blood flow impairment is established at this time (29). The experimental protocol was approved by the University of Western Ontario Council on Animal Care.

2.2.2 Intravital microscopy

Mice were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (4 mg/kg) at 5.5 h post-FIP (or post-saline injection in shams) and the extensor digitorum longus (EDL) muscle was prepared for intravital microscopy as a bioassay for capillary blood

flow impairment (29). To assess impairment, we used an upright microscope (Leitz) and video-recorded 5 random fields (0.61 x 0.87 mm) per muscle, counted capillaries with moving red blood cells (CD_{PER}) and stationary red blood cells (CD_{STAT}) in each field, normalized counts per mm of a test line drawn across the muscle fibers, averaged CD_{PER} and CD_{STAT} from 5 fields (29), and computed the percentage of stopped-flow capillaries as $100 \% \times CD_{STAT}/(CD_{PER} + CD_{STAT})$.

Platelets and fibrin were observed with a high resolution inverted microscope (Olympus IX81, 20x objective, N.A.= 0.75) yielding 0.35 x 0.47 mm field of view. To visualize platelets, fluorescent dye rhodamine 6G (0.08 mg/ml saline; Sigma, St. Louis, MO) was filtered (0.22 μ m) and then injected in a 0.05 ml bolus into the penile vein 15 min before visualization. We used fluorescent epi-illumination with TRITC filter and digital recording to determine adherent platelets in 3 random fields per muscle. Each field was recorded during two 10 s periods spaced 1 min apart. We verified that objects fluorescing under TRITC illumination were labeled platelets by viewing with a high-resolution 40x objective (N.A.= 0.90) under both TRITC and bright light illumination. Platelets were counted as adherent if they were stationary during both recording periods (counts were confirmed by a “blind” observer). In each field, we used bright light illumination to measure the total length of visible capillaries projected to the surface plane of the muscle. Platelet adherence was normalized to 1 mm capillary length. Since rhodamine 6G also labels leukocytes (identified by their large size under bright light illumination), we similarly measured the occurrence of adherent leukocytes in control and septic capillaries.

To visualize fibrin, a 0.1 ml saline bolus containing 200 µg/ml of Alexa 488-labeled anti-fibrin antibody (Fibrin II beta clone: T2G1, Accurate Chemical, Westbury, NY) was injected intravenously 15 min prior to visualization (25). This antibody does not bind fibrinogen. To assess fibrin deposition in capillaries, we recorded 3 - 6 random fields for 15 s, measured the total length of capillaries containing fibrin, and expressed it as the percentage of total capillary length.

In several experiments we determined platelet adhesion specifically in capillaries with formed fibrin. Here, we first injected the anti-fibrin antibody and then, 20-30 min later, rhodamine 6G.

2.2.3 Biochemical and blood platelet count analyses

Following intravital microscopy, 0.2 ml blood was collected by carotid artery puncture and analyzed for lactate (iStat analyzer and CG4+ cartridges, Abbott Laboratories, Mississauga, ON). Blood was also analyzed for platelet count by diluting 200-fold in saline, platelets were labelled by rhodamine 6G (0.4 µg/ml) and were counted in a hemocytometer chamber using an Olympus microscope.

2.2.4 Experimental design: (a) Role of platelet and coagulation functions in blood flow stoppage

We studied septic mice with lactate above 1 mmol/l at 6-7 h post-FIP. To determine if platelets are required for blood flow stoppage in septic capillaries, mice were injected intravenously with platelet-depleting antibody (AIAD31440, purchased from Accurate Chemical) at 0.5 h post-FIP (5 µl/mouse), P-selectin blocking antibody (2 mg/kg in 0.1

ml of saline, time 0; clone RB40.34 purchased from BD Biosciences) or control immunoglobulin (2 mg/kg; purchased from Accurate Chemical). P-selectin is a key adhesion protein mediating platelet-endothelial interaction (20). To examine the role of activated coagulation, mice were injected intravenously with antithrombin (250 U/kg, 0.5 h), a glycoprotein IIb/IIIa inhibitor, eptifibatid (180 µg/kg, 6 h), or saline. Thrombin and glycoprotein IIb/IIIa are required for fibrin and thrombus formation (18). Finally, to assess the thrombogenic potential of the septic capillary bed at 6 h, we flooded the EDL muscle with a FeCl₃ solution (50 mmol/l in saline, 0.1 ml volume, 5 min) and then examined capillary blood flow. Topical application of FeCl₃ is a conventional approach to assess thrombosis in microvessels (33).

2.2.5 Experimental design: (b) Roles ROS and NO in platelet adhesion in the septic capillary bed

To examine the role of ROS, mice were injected intravenously with the antioxidant ascorbate (10 mg/kg; freshly dissolved, 0 or 6 h) or saline. Alternatively, we used mice with genetically deleted gp91phox, a subunit of NADPH oxidase (major source of ROS in septic microvasculature (34)). To assess the role of iNOS-derived NO, we used iNOS^{-/-} mice. To examine the role of local NO available near capillaries, we flooded the EDL muscle with the NOS cofactor tetrahydrobiopterin (BH₄; 0.1 µmol/l, in repeated 0.1 ml bolus applications over 1 h), the NO donor S-nitroso-N-acetylpenicillamine (SNAP; 5 µmol/l in one 0.1 ml bolus), decomposed SNAP, or saline. The glass coverslip normally covering the muscle surface was slightly lifted to permit introduction of the bolus between the muscle surface and coverslip. To determine if the effects of ascorbate and

BH₄ treatments were eNOS-dependent, ascorbate/BH₄ treatments were done in eNOS^{-/-} mice.

2.2.6 Statistics

Data are presented as mean \pm SE; n indicates the number of mice (one muscle/mouse).

Data were analyzed by Student t-test or ANOVA followed by Bonferroni multiple comparisons test. Significance was assigned as $p < 0.05$.

2.3 RESULTS

2.3.1 Role of platelet and coagulation functions in blood flow stoppage

Sepsis markedly increased blood flow stoppage in capillaries in mice (Figure 2.1; control data in naive and sham-injected mice did not differ, and were therefore pooled here). No stoppage was seen in arterioles or venules. Sepsis also markedly increased platelet adherence and fibrin deposition in capillaries (Figure 2.1). We observed single-adhering platelets and aggregates up to 5 platelets. Platelets preferentially adhered in stopped-flow capillaries (4.5 ± 1.0 , $n = 7$) rather than in perfused capillaries (0.2 ± 0.1 platelets/mm, $n = 7$). The number of adherent leukocytes in capillaries was negligible (0.11 ± 0.04 leukocytes/mm in septic mice; not significantly different from zero in control mice, $n = 5$). Formed fibrin occurred only in stopped-flow capillaries; none was seen in arterioles/venules. We also determined platelet adhesion specifically in capillaries with formed fibrin. Platelet adhesion in these specific septic capillaries was more extensive (3.6 ± 0.8 platelets/mm, $n = 4$) than adhesion in any septic capillaries (1.6 /mm, Figure

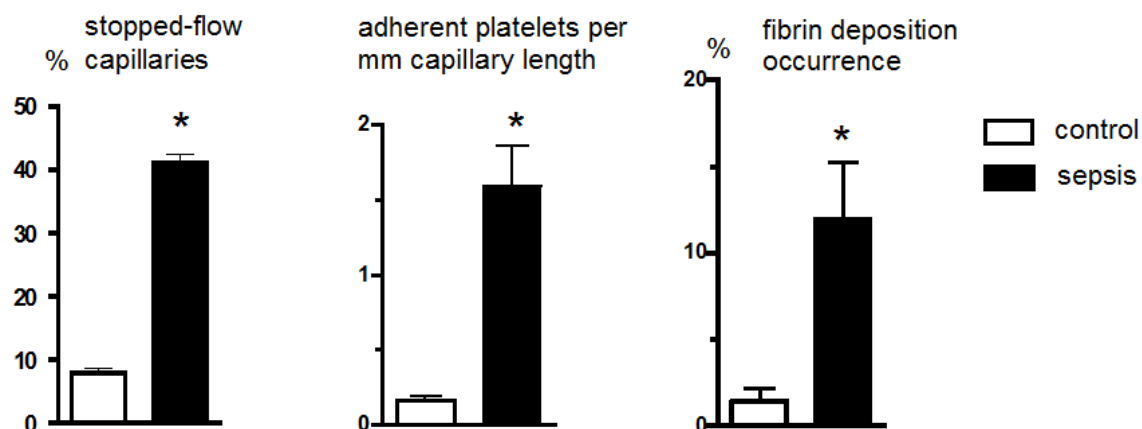


Figure 2.1. Sepsis increases blood flow stoppage, platelet adhesion and fibrin deposition in capillaries in mouse skeletal muscle. Sepsis was induced by feces injection into peritoneum (FIP). Flow stoppage was determined in 18 control and 16 septic mice at 6-7 h, adhesion in 9 control and 7 and septic mice at 7 h, and fibrin deposition in 2 control and 4 and septic mice at 7 h post-FIP. * Difference from control, $p < 0.05$.

2.1), confirming that septic platelet adhesion and fibrin deposition occurred in the same capillaries.

Platelet-depleting antibody dramatically lowered serum platelet counts in control (from 850 ± 50 to 40 ± 20) and septic mice (610 ± 40 to $60 \pm 20 \times 10^9/l$) ($n = 8-13$). In septic mice, the antibody decreased the abundance of stopped-flow capillaries (41 ± 2 to $28 \pm 2\%$, $p < 0.05$, $n = 16, 12$, respectively), implicating platelets in septic impairment of capillary flow.

P-selectin blockade, antithrombin and eptifibatide significantly reduced platelet adhesion and blood flow stoppage in septic capillaries (Figure 2.2), indicating that P-selectin and coagulation activation were required for this adhesion/stoppage. Injection of control immunoglobulin did not affect septic adhesion/stoppage (1.32 ± 0.10 platelets/mm; $41 \pm 3\%$ stopped-flow capillaries, $n = 5$). Finally, $FeCl_3$ was topically applied on the EDL surface. Topical application of $FeCl_3$ on microvessels is a conventional approach to assess the thrombogenic potential in these microvessels (33). Fifty micromolar of $FeCl_3$ in a 0.1 ml volume applied for 5 minutes stopped flow more extensively in septic than control capillaries (Figure 2.3), suggesting that sepsis increased the propensity of microthrombi formation in capillaries.

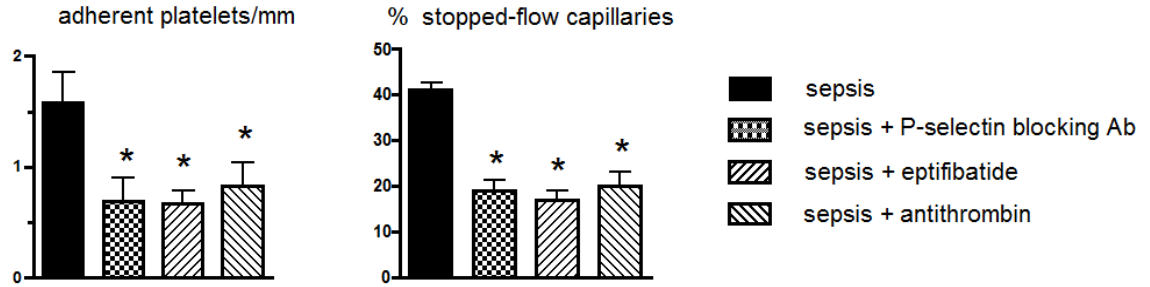


Figure 2.2. Effect of P-selectin blocking antibody, eptifibatide, and antithrombin on platelet adhesion and blood flow stopping. Injections of P-selectin blocking antibody at 0.5 h, eptifibatide at 6 h (glycoprotein IIb/IIIa inhibitor), and antithrombin at 0.5 h inhibited platelet adhesion and blood flow stopping in capillaries at 6-7 h post-FIP. * Difference from sepsis, $p < 0.05$, $n = 5-9$ for adhesion groups, and 5-16 for stopped-flow groups.

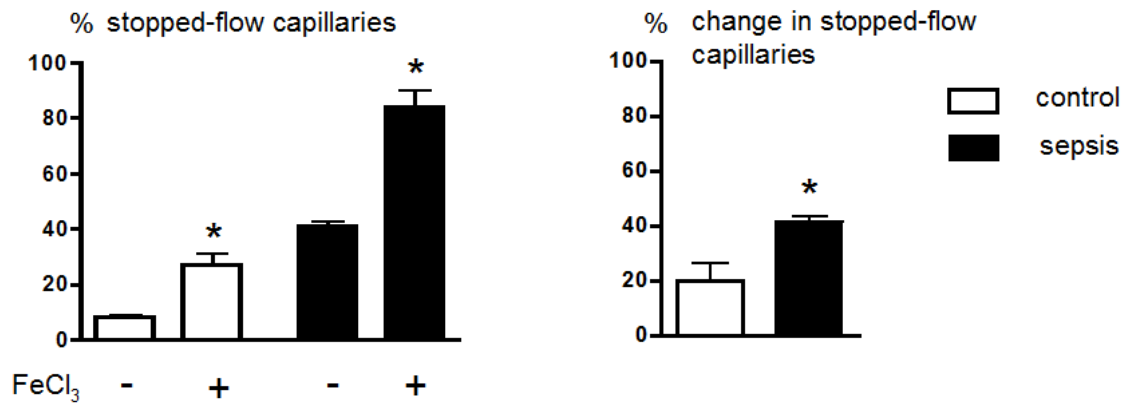


Figure 2.3. Sepsis increases thrombosis in capillaries. The thrombosis potential was tested by flooding the muscle with FeCl₃ at 6 h post-FIP. *Left panel:* * Difference between post-FeCl₃ and pre-FeCl₃ percentage of stopped-flow capillaries, $p < 0.05$, $n = 5-18$. *Right:* Change in stopped-flow percentage = (post-FeCl₃ percentage) - (pre-FeCl₃ percentage). * Difference from control, $p < 0.05$, $n = 5$ per group.

2.3.2 Role of ROS and NO

Prophylactic injection of ascorbate, or gp91phox deletion, prevented platelet adhesion and flow stoppage in septic capillaries (Figure 2.4). Moreover, delayed injection of ascorbate reversed septic adhesion/stoppage (Figure 2.5). These latter effects were eNOS-dependent, since they were absent in eNOS^{-/-} mice (Figure 2.5). BH₄ and NO donor SNAP at 6-7 h significantly reduced septic platelet adhesion and flow stoppage (Figure 2.6, top). Flooding of the muscle surface with decomposed SNAP or saline did not affect septic flow stoppage (44 ± 2 and 47 ± 2 % stopped-flow capillaries, n = 7, 3, respectively, versus 41 ± 2 %, n = 16 in non-flooded septic muscles). The reversal effects of BH₄ were eNOS-dependent, since they were absent in eNOS^{-/-} mice (Figure 2.6). Consistently, the effects of SNAP did not depend on eNOS (Figure 2.6). eNOS knockout did not affect blood flow stoppage and platelet adhesion in septic capillaries (Figure 2.5), but iNOS knockout inhibited platelet adhesion (Figure 2.6, bottom). Flow stoppage in control gp91phox^{-/-}, eNOS^{-/-} and iNOS^{-/-} mice (12 ± 2, 12 ± 5, and 5 ± 1 % stopped-flow capillaries, p < 0.05, n = 3, 5, 4, respectively) was not statistically different from that of control wild type mice (8 ± 1 %, n = 18) (Figure 2.1).

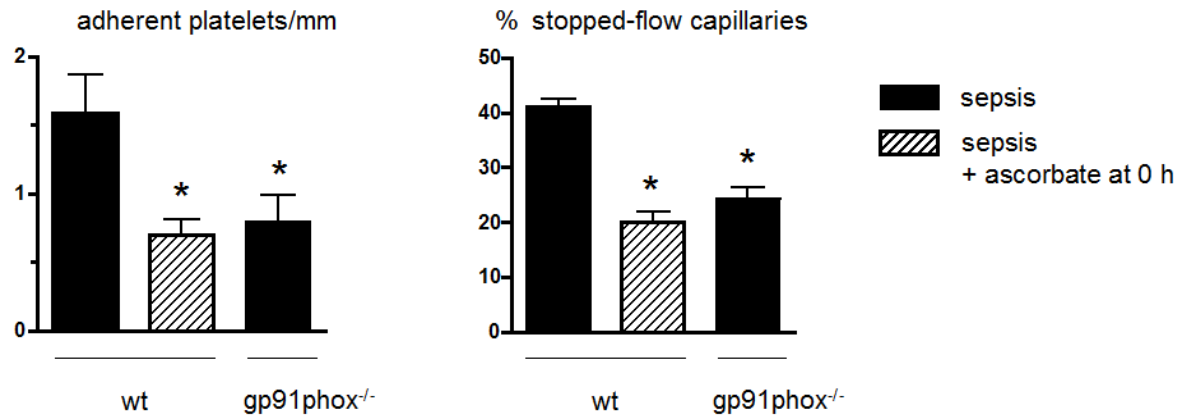


Figure 2.4. Effect of ascorbate and gp91phox knockout on platelet adhesion and blood flow stopping. Ascorbate injection at 0 h and gp91phox knockout prevent platelet adhesion and blood flow stopping in capillaries at 6-7 h post-FIP. * Difference from sepsis wild type (wt) group, $p < 0.05$, $n = 6-7$ for adhesion groups, and 5-16 for stopped-flow groups.

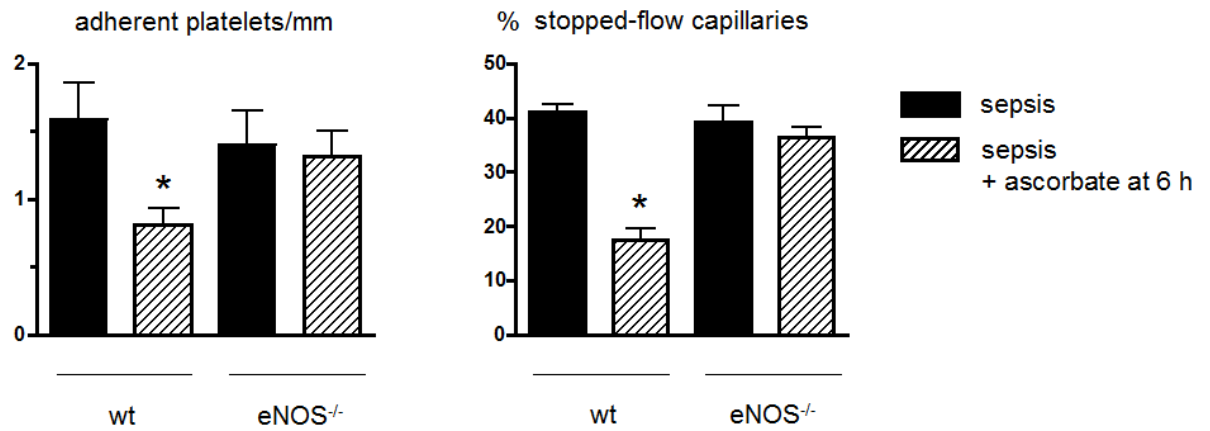


Figure 2.5. Effect of ascorbate on platelet adhesion and blood flow stoppage in wild type and eNOS knockout mice. Ascorbate injection at 6 h reverses platelet adhesion and blood flow stoppage in capillaries at 7 h post-FIP in wild type but not eNOS^{-/-} mice. * Difference from sepsis group, $p < 0.05$, $n = 6-17$ for adhesion groups, and $8-16$ for stopped-flow groups.

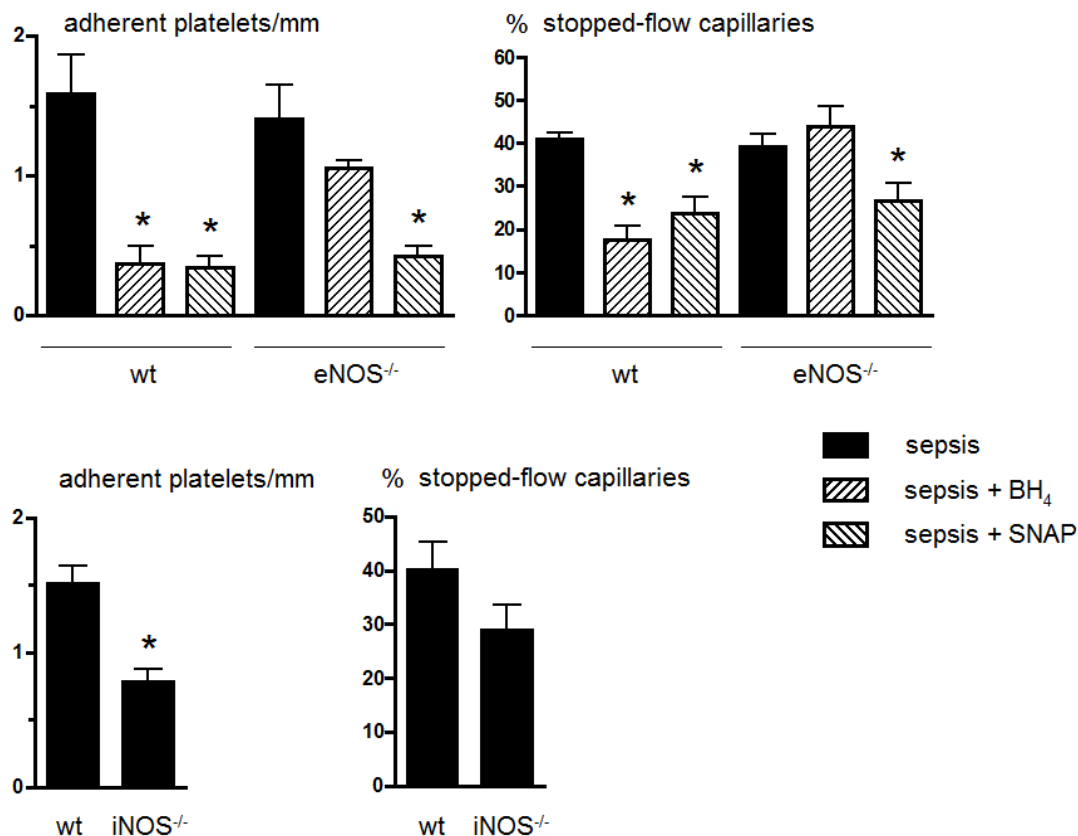


Figure 2.6. Effects of tetrahydrobiopterin (BH₄) and the NO donor SNAP on platelet adhesion and blood flow stoppage in capillaries of wild type and eNOS^{-/-} mice (top), and effect of iNOS knockout on adhesion/stoppage (bottom). At 6 h, 0.1 ml boluses of BH₄ (repeated over 1 h period) flooded the muscle surface to determine platelet adhesion and flow stoppage at 7 h post-FIP. Alternatively, a single 0.1 ml bolus of SNAP flooded the surface, and platelet adhesion and stoppage were determined 15 min later (when the temporary SNAP-induced vasodilation had ended). BH₄ reversed platelet adhesion and flow stoppage in septic capillaries of wild type but not eNOS^{-/-} mice. SNAP reversed platelet adhesion and flow stoppage in both types of mice. iNOS knockout inhibited septic platelet adhesion at 6 h post-FIP. * Difference from appropriate sepsis group, $p < 0.05$, $n = 5-10$ for adhesion groups, and 5-16 for stopped-flow groups in top row, and 6 and 10 for adhesion, and 6 and 10 for stopped-flow groups at bottom, respectively.

2.4 DISCUSSION

The present study addressed the mechanism of blood flow stoppage in septic capillaries. We report for the first time that (i) sepsis markedly increases platelet adhesion, fibrin deposition and propensity of thrombosis in capillaries, (ii) capillary flow stoppage requires platelets, P-selectin, and coagulation activation, and (iii) capillary platelet adhesion can be prevented or reversed by gp91phox and iNOS deficiencies, ascorbate, and local BH₄ and exogenous NO.

2.4.1 Role of platelet and coagulation functions in blood flow stoppage

In the present study, platelets adhered in septic capillaries and venules consistent with the literature (30). However, unlike in capillaries, adhering platelets in venules were not associated with fibrin deposition or stopped blood flow, indicating that rheological findings in larger microvessels cannot be extended to capillaries. In view of negligible leukocyte adhesion observed in capillaries, we sought leukocyte-independent mechanism(s) of capillary plugging in sepsis.

Possible mechanisms could be platelet activation, aggregation, and subsequent critical narrowing/plugging of the capillary lumen by adhering platelets (17, 32), or plugging by adhering platelets and formed microthrombi within the capillary. LPS and inflammatory cytokines increase expression of P-selectin and von Willebrand factor on endothelial cells to initiate platelet adhesion (4, 16). LPS and cytokines also increase tissue factor expression at the cell membrane of monocytes and endothelial cells (15), initiating coagulation. The present data are consistent with the mechanism of platelet

adhesion and microthrombi formation. Sepsis increased platelet adhesion in capillaries, whereas platelet depletion and blockade of P-selectin significantly reduced septic blood flow stoppage (Figure 2.2). Sepsis-induced platelet adhesion and fibrin deposition were seen to co-localize in stopped-flow capillaries, while anticoagulants decreased septic platelet adhesion and flow stoppage in capillaries (Figures 2.1, 2.2).

In critically ill patients, a 30 % drop in platelet count independently predicts death (21). In the present study, a comparable 28 % drop occurred in septic mice. Since the life span of platelets is longer than 6 h (22), we hypothesize that most of this platelet consumption is due to platelet adhesion in the microcirculation. To estimate how platelet adhesion in capillaries predicts septic blood flow stoppage, we used the present data to plot the mean values of stopped-flow capillaries (%) versus values of platelet adhesion/mm (Figure 2.7). The plot has a significant linear correlation, indicating that platelet adhesion in capillaries predicts ~ 90 % of capillary blood flow stoppage. If decreased platelet count were due to platelet adhesion in capillaries, then the 28 % drop implicates a substantial plugging of the capillary bed (e.g., 40 % plugging in skeletal muscle).

2.4.2 Role of ROS and NO in septic capillaries

We showed that sepsis increases ROS production in mouse skeletal muscle (35) and that septic capillary blood flow impairment depends on NADPH oxidase (29). ROS promote expression of P-selectin at the surface of platelets and endothelial cells, and enhance platelet adhesion to the endothelium and coagulation (12, 15, 24). Our data are consistent with ROS-mediated blood flow stoppage due to enhanced platelet adhesion in capillaries. The

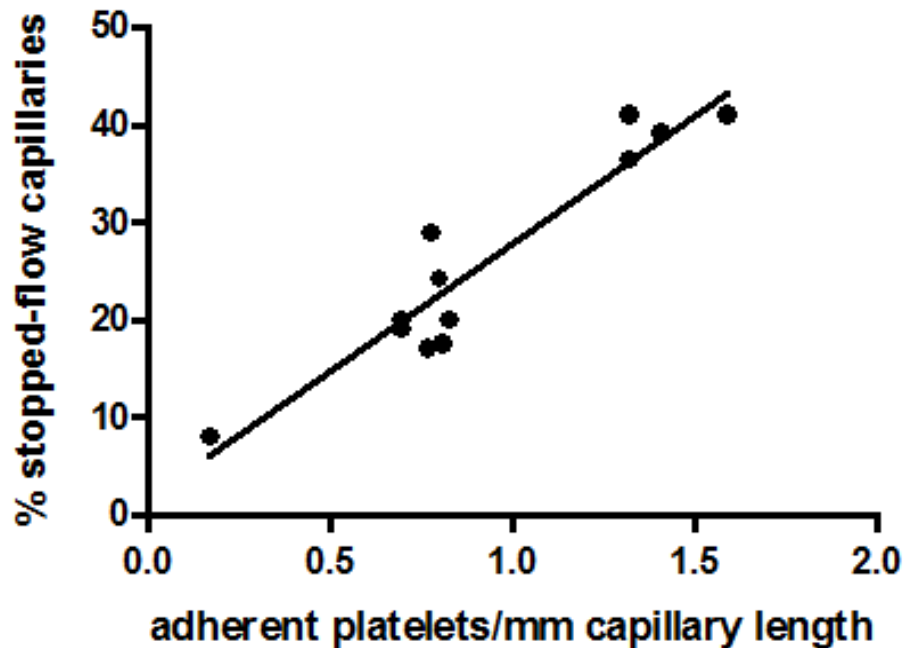


Figure 2.7. Platelet adhesion in septic capillaries predicts capillary blood flow

impairment. The average values of percent stopped-flow capillaries in experiments of Figures 2.1, 2.2, 2.4, 2.5 and 2.6 (bottom) were plotted against the corresponding average values of adherent platelets/mm. In these experiments, no systematic difference existed between the treatment/concentration of agents in surface and deep capillaries (e.g., agents were injected intravenously, rather than introduced by flooding of the muscle surface). Computed line of linear regression has $r^2 = 0.89$ and slope different from 0, $p < 0.05$.

antioxidant ascorbate and gp91phox knockout prevented/reversed septic platelet adhesion and flow stoppage (Figures 2.4, 2.5) (an effect consistent with ascorbate's ability to significantly improve septic mouse survival at 24 h) (29, 35).

Recently we proposed that increased ROS level in sepsis oxidizes the eNOS cofactor BH₄, uncouples it from eNOS in platelets and endothelial cells and thus stops NO production in these cells (29). Because low physiological levels of NO are anti-aggregatory and anti-adhesive (24), uncoupled eNOS promotes platelet adhesion/aggregation and flow cessation in capillaries. The present data are consistent with this proposed mechanism. BH₄ or NO applied locally reversed platelet adhesion and flow stoppage in septic capillaries (Figure 2.6). Further, the beneficial effects of ascorbate and BH₄ were eNOS-dependent (Figures 2.5, 2.6).

Knockout of iNOS reduced septic platelet adhesion (Figure 2.6, bottom), suggesting that iNOS-derived NO is pro-adhesive. At first glance, this result contradicts the beneficial effect of local NO (Figure 2.6, top). To reconcile these observations, we note that iNOS enzymatic activity is negligible in the mouse skeletal muscle at 6 h of sepsis (29) and hypothesize that (i) iNOS activity is higher in other septic tissues and (ii) NO overproduction here could promote platelet adhesion in the skeletal muscle. Excess NO could react with superoxide, form peroxynitrite (24), and lead to activation/priming of blood-borne platelets to adhere in tissues. Increased iNOS expression results in decreased eNOS activity and expression (27) further reducing the local production of NO. The apparent opposite effects of NO (Figure 2.6) underscore the complex role NOS/NO may play during sepsis (5, 14).

2.4.3 Methodological limitations

The use of the platelet-depleting antibody to study the role of platelets in septic blood flow stoppage was problematic. Both control and septic mice injected with the antibody were noticeably sicker than their non-injected counterparts (mice hunched in the cage, had erected fur, and did not respond to tactile stimuli). Further, the antibody significantly increased (40 %) capillary flow stoppage in control mice. Consistent with reported increased mortality in mice by platelet-depleting antibody (10), the present deleterious effects of the antibody on animal health might have obscured the full beneficial effect of platelet depletion against septic capillary flow stoppage in skeletal muscle.

Finally, the present value of adherent platelets/mm in sepsis may underestimate the actual platelet adherence, since plugged septic capillaries may not permit plasma flow and detection of platelets with a fluorescent dye. Capillary obstructions could also limit the full impact of agents injected at 6 h on platelet adhesion and blood flow stoppage studied at 7 h.

In conclusion, we demonstrated that polymicrobial sepsis increases platelet adhesion and fibrin deposition in skeletal muscle capillaries and that septic impairment of capillary blood flow requires platelet adhesion, P-selectin and activated coagulation. Since platelet adhesion and capillary flow impairment can be inhibited by the antioxidant ascorbate and exogenous NO, administration of ascorbate and/or NO donors to attenuate platelet accumulation in septic capillaries is an important consideration for future development of novel adjuvant therapies for sepsis.

2.5 REFERENCES

1. Al-Bahrani A, Taha S, Shaath H, Bakhiet M. TNF-alpha and IL-8 in acute stroke and the modulation of these cytokines by antiplatelet agents. *Curr Neurovasc Res* 4(1): 31-37, 2007.
2. Amaral A, Opal SM, Vincent JL. Coagulation in sepsis. *Intensive Care Med* 30(6): 1032-1040, 2004.
3. Bateman RM, Walley KR. Microvascular resuscitation as a therapeutic goal in severe sepsis. *Crit Care* 9 Suppl 4: S27-32, 2005.
4. Bergmeier W, Chauhan AK, Wagner DD. Glycoprotein Ibalpha and von Willebrand factor in primary platelet adhesion and thrombus formation: lessons from mutant mice. *Thromb Haemost* 99(2): 264-270, 2008.
5. Cauwels A. Nitric oxide in shock. *Kidney Int* 72(5): 557-565, 2007.
6. Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, Patel KD, Chakrabarti S, McAvoy E, Sinclair GD, Keys EM, Allen-Vercoe E, Devinney R, Doig CJ, Green FH, Kubes P. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med* 13(4): 463-469, 2007.
7. Daudel F, Kessler U, Folly H, Lienert JS, Takala J, Jakob SM. Thromboelastometry for the assessment of coagulation abnormalities in early and established adult sepsis: a prospective cohort study. *Crit Care* 13(2): R42, 2009.

8. Draisma A, Bemelmans R, van der Hoeven JG, Spronk P, Pickkers P. Microcirculation and vascular reactivity during endotoxemia and endotoxin tolerance in humans. *Shock* 31(6): 581-585, 2009.
9. Ellis CG, Bateman RM, Sharpe MD, Sibbald WJ, Gill R. Effect of a maldistribution of microvascular blood flow on capillary O₂ extraction in sepsis. *Am J Physiol Heart Circ Physiol* 282(1): H156-64, 2002.
10. Fujimi S, MacConmara MP, Maung AA, Zang Y, Mannick JA, Lederer JA, Lapchak PH. Platelet depletion in mice increases mortality after thermal injury. *Blood* 107(11): 4399-4406, 2006.
11. Jeong YI, Jung ID, Lee CM, Chang JH, Chun SH, Noh KT, Jeong SK, Shin YK, Lee WS, Kang MS, Lee SY, Lee JD, Park YM. The novel role of platelet-activating factor in protecting mice against lipopolysaccharide-induced endotoxic shock. *PLoS One* 4(8): e6503, 2009.
12. Krotz F, Sohn HY, Pohl U. Reactive oxygen species: players in the platelet game. *Arterioscler Thromb Vasc Biol* 24(11): 1988-1996, 2004.
13. Lam C, Tyml K, Martin C, Sibbald W. Microvascular perfusion is impaired in a rat model of normotensive sepsis. *J Clin Invest* 94(5): 2077-2083, 1994.
14. Lange M, Enkhbaatar P, Nakano Y, Traber DL. Role of nitric oxide in shock: the large animal perspective. *Front Biosci* 14: 1979-1989, 2009.

15. Levi M, van der Poll T, Buller HR. Bidirectional relation between inflammation and coagulation. *Circulation* 109(22): 2698-2704, 2004.
16. Lindenblatt N, Schareck W, Belusa L, Nickels RM, Menger MD, Vollmar B. Anti-oxidant ebselen delays microvascular thrombus formation in the rat cremaster muscle by inhibiting platelet P-selectin expression. *Thromb Haemost* 90(5): 882-892, 2003.
17. Lou J, Donati YR, Juillard P, Giroud C, Vesin C, Mili N, Grau GE. Platelets play an important role in TNF-induced microvascular endothelial cell pathology. *Am J Pathol* 151(5): 1397-1405, 1997.
18. Mannel DN, Grau GE. Role of platelet adhesion in homeostasis and immunopathology. *Mol Pathol* 50(4): 175-185, 1997.
19. Mariscalco MM. Unlocking (perhaps unblocking) the microcirculation in sepsis. *Crit Care Med* 34(2): 561-562, 2006.
20. McEver RP. P-selectin and PSGL-1: exploiting connections between inflammation and venous thrombosis. *Thromb Haemost* 87(3): 364-365, 2002.
21. Moreau D, Timsit JF, Vesin A, Garrouste-Orgeas M, de Lassence A, Zahar JR, Adrie C, Vincent F, Cohen Y, Schlemmer B, Azoulay E. Platelet count decline: an early prognostic marker in critically ill patients with prolonged ICU stays. *Chest* 131(6): 1735-1741, 2007.
22. Najean Y, Ardaillou N, Dresch C. Platelet lifespan. *Annu Rev Med* 20: 47-62, 1969.

23. Otterdal K, Smith C, Oie E, Pedersen TM, Yndestad A, Stang E, Endresen K, Solum NO, Aukrust P, Damas JK. Platelet-derived LIGHT induces inflammatory responses in endothelial cells and monocytes. *Blood* 108(3): 928-935, 2006.
24. Ovechkin AV, Lominadze D, Sedoris KC, Robinson TW, Tyagi SC, Roberts AM. Lung ischemia-reperfusion injury: implications of oxidative stress and platelet-arteriolar wall interactions. *Arch Physiol Biochem* 113(1): 1-12, 2007.
25. Reheman A, Gross P, Yang H, Chen P, Allen D, Leytin V, Freedman J, Ni H. Vitronectin stabilizes thrombi and vessel occlusion but plays a dual role in platelet aggregation. *J Thromb Haemost* 3(5): 875-883, 2005.
26. Sakr Y, Dubois MJ, De Backer D, Creteur J, Vincent JL. Persistent microcirculatory alterations are associated with organ failure and death in patients with septic shock. *Crit Care Med* 32(9): 1825-1831, 2004.
27. Scott JA, Mehta S, Duggan M, Bihari A, McCormack DG. Functional inhibition of constitutive nitric oxide synthase in a rat model of sepsis. *Am J Respir Crit Care Med* 165(10): 1426-1432, 2002.
28. Stamme C, Bundschuh DS, Hartung T, Gebert U, Wollin L, Nusing R, Wendel A, Uhlig S. Temporal sequence of pulmonary and systemic inflammatory responses to graded polymicrobial peritonitis in mice. *Infect Immun* 67(11): 5642-5650, 1999.
29. Tyml K, Li F, Wilson JX. Septic impairment of capillary blood flow requires nicotinamide adenine dinucleotide phosphate oxidase but not nitric oxide synthase and is

rapidly reversed by ascorbate through an endothelial nitric oxide synthase-dependent mechanism. *Crit Care Med* 36(8): 2355-2362, 2008.

30. Vachharajani V, Vital S, Russell J, Granger DN. Hypertonic saline and the cerebral microcirculation in obese septic mice. *Microcirculation* 14(3): 223-231, 2007.

31. Vincent JL. Clinical sepsis and septic shock--definition, diagnosis and management principles. *Langenbecks Arch Surg* 393(6): 817-824, 2008.

32. Wassmer SC, de Souza JB, Frere C, Candal FJ, Juhan-Vague I, Grau GE. TGF-beta1 released from activated platelets can induce TNF-stimulated human brain endothelium apoptosis: a new mechanism for microvascular lesion during cerebral malaria. *J Immunol* 176(2): 1180-1184, 2006.

33. Westrick RJ, Winn ME, Eitzman DT. Murine models of vascular thrombosis (Eitzman series). *Arterioscler Thromb Vasc Biol* 27(10): 2079-2093, 2007.

34. Wu F, Tysl K, Wilson JX. iNOS expression requires NADPH oxidase-dependent redox signaling in microvascular endothelial cells. *J Cell Physiol* 217(1): 207-214, 2008.

35. Wu F, Wilson JX, Tysl K. Ascorbate protects against impaired arteriolar constriction in sepsis by inhibiting inducible nitric oxide synthase expression. *Free Radic Biol Med* 37(8): 1282-1289, 2004.

Chapter 3

Ascorbate reduces mouse platelet aggregation and surface P-selectin expression in an *ex vivo* model of sepsis

A version of this chapter has been published previously

Secor D, Swarbreck S, Ellis CG, Sharpe MD and Tymi K. Ascorbate reduces mouse platelet aggregation and surface P-selectin expression in an *ex vivo* model of sepsis.

Microcirculation, [ePub ahead of print], 2013.

3.1 INTRODUCTION

Sepsis is a systemic inflammatory response due to an infection. Sepsis results in circulatory dysfunction that can lead to multi-organ failure and death (6, 60). An exaggerated coagulation response contributes to the severity of the disease in the form of microvascular thrombosis, leading to disseminated intravascular coagulation (DIC) and organ dysfunction (28). Furthermore, it has been shown that a reduced platelet count in blood (i.e., an event contributing to DIC) correlates with higher mortality (27, 37). Adhering platelets and coagulation (fibrin deposition) in capillaries may lead to cessation of blood flow in these microvessels, an event frequently observed during sepsis (1, 26, 56, 57, Chapter 2). Cessation of flow will result in increased diffusional distance for oxygen to reach cell mitochondria, leading to tissue hypoxia and organ failure (14, 17).

It is known that septic plasma potentiates platelet activation (22). The resulting platelet aggregation and platelet-endothelial adhesion may be critical in capillary flow impairment in sepsis. Platelets can be activated by a large number of agonists including thrombin, adenosine diphosphate (ADP), thromboxane, serotonin, and collagen (15, 29). Thromboxane, serotonin, and ADP are subsequently released from activated platelets to further aggregation (42). Recently, reactive oxygen species (ROS) have been implicated to modulate platelet function (25). The levels of platelet-activating agonists and the level of ROS are increased during sepsis (12, 20, 32, 35, 36, 39, 55). The excess of ROS during sepsis will oxidize tetrahydrobiopterin which is a cofactor for endothelial nitric oxide synthase (eNOS). When tetrahydrobiopterin is oxidized, eNOS produces superoxide instead of nitric oxide (NO). Superoxide will interact with NO to form peroxynitrite, further reducing the bioavailability of NO. NO is an important factor in the homeostasis

of platelets and is a known inhibitor of platelet activation/adhesion (41). The antioxidant ascorbate (reduced vitamin C) has been shown to prevent and reverse the oxidation of tetrahydrobiopterin thus restoring the NO production via eNOS (23, 38).

Clinically, there is no effective pharmacological treatment for sepsis. Current management strategies aim to increase oxygen delivery to counteract the rising levels of lactate (13). One possible treatment that has been under investigation is the use of ascorbate (33). An intravenous bolus of ascorbate has been shown to increase survival of septic mice as well as to prevent and reverse the capillary blood flow impairment (1). Ascorbate also inhibits the sepsis-induced increase in adhesion of single platelets and their aggregates in capillaries (Chapter 2). Although several mechanisms have been proposed to explain the beneficial effect of ascorbate in the septic microvasculature (55), the precise mechanism of this effect is unclear. Ascorbate could act directly, because oxidative stress of the platelet plays an important role in the platelet activation (54), and also indirectly because it could restore NO production by the aforementioned eNOS pathway and thus inhibit platelet adhesion/aggregation (55, 56, Chapter 2).

We have recently reported that the treatment of septic mice with a blocking antibody against P-selectin reduces adhesion of single platelets/aggregates in capillaries (Chapter 2). P-selectin is a key platelet-endothelium and platelet-platelet adhesion molecule (5). In our study, it was not possible to determine whether the platelet and/or endothelial P-selectin are involved in platelet adhesion to capillaries. To address this issue, the present study employed a simpler *ex vivo* model of isolated platelets to examine (i) the effect of sepsis on the platelet surface P-selectin expression, and (ii) the effect of ascorbate on this expression. We have also reported that the inhibitory effect of ascorbate

against adhesion of platelet aggregates in septic capillaries was eNOS-dependent (Chapter 2). However, we could not differentiate whether the endothelial and/or platelet eNOS were involved. In the present study, we used the *ex vivo* model to examine the platelet eNOS involvement. Overall, we hypothesized that the surface P-selectin expression and aggregability of activated platelets are reduced by ascorbate in an eNOS-dependent manner.

3.2 METHODS

3.2.1 Reagents used

Lipopolysaccharide (LPS), tumor necrosis factor alpha (TNF α), ADP, N-Nitro-L-arginine methyl ester (LNAME), N-Nitro-D-arginine methyl ester (DNAME), S-Nitroso-N-acetyl-DL-penicillamine (SNAP), and ascorbate were purchased from Sigma-Aldrich (St. Louis, MO). U46619, a thromboxane A₂ receptor agonist, was purchased from Enzo Life Sciences (Farmingdale, NY). Serotonin was purchased from Alfa Aesar (Ward Mill, MA). The phycoerythrin (PE) conjugated P-selectin antibody (clone: Wug.E9) and PE conjugated IgG control were purchased from Emfret Analytics (Eibelstadt, Germany). A fluorescein isothiocyanate (FITC) conjugated CD41 antibody was purchased from BD Pharmingen (Mississauga, ON). Eptifibatide was from Shering-Plough (Kenilworth, NJ).

3.2.2 Experimental design

To investigate the effect of sepsis on platelet aggregation and P-selectin surface expression *ex vivo*, we used plasma from septic mice and the conventional septic

mediators LPS and TNF α . We also used platelet-activating agents known to be released into bloodstream during sepsis, namely thrombin, ADP, serotonin and U46619 (thromboxane A2 analog) (12, 20, 35, 37, 39). The involvement of NOS in the responses to the activating agents was examined by using the non-selective inhibitor LNAME.

3.2.3 Platelet collection and isolation, and plasma collection.

Experimental protocols were approved by the University of Western Ontario Council on Animal Care. Wild type C57BL/6 mice (weight: 18-25 g, age: 1.5 - 4 months) were obtained from Charles River (Sherbrooke, QE) and bred in our animal facility. We collected platelets from control male mice anaesthetized with ketamine and xylazine (Chapter 2). Blood was collected via carotid artery puncture into an acid citrate dextrose solution in a 6:1 blood to solution ratio. Platelets were isolated as described previously (53). Briefly, platelet rich plasma was collected by centrifuging the whole blood at 120 g for 8 min. The plasma was then re-spun to remove any collected leukocytes. Finally, the platelets were pelleted at 735 g for 10 min and resuspended in calcium free Tyrode's buffer. Immediately before testing, normal Tyrode's buffer was added (1:5 ratio) to the platelet solution.

We also used the blood collection procedure to obtain plasma from septic mice. Mice were made septic by fecal injection into peritoneum as previously described (56, Chapter 2). Blood was collected 4 h post fecal injection.

3.2.4 Platelet aggregation

Platelet aggregation was measured using a custom built Born aggregometer (7). Briefly,

isolated platelets were placed in a specialized cuvette and treated with thrombin at 0.075 U/ml (i.e., the EC₅₀ value from dose-response curve, Figure 3.1) or 0.0375 U/ml, ADP (1 μM), U46619 (320 nM), serotonin (10 μM), LPS (10 μg/ml), or TNFα (10 ng/ml) at effective concentrations published in the literature (3, 19, 30, 48, 58, 62). Some experimental groups of platelets were treated simultaneously with ascorbate (100 μM), LNAME (5 mM), DNAME (non-functional LNAME analogue, 5 mM), SNAP (1 μM), inactive SNAP (10 μM, exposed to room air for at least 24 h), eptifibatide (clinically used anti-aggregatory agent, 1.8 μg/ml), and/or septic plasma (1:1 ratio with normal Tyrode's buffer solution with platelets). The platelets were continuously stirred and the light transmittance was recorded. Aggregation was determined as the change in light transmittance in the aggregometer from the initial light transmittance reading prior to adding any platelet-activating agent.

3.2.5 Flow cytometry

Isolated platelets were treated with thrombin (0.0075 - 7.5 U/ml), LPS (10 μg/ml), or TNFα (10 ng/ml) for 5 min at room temperature. Some experimental groups of platelets were treated simultaneously with ascorbate (100 μM), LNAME (5 mM), and/or DNAME (5 mM). Platelets were then incubated with a PE conjugated P-selectin antibody (clone: Wug.E9) and a FITC conjugated CD41 antibody (platelet specific marker) for 15 min prior to stopping the experiment by addition of phosphate buffered solution. As a negative control, a PE conjugated IgG was used. The labeled platelets were then analyzed with a flow cytometer (Coulter EPICS XL-MCL, Beckman Coulter, Brea, CA, USA). The percent of total events indicated the number of events that were both P-selectin and

CD41 positive over the total number of detectable signals within the assigned region of the scatter plot (forward scatter 70 – 400, side scatter 6 – 60).

3.2.6 *Statistical analysis*

Data are presented as mean \pm SE; n indicates the number of separate platelet isolations.

Blood from one mouse was used for each isolation. Data were analyzed by student t test or ANOVA followed by Bonferroni multiple comparisons test. Significance was assigned as $p < 0.05$.

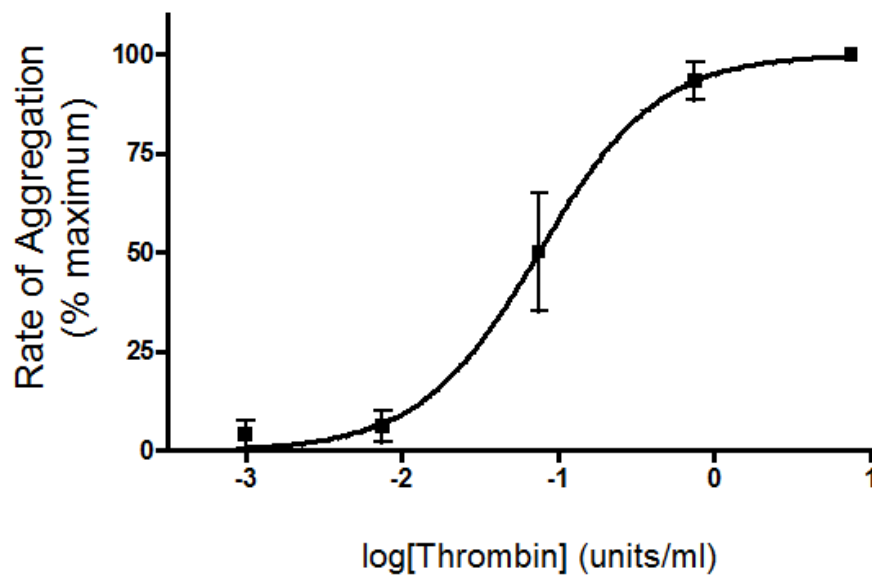


Figure 3.1. Dose response to thrombin on mouse platelet aggregation ex vivo.

Isolated platelets were treated with thrombin (0.001, 0.0075, 0.075, 0.75, 7.5 units/ml) and then placed in an aggregometer. Aggregation was defined as the change in light transmittance normalized to transmittance at time 0 for each experiment. The initial rate of aggregation is shown as the percentage of the maximum rate (i.e., caused by thrombin) per experiment. EC₅₀ value is 0.075 units/ml. n = 6 per group.

3.3 RESULTS

3.3.1 LPS and TNF α do not initiate platelet aggregation nor increase P-selectin surface expression

LPS or TNF α did not affect platelet aggregation (Figure 3.2). This figure shows a strong effect of thrombin (0.075 U/ml) used as positive control. The standard error bars represent the day-to-day variability of the platelet aggregation. LPS did not potentiate the aggregation caused by thrombin at a sub-threshold concentration of 0.0075 U/ml (i.e., initial rates of aggregation were negligible for thrombin at 0.25 ± 0.16 and for thrombin+LPS at 0.21 ± 0.15 % maximum; n = 5 per group). Furthermore, collected septic plasma applied for 5 min did not cause aggregation and also it did not potentiate platelet aggregation caused by thrombin (initial rate of aggregation for septic plasma was 0.01 ± 0.01 and for septic plasma+thrombin (0.0075 U/ml) it was 0.01 ± 0.01 % maximum; n = 8 per group). Septic plasma applied for 1 h did not cause aggregation and did not potentiate the effect of thrombin at 0.0075 U/ml (initial rate of aggregation for septic plasma was 0.01 ± 0.01 and for septic plasma+thrombin it was 0.01 ± 0.01 % maximum; n = 6 per group).

Using flow cytometry, we measured the P-selectin surface expression on the platelets after treatment with LPS and TNF α . Neither LPS nor TNF α increased the P-selectin expression (Figure 3.3). Again, thrombin (0.075 U/ml) was used as positive control.

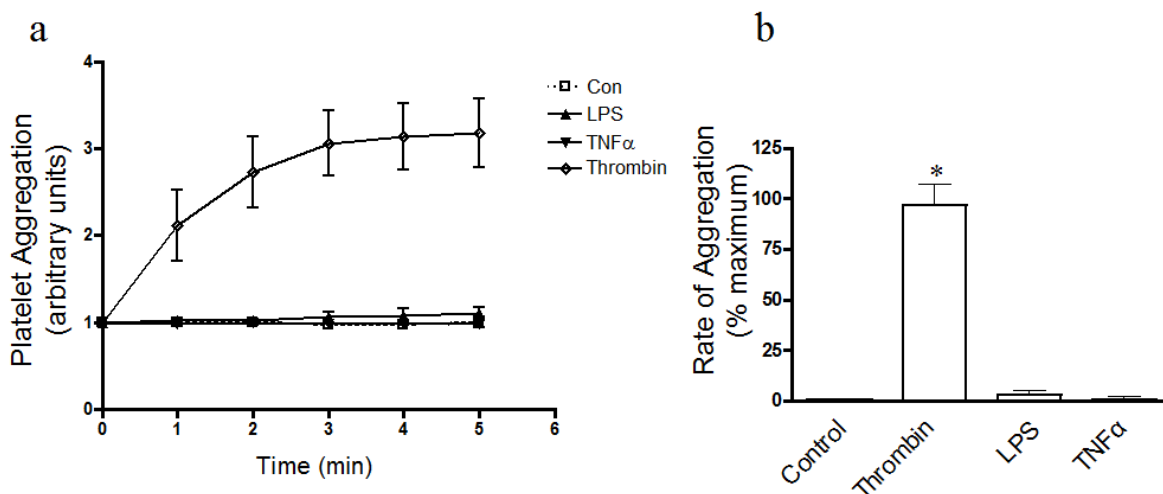


Figure 3.2. LPS and TNF α have no effect on mouse platelet aggregation *ex vivo*.

Panel a: Isolated platelets were treated with LPS (10 μ g/ml), TNF α (10 ng/ml), or thrombin (0.075 units/ml) and were placed in an aggregometer. Aggregation was defined as the change in light transmittance normalized to transmittance at time 0 for each experiment. Platelet aggregation was measured over 5 minutes. Panel b: The initial rate of aggregation is shown as the percentage of the maximum rate (i.e., caused by thrombin) per experiment. * $P < 0.05$ compared to control, $n = 5$ per group.

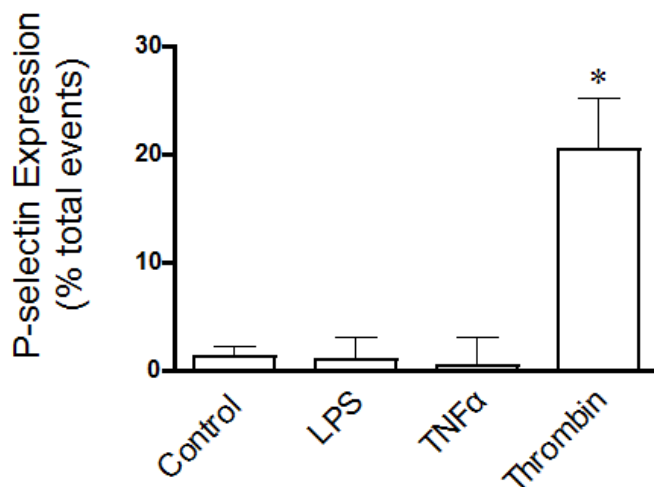


Figure 3.3. Effect of LPS, TNF α , and thrombin on P-selectin surface expression on isolated platelets. Isolated platelets treated with LPS (10 μ g/ml), TNF α (10 ng/ml), or thrombin (0.075 units/ml) for 20 min were examined for surface P-selectin expression by flow cytometry. LPS or TNF α had no effect. Thrombin significantly increased P-selectin surface expression. P-selectin surface expression is represented by percent of events that were both positive for P-selectin and CD41 (platelet marker). * $P < 0.05$ compared to control, $n = 5$ per group.

3.3.2 Ascorbate reduces platelet aggregation to thrombin, ADP, and U46619

Thrombin, ADP, and U46619 (thromboxane A2 analog) caused platelet aggregation as expected (Figures 3.4 a-f). Serotonin did not initiate aggregation in our model (initial rate of aggregation was 2.09 ± 1.22 versus 1.54 ± 0.86 % maximum in untreated controls, n = 5 and 8, respectively). Ascorbate inhibited the initial rate of aggregation due to thrombin, ADP, and U46619 by 44, 20.4, and 51.5 %, respectively (Figure 3.4). In comparison with ascorbate, eptifibatide similarly inhibited the thrombin-induced aggregation (initial rate of aggregation was 40.2 ± 4.50 % maximum, n = 6). The inhibition by ascorbate depended on the concentration of thrombin employed to aggregate platelets. When the lower concentration was used (i.e., 0.0375 U/ml), ascorbate inhibited aggregation by 74 % (initial rate of aggregation was 25.8 ± 9.3 % compared to thrombin alone, n = 5). Ascorbate alone had no effect on platelet aggregation (initial rate of aggregation was 2.38 ± 1.50 versus 1.54 ± 0.86 % maximum in untreated controls; n = 4 and 8, respectively).

3.3.3 Ascorbate reduces the sensitivity of P-selectin surface expression to thrombin

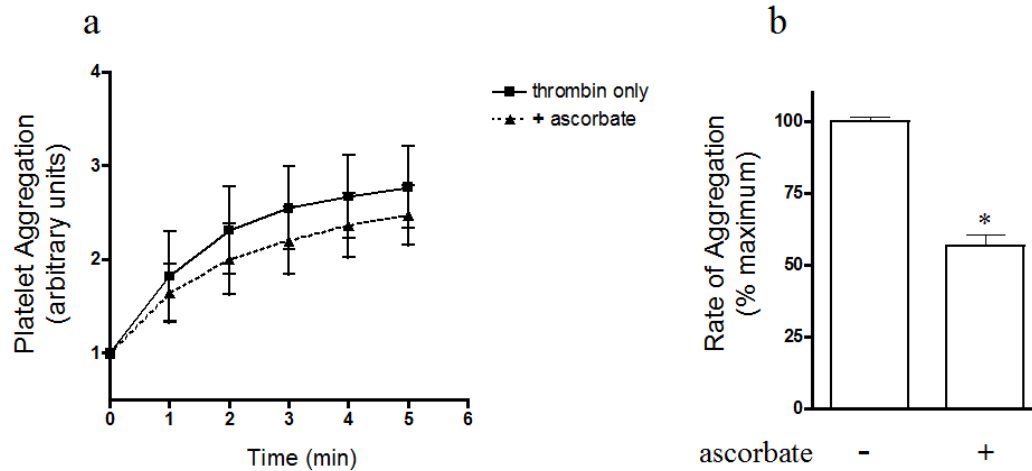
Since it is difficult to determine the concentration of thrombin found within the septic microvasculature in vivo and then test the effect of ascorbate at this concentration, we examined the effect of ascorbate across multiple concentrations of thrombin. Figure 3.5 represents a dose-response of P-selectin surface expression to thrombin and to thrombin in the presence of ascorbate. Ascorbate caused a significant increase in the EC₅₀ value from 0.26 ± 0.03 to 0.40 ± 0.04 units/ml (Figure 3.5 insert), indicating that it reduced the

sensitivity of platelets to thrombin. Platelets treated only with ascorbate had P-selectin surface levels similar to controls (ascorbate = 1.16 ± 0.86 , control = 0.79 ± 0.65 % of total events detected by flow cytometer, n = 8 per group).

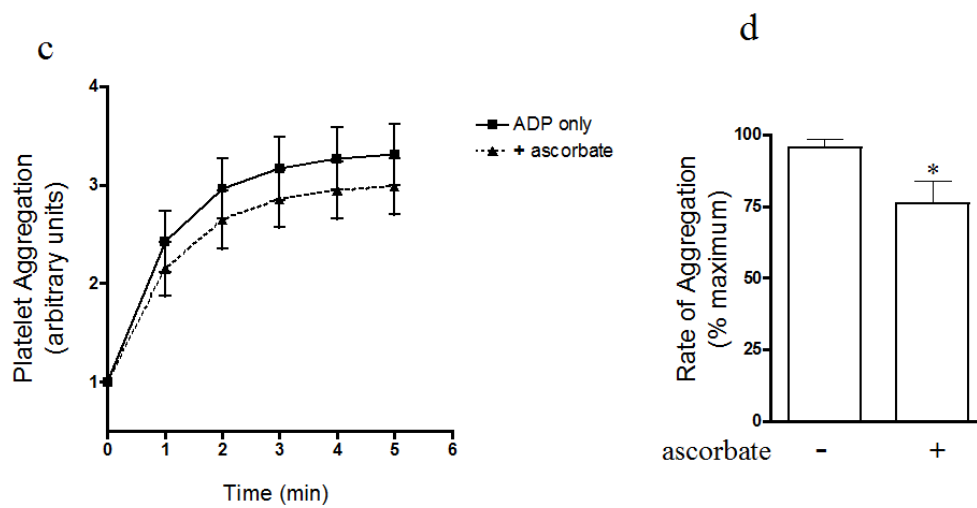
3.3.4 Ascorbate reduces thrombin-induced platelet aggregation independent of NOS

Figure 3.6 shows that the non-specific NOS inhibitor LNAME did not alter the effect of ascorbate on platelet aggregation due to thrombin. This indicates that the effect of ascorbate was not mediated by eNOS in our ex vivo model. Importantly, platelets in this model had their ability to respond to exogenous NO, because SNAP significantly inhibited thrombin-induced aggregation. In the thrombin+SNAP group (n= 5) the initial rate of aggregation was 66.2 ± 6.6 % of the control group (thrombin+inactive SNAP), (n = 4, p< 0.05). L-NAME had no effect on thrombin-induced aggregation in the absence of ascorbate (0.075 U thrombin: 2.67 ± 0.15 , thrombin + LNAME: 2.65 ± 0.10 units after 5 min of stimulation, n = 3 per group). L-NAME did not alter the effect of ascorbate on thrombin-induced P-selectin expression (Figure 3.7). EC₅₀ for thrombin+ascorbate+LNAME was 0.341 ± 0.025 and EC₅₀ for thrombin+ascorbate+DNAME was 0.339 ± 0.021 units/ml. L-NAME had no effect on thrombin-induced P-selectin surface expression in the absence of ascorbator (0.075 U thrombin: 64.8 ± 5.2 , thrombin + LNAME: 57.4 ± 8.0 % of total events detected by flow cytometer, n = 4 per group).

Thrombin



ADP



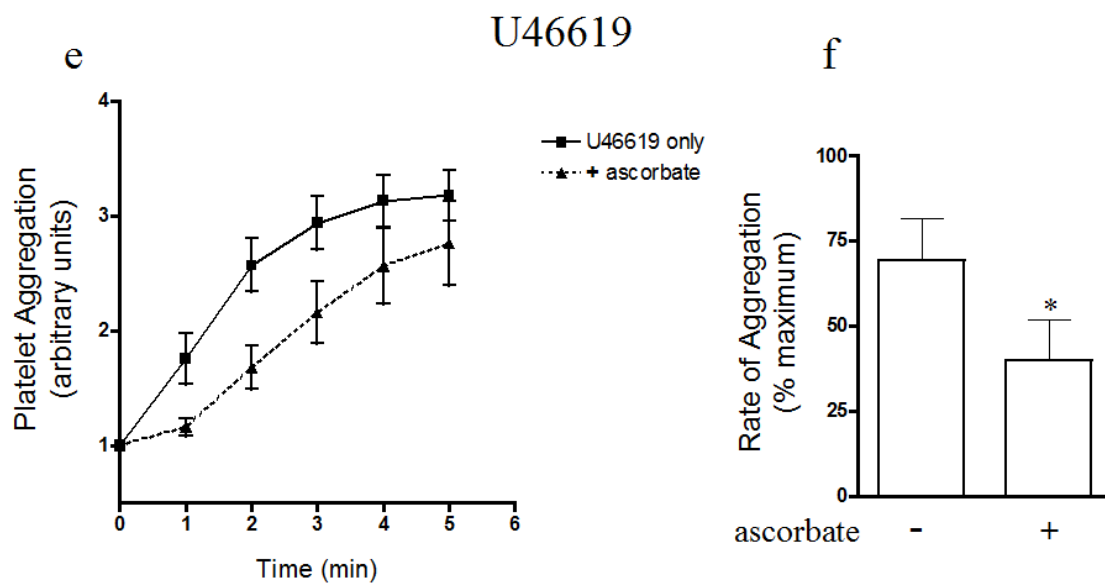


Figure 3.4. Ascorbate reduces thrombin-, ADP-, and U46619-induced platelet aggregation. Isolated platelets were treated with thrombin (0.075 units/ml, Panels a and b), ADP (1 μ M, Panels c and d) or U46619 (320 nM, Panels e and f) with or without ascorbate (100 μ M) and then placed in an aggregometer. * $P < 0.05$ compared to thrombin alone group, $n = 5$ (Panel b), compared to ADP alone group, $n = 6$ (Panel d), and compared to U46619 alone group, $n = 5$ (Panel f).

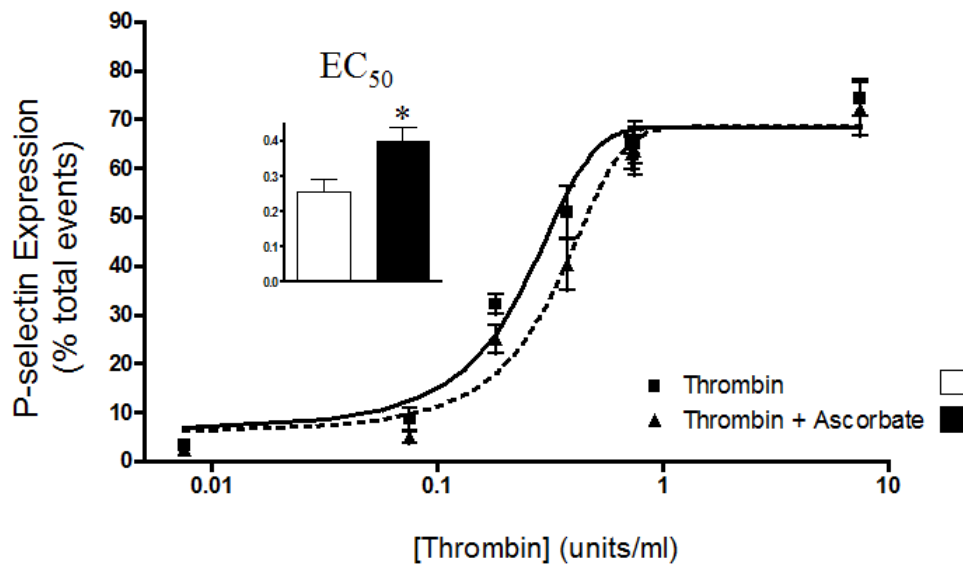


Figure 3.5. Dose response to thrombin and to thrombin plus ascorbate on P-selectin surface expression on isolated platelets. Isolated platelets were treated for 20 min with thrombin (0.0075, 0.075, 0.181, 0.375, 0.7, 0.75, 7.5 units/ml), concurrently with or without ascorbate (100 μ M), and then they were examined for surface P-selectin expression by flow cytometry. P-selectin surface expression was represented by the percent of events that were both positive for P-selectin and CD41 (platelet marker). Ascorbate significantly increased the EC_{50} value from 0.26 to 0.40 units/ml (inset). * $P < 0.05$ compared to thrombin alone, $n = 5$ per group.

Thrombin

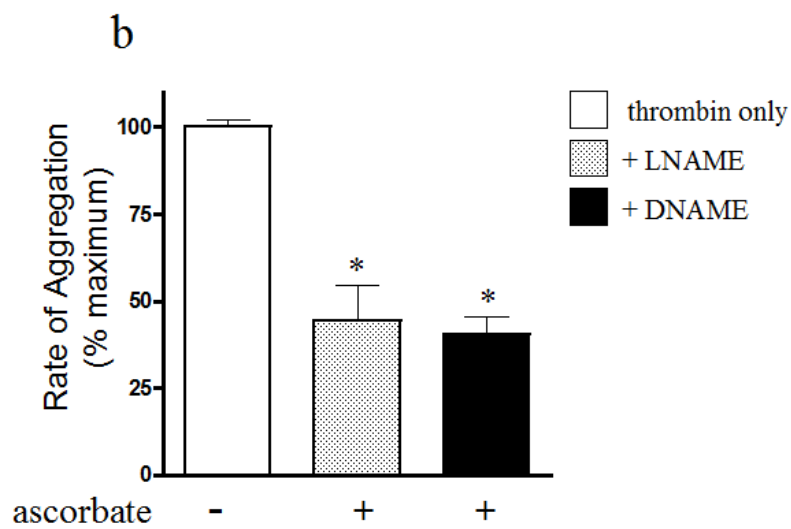
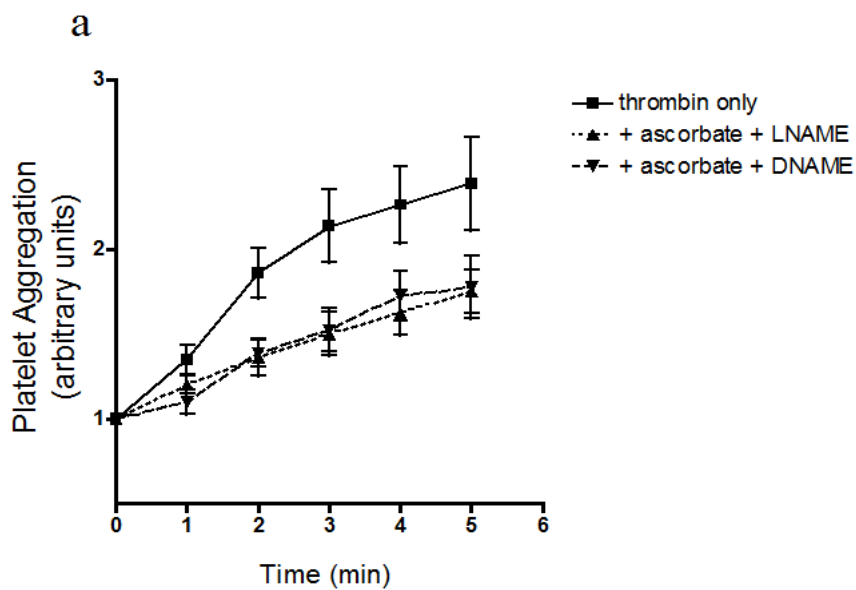


Figure 3.6. Effect of ascorbate on thrombin-induced platelet aggregation is

independent of platelet NOS. Panel a: Aggregation of isolated platelets treated with thrombin (0.075 units/ml) alone or concurrently with ascorbate (100 μ M) plus LNAME (5 mM) or ascorbate plus DNAME (5 mM). Panel b: The initial rate of aggregation for thrombin, thrombin+ascorbate+LNAME and thrombin+ascorbate+DNAME, shown as the percentage of the maximum rate per experiment. There was no difference between the thrombin+ascorbate+LNAME and thrombin+ascorbate+DNAME groups. * $P < 0.05$ compared thrombin alone group, $n = 4$ per group.

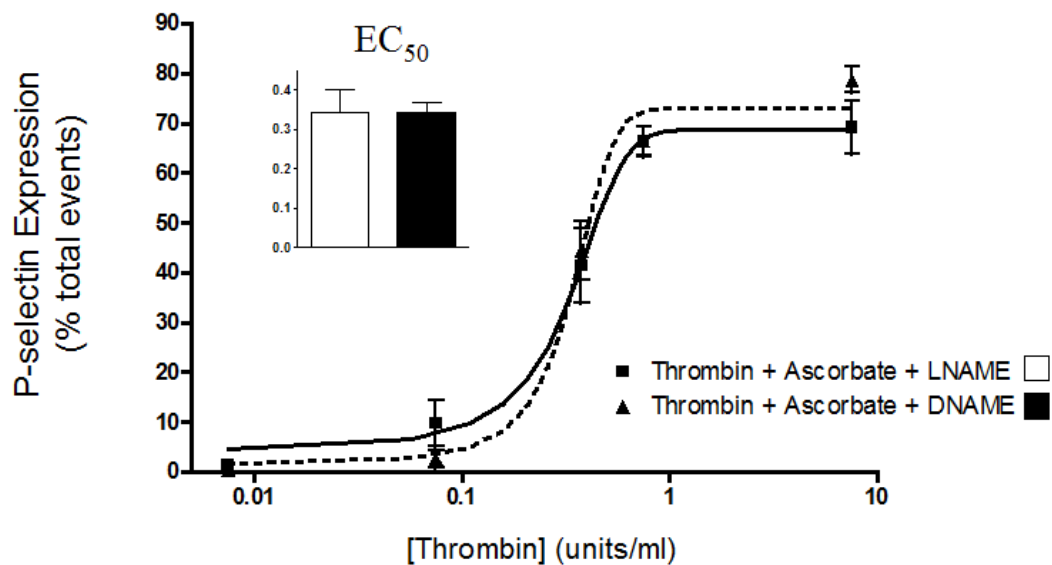


Figure 3.7. Effect of ascorbate on thrombin-induced P-selectin surface expression on isolated platelets is independent of platelet NOS. Isolated platelets were treated for 20 min with thrombin, ascorbate, LNAME, and/or DNAME. P-selectin surface expression was then measured by flow cytometry. There was no difference in EC₅₀ between the thrombin+ascorbate+LNAME and thrombin+ascorbate+DNAME groups (inset), n= 4 per group.

3.4 DISCUSSION

In the present *ex-vivo* study we found that ascorbate reduced platelet aggregation and P-selectin surface expression on platelets activated with thrombin, ADP and U46619. This effect of ascorbate was NOS independent. In contrast to these agents, LPS, TNF α and plasma from septic mice did not alter platelet aggregation nor P-selectin surface expression.

Our findings that LPS does not alter platelet aggregation and expression of P-selectin are consistent with earlier reports (10, 47, 63). However, recent studies have shown otherwise, namely that LPS enhances thrombin-induced platelet aggregation and increases P-selectin expression (11, 52, 64). The reason for these discrepancies is unclear. We suspect that inter-species differences may be important here because our data agree with the study of Rumbaut and co-workers (47) who also used platelets from mice. Consistent with previous reports (8, 40), treatment with TNF α also did not initiate aggregation or increased P-selectin expression. Regarding the lack of effect of plasma from septic mice, it should be noted that the present protocol for plasma collection from septic mice included an inevitable delay before its application to the platelet-Tyrode suspension (about a 30 min delay). During this delay the concentration or effectiveness of these platelet-activating agents might have been minimized (e.g. half-life of thrombin in plasma is 56 sec (46)), thus explaining the lack of effect of septic plasma on platelet aggregation or P-selectin surface expression. To our knowledge, there are no reports addressing the direct effect of septic plasma on this aggregation/expression under similar *ex-vivo* experimental conditions.

There are numerous reports that sepsis increases the plasma levels of thrombin, ADP, and thromboxane (12, 20, 32, 35, 36, 39, 55). The present data showing that thrombin, ADP, and thromboxane promote platelet aggregation are consistent with the literature (15, 29). Furthermore, thrombin-induced P-selectin expression on platelets has been reported in humans and dogs where the EC_{50} value was approximately 0.05 units/ml (21, 44). Our higher EC_{50} of 0.26 units/ml is most likely due to the differences between species and experimental protocol used.

The novel finding of this chapter is that ascorbate reduces platelet aggregation and P-selectin surface expression. Administration of anti-oxidants ebselen, resveratrol and trolox have also similar effects (31, 54). Pignatelli and coworkers (45) reported that vitamin C reduces platelet CD40 expression. However, vitamin C had no effect on platelet aggregation and P-selectin expression in this report. This discrepancy may be due to the use of human platelets instead of mouse platelets.

We observed that the inhibitory effect of ascorbate was larger against thrombin-induced than against ADP-induced platelet aggregation (Figure 3.4). This could be explained by 2 mechanisms. First, platelets produce ROS when exposed to various agonists and these ROS augment the aggregation response (18, 24, 49, 61). The various agonists will produce different amounts of ROS (2, 4, 25). Therefore, the aggregation response to a particular agonist may be more sensitive to antioxidant treatment than the response to another agonist. Second, stimulations with various agonists follow different pathways to activate platelets. The receptors for thrombin and thromboxane are G_q coupled, leading to the activation of protein kinase C (PKC) whereas the receptor for ADP is G_i coupled (29). Both antioxidants vitamin E and gallic acid inhibit PKC activity

in stimulated platelets (9, 16) although vitamin E may have effects that are independent of its antioxidant ability. Furthermore, PKC activation in platelets increases the expression of the ascorbate transporter SVCT2, and therefore increases ascorbate levels in the platelets (50). This would explain the greater inhibition by ascorbate in thrombin- and U46619- stimulated platelets as compared with ADP-stimulated.

Since P-selectin plays a role in both aggregation and platelet-endothelial adhesion, reducing P-selectin expression/activity would decrease the formation of microthrombi, leading to reduced capillary plugging. In the previous chapter, we found that blocking P-selectin reduces the capillary blood flow impairment (Chapter 2). The ability of ascorbate to reduce the surface expression of P-selectin on platelets may be one of the mechanisms by which ascorbate reduces the plugging of capillaries.

Our data suggest that, despite the ability of platelets to respond to exogenous NO, the effect of ascorbate on thrombin-induced platelet aggregation and P-selectin surface expression is independent of platelet-derived NO. Others have reported that platelet-derived NO may only have a minor role in aggregation and P-selectin surface expression and have indicated that non-platelet sources of NO within the microvasculature may still have an important inhibitory role in platelet function (43, 51, 59).

The effect of ascorbate *in vivo* has been shown to be eNOS-dependent through a mechanism that restores eNOS function during sepsis (23, 55, 56, Chapter 2). The restored eNOS produces NO that could reduce platelet aggregation in 2 ways. First, NO reduces platelet activation by the classical cyclic GMP pathway (41). Second, NO reduces platelet adhesion by reducing P-selectin surface expression via a reduction in granule secretion. This second pathway includes the nitrosylation of N-ethylmaleimide

sensitive factor (NSF) which is necessary for proper granule docking to the plasma membrane (34). Nitrosylated NSF has a diminished function leading to reduced granular exocytosis and reduced P-selectin surface expression. Our data show that ascorbate works independently of platelet-derived NOS and imply that ascorbate could reduce platelet adhesion/aggregation in the absence of restored eNOS function within the microvasculature. Thus, in the context of plugging of septic capillaries, the inhibition of platelet aggregation directly by ascorbate may not be enough to fully restore capillary blood flow. We propose that, in addition to the reduced adhesion/aggregation caused directly by ascorbate, NO-derived from non-platelet sources (e.g., endothelial eNOS) may also be required for the full *in vivo* effect of ascorbate.

Although the inhibitory effect of ascorbate seemed modest (Figures 3.4 and 3.5), this finding might have reflected the particular conditions of our model. The conditions were pro-aggregatory with thrombin at 0.075 U/ml, since the anti-aggregatory agent eptifibatid inhibited aggregation to a similar modest degree as ascorbate (44% inhibition). Using a lower concentration of thrombin at 0.0375 U/ml, ascorbate inhibited aggregation more robustly (74% inhibition). Furthermore, the present conditions did not exclude the effect of endogenous ascorbate remaining in the platelets after isolation. This remaining ascorbate could have masked the true difference between the control untreated and ascorbate-treated aggregation of platelets.

In conclusion, we used an *ex vivo* model of sepsis in mice to demonstrate that ascorbate (i) reduces platelet aggregation stimulated by thrombin, ADP, and U46619, and (ii) lowers platelet surface P-selectin expression induced by thrombin, in NOS-independent manner. The ability of ascorbate to inhibit platelet P-selectin expression and

platelet aggregation could be an important mechanism by which ascorbate inhibits capillary plugging. Further studies are needed to determine if this inhibition of platelet aggregation/plugging reduces the organ dysfunction occurring during sepsis.

3.5 REFERENCES

1. Armour J, Tyml K, Lidington D, Wilson JX. Ascorbate prevents microvascular dysfunction in the skeletal muscle of the septic rat. *J Appl Physiol* 90(3): 795-803, 2001.
2. Bakdash N, Williams MS. Spatially distinct production of reactive oxygen species regulates platelet activation. *Free Radic Biol Med* 45(2): 158-166, 2008.
3. Baudry N, Rasetti C, Vicaut E. Differences between cytokine effects in the microcirculation of the rat. *Am J Physiol* 271(3 Pt 2): H1186-92, 1996.
4. Begonja AJ, Teichmann L, Geiger J, Gambaryan S, Walter U. Platelet regulation by NO/cGMP signaling and NAD(P)H oxidase-generated ROS. *Blood Cells Mol Dis* 36(2): 166-170, 2006.
5. Blann AD, Nadar SK, Lip GY. The adhesion molecule P-selectin and cardiovascular disease. *Eur Heart J* 24(24): 2166-2179, 2003.
6. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* 101(6): 1644-1655, 1992.
7. BORN GV. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature* 194: 927-929, 1962.

8. Cambien B, Bergmeier W, Saffaripour S, Mitchell HA, Wagner DD. Antithrombotic activity of TNF-alpha. *J Clin Invest* 112(10): 1589-1596, 2003.
9. Chang SS, Lee VS, Tseng YL, Chang KC, Chen KB, Chen YL, Li CY. Gallic Acid Attenuates Platelet Activation and Platelet-Leukocyte Aggregation: Involving Pathways of Akt and GSK3beta. *Evid Based Complement Alternat Med* 2012: 683872, 2012.
10. Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, Patel KD, Chakrabarti S, McAvoy E, Sinclair GD, Keys EM, Allen-Vercoe E, Devinney R, Doig CJ, Green FH, Kubes P. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med* 13(4): 463-469, 2007.
11. Cognasse F, Hamzeh-Cognasse H, Lafarge S, Delezay O, Pozzetto B, McNicol A, Garraud O. Toll-like receptor 4 ligand can differentially modulate the release of cytokines by human platelets. *Br J Haematol* 141(1): 84-91, 2008.
12. Cook JA, Wise WC, Halushka PV. Elevated thromboxane levels in the rat during endotoxic shock: protective effects of imidazole, 13-azaprostanic acid, or essential fatty acid deficiency. *J Clin Invest* 65(1): 227-230, 1980.
13. Dellinger RP, Levy MM, Carlet JM, Bion J, Parker MM, Jaeschke R, Reinhart K, Angus DC, Brun-Buisson C, Beale R, Calandra T, Dhainaut JF, Gerlach H, Harvey M, Marini JJ, Marshall J, Ranieri M, Ramsay G, Sevransky J, Thompson BT, Townsend S, Vender JS, Zimmerman JL, Vincent JL, International Surviving Sepsis Campaign Guidelines Committee, American Association of Critical-Care Nurses, American College of Chest Physicians, American College of Emergency Physicians, Canadian Critical Care

Society, European Society of Clinical Microbiology and Infectious Diseases, European Society of Intensive Care Medicine, European Respiratory Society, International Sepsis Forum, Japanese Association for Acute Medicine, Japanese Society of Intensive Care Medicine, Society of Critical Care Medicine, Society of Hospital Medicine, Surgical Infection Society, World Federation of Societies of Intensive and Critical Care Medicine. Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2008. *Crit Care Med* 36(1): 296-327, 2008.

14. Ellis CG, Bateman RM, Sharpe MD, Sibbald WJ, Gill R. Effect of a maldistribution of microvascular blood flow on capillary O₂ extraction in sepsis. *Am J Physiol Heart Circ Physiol* 282(1): H156-64, 2002.

15. Flaumenhaft R. Molecular basis of platelet granule secretion. *Arterioscler Thromb Vasc Biol* 23(7): 1152-1160, 2003.

16. Freedman JE, Keaney JF, Jr. Vitamin E inhibition of platelet aggregation is independent of antioxidant activity. *J Nutr* 131(2): 374S-7S, 2001.

17. Goldman D, Bateman RM, Ellis CG. Effect of sepsis on skeletal muscle oxygen consumption and tissue oxygenation: interpreting capillary oxygen transport data using a mathematical model. *Am J Physiol Heart Circ Physiol* 287(6): H2535-44, 2004.

18. Handin RI, Karabin R, Boxer GJ. Enhancement of platelet function by superoxide anion. *J Clin Invest* 59(5): 959-965, 1977.

19. Heptinstall S, Glenn JR, Losche W, Spangenberg P. ADP-induced platelet aggregation and actin polymerization. Involvement of GpIIb/IIIa and the effect of Mg²⁺. *Semin Thromb Hemost* 21(2): 137-145, 1995.
20. Jabs CM, Ferrell WJ, Robb HJ. Plasma changes in endotoxin and anaphylactic shock (ATP, ADP and Creatine phosphorus). *Ann Clin Lab Sci* 9(2): 121-132, 1979.
21. Keh D, Gerlach M, Kurer I, Seiler S, Kerner T, Falke KJ, Gerlach H. The effects of nitric oxide (NO) on platelet membrane receptor expression during activation with human alpha-thrombin. *Blood Coagul Fibrinolysis* 7(6): 615-624, 1996.
22. Khan R, Kirschenbaum LA, LaRow C, Berna G, Griffin K, Astiz ME. Augmentation of platelet and endothelial cell eNOS activity decreases sepsis-related neutrophil-endothelial cell interactions. *Shock* 33(3): 242-246, 2010.
23. Kim HJ, Lee SI, Lee DH, Smith D, Jo H, Schellhorn HE, Boo YC. Ascorbic acid synthesis due to L-gulono-1,4-lactone oxidase expression enhances NO production in endothelial cells. *Biochem Biophys Res Commun* 345(4): 1657-1662, 2006.
24. Krotz F, Sohn HY, Gloe T, Zahler S, Riexinger T, Schiele TM, Becker BF, Theisen K, Klauss V, Pohl U. NAD(P)H oxidase-dependent platelet superoxide anion release increases platelet recruitment. *Blood* 100(3): 917-924, 2002.
25. Krotz F, Sohn HY, Pohl U. Reactive oxygen species: players in the platelet game. *Arterioscler Thromb Vasc Biol* 24(11): 1988-1996, 2004.

26. Lam C, Tyml K, Martin C, Sibbald W. Microvascular perfusion is impaired in a rat model of normotensive sepsis. *J Clin Invest* 94(5): 2077-2083, 1994.
27. Levi M. Platelets in sepsis. *Hematology* 10 Suppl 1: 129-131, 2005.
28. Levi M, Ten Cate H. Disseminated intravascular coagulation. *N Engl J Med* 341(8): 586-592, 1999.
29. Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. *Arterioscler Thromb Vasc Biol* 30(12): 2341-2349, 2010.
30. Lidington D, Ouellette Y, Tyml K. Endotoxin increases intercellular resistance in microvascular endothelial cells by a tyrosine kinase pathway. *J Cell Physiol* 185(1): 117-125, 2000.
31. Lindenblatt N, Schareck W, Belusa L, Nickels RM, Menger MD, Vollmar B. Anti-oxidant ebselen delays microvascular thrombus formation in the rat cremaster muscle by inhibiting platelet P-selectin expression. *Thromb Haemost* 90(5): 882-892, 2003.
32. Llesuy S, Evelson P, Gonzalez-Flecha B, Peralta J, Carreras MC, Poderoso JJ, Boveris A. Oxidative stress in muscle and liver of rats with septic syndrome. *Free Radic Biol Med* 16(4): 445-451, 1994.
33. Long CL, Maull KI, Krishnan RS, Laws HL, Geiger JW, Borghesi L, Franks W, Lawson TC, Sauberlich HE. Ascorbic acid dynamics in the seriously ill and injured. *J Surg Res* 109(2): 144-148, 2003.

34. Lowenstein CJ, Tsuda H. N-ethylmaleimide-sensitive factor: a redox sensor in exocytosis. *Biol Chem* 387(10-11): 1377-1383, 2006.
35. Luna A, Villanueva E, Hernandez-Cueto C, Morales I. Study of 5-hydroxytryptamine (serotonin) in pericardial fluid in different causes of death (II.). Experimental study of 5-HT levels in two types of shocks (hemorrhagic and septic) in dogs. *Z Rechtsmed* 89(4): 227-236, 1983.
36. Mavrommatis AC, Theodoridis T, Economou M, Kotanidou A, El Ali M, Christopoulou-Kokkinou V, Zakynthinos SG. Activation of the fibrinolytic system and utilization of the coagulation inhibitors in sepsis: comparison with severe sepsis and septic shock. *Intensive Care Med* 27(12): 1853-1859, 2001.
37. Mavrommatis AC, Theodoridis T, Orfanidou A, Roussos C, Christopoulou-Kokkinou V, Zakynthinos S. Coagulation system and platelets are fully activated in uncomplicated sepsis. *Crit Care Med* 28(2): 451-457, 2000.
38. Mittermayer F, Pleiner J, Schaller G, Zorn S, Namiranian K, Kapiotis S, Bartel G, Wolfrum M, Brugel M, Thiery J, Macallister RJ, Wolzt M. Tetrahydrobiopterin corrects *Escherichia coli* endotoxin-induced endothelial dysfunction. *Am J Physiol Heart Circ Physiol* 289(4): H1752-7, 2005.
39. Morris KM, Moon RJ. Quantitative analysis of serotonin biosynthesis in endotoxemia. *Infect Immun* 10(2): 340-346, 1974.

40. Nakatsuji K, Kii Y, Fujitani B, Ito T. General pharmacology of recombinant human tumor necrosis factor. 1st communication: effects on cardiovascular, gastrointestinal, renal and blood functions. *Arzneimittelforschung* 40(2 Pt 1): 218-225, 1990.
41. Naseem KM, Roberts W. Nitric oxide at a glance. *Platelets* 22(2): 148-152, 2011.
42. Offermanns S. Activation of platelet function through G protein-coupled receptors. *Circ Res* 99(12): 1293-1304, 2006.
43. Ozuyaman B, Godecke A, Kusters S, Kirchhoff E, Scharf RE, Schrader J. Endothelial nitric oxide synthase plays a minor role in inhibition of arterial thrombus formation. *Thromb Haemost* 93(6): 1161-1167, 2005.
44. Peng J, Friese P, Wolf RF, Harrison P, Downs T, Lok S, Dale GL, Burstein SA. Relative reactivity of platelets from thrombopoietin- and interleukin-6-treated dogs. *Blood* 87(10): 4158-4163, 1996.
45. Pignatelli P, Sanguigni V, Paola SG, Lo Coco E, Lenti L, Violi F. Vitamin C inhibits platelet expression of CD40 ligand. *Free Radic Biol Med* 38(12): 1662-1666, 2005.
46. Ruhl H, Muller J, Harbrecht U, Fimmers R, Oldenburg J, Mayer G, Potzsch B. Thrombin inhibition profiles in healthy individuals and thrombophilic patients. *Thromb Haemost* 107(5): 848-853, 2012.
47. Rumbaut RE, Bellera RV, Randhawa JK, Shrimpton CN, Dasgupta SK, Dong JF, Burns AR. Endotoxin enhances microvascular thrombosis in mouse cremaster venules

via a TLR4-dependent, neutrophil-independent mechanism. *Am J Physiol Heart Circ Physiol* 290(4): H1671-9, 2006.

48. Saklatvala J, Rawlinson L, Waller RJ, Sarsfield S, Lee JC, Morton LF, Barnes MJ, Farndale RW. Role for p38 mitogen-activated protein kinase in platelet aggregation caused by collagen or a thromboxane analogue. *J Biol Chem* 271(12): 6586-6589, 1996.

49. Salvemini D, de Nucci G, Sneddon JM, Vane JR. Superoxide anions enhance platelet adhesion and aggregation. *Br J Pharmacol* 97(4): 1145-1150, 1989.

50. Savini I, Catani MV, Arnone R, Rossi A, Frega G, Del Principe D, Avigliano L. Translational control of the ascorbic acid transporter SVCT2 in human platelets. *Free Radic Biol Med* 42(5): 608-616, 2007.

51. Schafer A, Alp NJ, Cai S, Lygate CA, Neubauer S, Eigenthaler M, Bauersachs J, Channon KM. Reduced vascular NO bioavailability in diabetes increases platelet activation in vivo. *Arterioscler Thromb Vasc Biol* 24(9): 1720-1726, 2004.

52. Shashkin PN, Brown GT, Ghosh A, Marathe GK, McIntyre TM. Lipopolysaccharide is a direct agonist for platelet RNA splicing. *J Immunol* 181(5): 3495-3502, 2008.

53. Singer G, Urakami H, Specian RD, Stokes KY, Granger DN. Platelet recruitment in the murine hepatic microvasculature during experimental sepsis: role of neutrophils. *Microcirculation* 13(2): 89-97, 2006.

54. Sobotkova A, Masova-Chrastinova L, Suttnar J, Stikarova J, Majek P, Reicheltova Z, Kotlin R, Weisel JW, Maly M, Dyr JE. Antioxidants change platelet responses to various stimulating events. *Free Radic Biol Med* 47(12): 1707-1714, 2009.
55. Tymk K. Critical role for oxidative stress, platelets, and coagulation in capillary blood flow impairment in sepsis. *Microcirculation* 18(2): 152-162, 2011.
56. Tymk K, Li F, Wilson JX. Septic impairment of capillary blood flow requires nicotinamide adenine dinucleotide phosphate oxidase but not nitric oxide synthase and is rapidly reversed by ascorbate through an endothelial nitric oxide synthase-dependent mechanism. *Crit Care Med* 36(8): 2355-2362, 2008.
57. Tymk K, Li F, Wilson JX. Delayed ascorbate bolus protects against maldistribution of microvascular blood flow in septic rat skeletal muscle. *Crit Care Med* 33(8): 1823-1828, 2005.
58. Tymk K, Wang X, Lidington D, Ouellette Y. Lipopolysaccharide reduces intercellular coupling in vitro and arteriolar conducted response in vivo. *Am J Physiol Heart Circ Physiol* 281(3): H1397-406, 2001.
59. Tymvios C, Moore C, Jones S, Solomon A, Sanz-Rosa D, Emerson M. Platelet aggregation responses are critically regulated in vivo by endogenous nitric oxide but not by endothelial nitric oxide synthase. *Br J Pharmacol* 158(7): 1735-1742, 2009.
60. Vincent JL. Clinical sepsis and septic shock--definition, diagnosis and management principles. *Langenbecks Arch Surg* 393(6): 817-824, 2008.

61. Wachowicz B, Olas B, Zbikowska HM, Buczynski A. Generation of reactive oxygen species in blood platelets. *Platelets* 13(3): 175-182, 2002.
62. Walther DJ, Peter JU, Winter S, Holtje M, Paulmann N, Grohmann M, Vowinckel J, Alamo-Bethencourt V, Wilhelm CS, Ahnert-Hilger G, Bader M. Serotonylation of small GTPases is a signal transduction pathway that triggers platelet alpha-granule release. *Cell* 115(7): 851-862, 2003.
63. Ward JR, Bingle L, Judge HM, Brown SB, Storey RF, Whyte MK, Dower SK, Buttle DJ, Sabroe I. Agonists of toll-like receptor (TLR)2 and TLR4 are unable to modulate platelet activation by adenosine diphosphate and platelet activating factor. *Thromb Haemost* 94(4): 831-838, 2005.
64. Zhang G, Han J, Welch EJ, Ye RD, Voyno-Yasenetskaya TA, Malik AB, Du X, Li Z. Lipopolysaccharide stimulates platelet secretion and potentiates platelet aggregation via TLR4/MyD88 and the cGMP-dependent protein kinase pathway. *J Immunol* 182(12): 7997-8004, 2009.

Chapter 4

Ascorbate reduces P-selectin mediated platelet-endothelial adhesion in an *in vitro* model of sepsis via reduced endothelial granule secretion

A version of this chapter has been submitted to *Microcirculation*

4.1 INTRODUCTION

Sepsis is the tenth leading cause of mortality in the US (19) with a mortality rate near 40% (25). Circulatory dysfunctions including cessation of blood flow in septic capillaries can lead to multiple organ failure (6, 38). Contributing to the capillary blood flow impairment is increased platelet adhesion and fibrin deposition in the capillary suggesting the formation of microthrombi (Chapter 2). This could explain the reduced platelet counts seen in septic patients which correlate with increased mortality (27). The cessation of capillary blood flow leads to increased diffusional distance for oxygen to reach cell mitochondria, tissue hypoxia and organ failure (10, 14).

Enhanced coagulation contributes to the severity of sepsis resulting in microvascular thrombosis and disseminated intravascular coagulation (DIC, (20)). During sepsis, increased platelet-endothelial adhesion may be critical in capillary flow impairment (Chapter 2). P-selectin is a key platelet-endothelium adhesion molecule (4) as inhibition of P-selectin results in decreased platelet adhesion in the septic capillary. Inhibition also prevents the cessation of capillary blood flow (Chapter 2). P-selectin is stored in Weibel-Palade bodies in endothelial cells and, upon stimulation, it is expressed at the surface of the cell where it can facilitate platelet adhesion through binding its counter receptor P-selectin glycoprotein ligand-1 (PSGL1) (23). Reactive oxygen species have also been implicated in the capillary blood flow impairment as sepsis increases the level of ROS (21, 36). ROS have been shown to modulate platelet function by enhancing platelet activation (18) and indirectly, by altering endothelial function (3). Furthermore, ROS can reduce local nitric oxide production by oxidizing the eNOS cofactor BH₄. When

BH₄ is oxidized, eNOS production of nitric oxide (NO) is reduced. NO is an important factor in the homeostasis of the microcirculation and is a known inhibitor of platelet activation/adhesion (30). Furthermore, NO can prevent expression of P-selectin (2), and it can inhibit P-selectin surface expression by reducing the exocytosis of the Weibel-Palade bodies in endothelial cells (24). The antioxidant ascorbate (reduced vitamin C) prevents and reverses the oxidation of BH₄ thus restoring local NO production via eNOS (16, 29).

Clinically, there is no effective pharmacological treatment for the systemic inflammation component of sepsis. Ascorbate has been under investigation as a possible treatment (22). An intravenous bolus of ascorbate has been shown to prevent and reverse the cessation of blood flow in septic capillaries as well as increase survival of septic mice (1, 37). Sepsis-induced increase in adhesion of single platelets and their aggregates in capillaries are also inhibited by ascorbate treatment (Chapter 2). Although several mechanisms have been proposed to explain the beneficial effect of ascorbate in the septic microvasculature (36), the precise mechanism of this effect is unclear.

Our recent study showed that ascorbate could reduce platelet adhesion during sepsis as well as improve capillary blood flow (Chapter 2). However, it was not possible to determine if ascorbate was acting directly on the platelet adhesion or that the reduced platelet adhesion was a consequence of other effects of ascorbate. To address this issue, the present study employed a simple *in vitro* platelet-endothelial cell adhesion assay. This *in vitro* model could determine (i) the effect of sepsis on the endothelial P-selectin expression, and (ii) the effect of ascorbate on this expression. We hypothesized that sepsis-induced platelet-endothelial adhesion and endothelial P-selectin genomic expression and surface expression are reduced by ascorbate.

4.2 METHODS

4.2.1 Reagents used

Lipopolysacchride (LPS), tumor necrosis factor alpha (TNF α), calcein-AM, protease inhibitor cocktail, Superscript II, and ascorbate were purchased from Sigma-Aldrich (St. Louis, MO). The phycoerythrin (PE) conjugated P-selectin antibody (clone: Wug.E9) and PE conjugated IgG control were purchased from Emfret Analytics (Eibelstadt, Germany). A P-selectin blocking antibody (clone: RB40.34) and a rat IgG1 lambda isotype control were purchased from BD Pharmingen (Mississauga, ON). TRIZOL was purchased from Invitrogen (Burlington, ON). A horse radish peroxidase (HRP) tagged anti-rat IgG antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). A GAPDH antibody and a HRP anti-rabbit IgG antibody were purchased from Cell Signalling Technology (Danvers, MA). Quantifast SYBR Green master mix was purchased from Qiagen (Mississauga, ON).

4.2.2 Endothelial cell harvest and culture

All experimental protocols were approved by the University of Western Ontario Council on Animal Care. Skeletal muscle microvascular endothelial cells were harvested from wild type (C57BL/6) mice and isolated as performed previously in our lab (5, 41). Briefly, skeletal muscle from the hind limb of mice was collected and digested. The digest was plated and grown to confluency. Endothelial cells were isolated by lectin

coated magnetic beads. Cells were cultured to confluence and cell type was confirmed by immunocytochemistry, checking for the endothelial marker von Willebrand factor. To model sepsis, endothelial cells were treated with LPS (10 $\mu\text{g/ml}$) or $\text{TNF}\alpha$ (10 ng/ml) for 1h. Ascorbate (100 μM) was applied to cells as a pre-treatment 4 h prior to LPS or $\text{TNF}\alpha$ treatment. In some experiments, a P-selectin blocking antibody (clone: RB40.34, 1:1000) or a control IgG was added cells concurrently with LPS or $\text{TNF}\alpha$.

4.2.3 Platelet collection and isolation

Blood (600 μl) was collected from anaesthetized naive male mice via carotid artery puncture into a vial containing 100 μl of acid citrate dextrose solution. Platelets were isolated by the method describe by Singer *et al* (32). Briefly, platelet rich plasma was collected by centrifuging the whole blood at 120 g for 8 min. The plasma was then re-spun to remove any collected leukocytes. Finally the platelets were pelleted at 735 g for 10 min and resuspended in phosphate buffer solution (PBS).

4.2.4 In vitro adhesion model to mimic the platelet-endothelial cell interaction

To visualize platelets with fluorescence microscopy, isolated platelets were incubated with 8 μM calcein-AM for 10 minutes. The platelets were pelleted at 735 g for 10 min to remove excess calcein-AM and resuspended in fresh PBS. Following endothelial cell treatment, $\sim 3 \times 10^6$ platelets were added to the a confluent monolayer of 0.25×10^6 endothelial cells and co-incubated under static conditions for 1h, and then washed. The remaining platelets adhering to the endothelial cells were visualized with a Zeiss

fluorescence microscope using a 20 x magnification objective. Labelled platelets were then counted in the entire area of the microscopic field of view (0.43 mm x 0.32 mm). Each treatment group was done in triplicate and 5 arbitrary microscopic fields of view (chosen blindly) were used per replicate. Counts of attached platelets were repeated by a separate individual blinded to the conditions.

4.2.5 Septic mouse model

Male mice were anaesthetized with an intraperitoneal (i.p.) injection of a mixture of ketamine (80 mg/kg) and xylazine (4 mg/kg). Sepsis was induced by feces injected into peritoneum (FIP) method previously described by our lab (37). Briefly, mice were given an i.p. injection of 50 ml/kg volume of saline containing 75 mg/ml of feces. Control (sham) mice received sterile saline i.p. injection 50 ml/kg. Both FIP and sham mice were given 1 ml of saline containing 4µg/ml buprenorphine sub-cutaneously, providing fluid resuscitation and analgesics.

4.2.6 P-selectin and PSGL1 mRNA expression in cultured endothelial cells and skeletal muscle homogenates

P-selectin and PSGL1 mRNA were measured by real time qPCR. After treatment, mRNA from the endothelial cells was collected by TRIZOL according to the manufacturer's instructions. Hindlimb skeletal muscle was collected from sham and septic mice at 7 h post-FIP. Prior to this, septic mice were injected intravenously (penile vein) with 1 ml of sterile saline or with 1 ml saline containing ascorbate (10 mg/kg) at 6 h (i.e., when capillary plugging is prevalent (37)). Similarly, sham mice were injected with saline at 6

h. The muscle was homogenized and mRNA was collected by TRIZOL. mRNA was then reversed transcribed to cDNA by Superscript II. We used qPCR to determine the mRNA expression levels of P-selectin (primers: forward 5'-GTCCACGGAGAGTTTGGTGT-3' and reverse 5'-AAGTGGTGTTCGGACCAAAG-3') and PSGL1 (primers: forward 5'-CTTCCTTGCTGCTGACCAT-3' and reverse 5'-TCAGGGTCCTCAAATCGTCATC-3') and used β -actin (primers: forward 5'-TCGTGGGCCGCTCTAGGCACCA-3' and reverse 5'-GTTGGCCTTAGGGTTCAGGGGGG-3') as our reference gene. The qPCR was carried out using Quantifast SYBR Green master mix on a Mini-opticon cycler (Bio-Rad, Mississauga, ON). We used the following cycling protocol: 95°C for 5 min and then 40 cycles of 95°C for 10 s, 60°C for 30 s and 80°C for 30 s. SYBR green fluorescence was acquired at 80°C in each amplification cycle. Subsequently a melt curve was generated, starting at 60°C and taking measurements every 0.2°C until 95°C was reached.

4.2.7 Von Willebrand factor (vWF) ELISA

The purpose of this procedure was to assess indirectly P-selectin protein expression at the endothelial cell surface. Originally, we carried out pilot experiments using immunocytochemistry and flow cytometry to detect P-selectin. Unfortunately, for unknown reasons these procedures failed to detect P-selectin under any experimental conditions. For this reason we have used the vWF ELISA as a surrogate measure for P-selectin surface protein detection. P-selectin is contained together with vWF in Weibel-Palade granules under the surface of endothelial cells. Exocytosis of these granules delivers to the surface both vWF and P-selectin (23).

Endothelial cells grown to confluence were treated with dialyzed serum medium with or without ascorbate (100 μ M) for 4 h. The medium was replaced by fresh medium without ascorbate and the cells were treated with LPS (10 μ g/ml) or TNF α (10 ng/ml) for 1 h. The cell culture media after the endothelial cell treatments was collected and measured for von Willebrand factor using a commercially available ELISA kit (Cusabio, Wuhan, China).

4.2.8 Western blots

Hindlimb extensor digitorum longus skeletal muscle was collected and homogenized in SDS lysis buffer with protease inhibitor cocktail. The collected protein was run on an acrylamide gel and transferred to a PVDF membrane. The membrane was probed with a P-selectin antibody (Clone: Wug.9, at 1:1000) and a HRP tagged anti-rat IgG secondary antibody (1:5000). To control for protein loading, the membrane was also probed with a GAPDH antibody (Clone: 14C10, 1:1000) followed by a HRP anti-rabbit IgG secondary antibody (1:2000).

4.2.9 Statistical analysis

Data are represented as mean \pm standard error and n represents the number of mice or separate cell culture experiments where cells were harvested from at least 3 mice. Data were analyzed by one-way ANOVA followed by Bonferroni comparison. P values less than 0.05 were considered significant.

4.3 RESULTS

4.3.1 Ascorbate prevents increased platelet-endothelial cell adhesion in an in vitro model of sepsis

Both LPS and TNF α increased platelet-endothelial cell adhesion under static conditions (Figures 4.1, 4.2). LPS or TNF α -induced increase in adhesion was prevented by blocking P-selectin (Figure 4.1). The control IgG had no effect (LPS-induced platelet adhesion with control IgG and TNF α -induced platelet adhesion with control IgG were 2.4 ± 0.7 and 2.5 ± 0.9 fold, respectively, over vehicle treated control cells with control IgG, n = 3 per group). Pretreatment of endothelial cells with ascorbate prevented both the LPS-induced and TNF α -induced increases in platelet-endothelial cell adhesion (Figure 4.2). Ascorbate has no effect in vehicle treated cells.

4.3.2 LPS and TNF α increase P-selectin mRNA expression but not PSGL1 mRNA

LPS and TNF α increased P-selectin mRNA 3 and 4 fold, respectively, in endothelial cells. However, pre-treatment of the endothelial cells with ascorbate did not affect this increase (Figure 4.3). Ascorbate has no effect in vehicle treated cells. LPS or TNF α treatments did not alter PSGL1 mRNA (Figure 4.4).

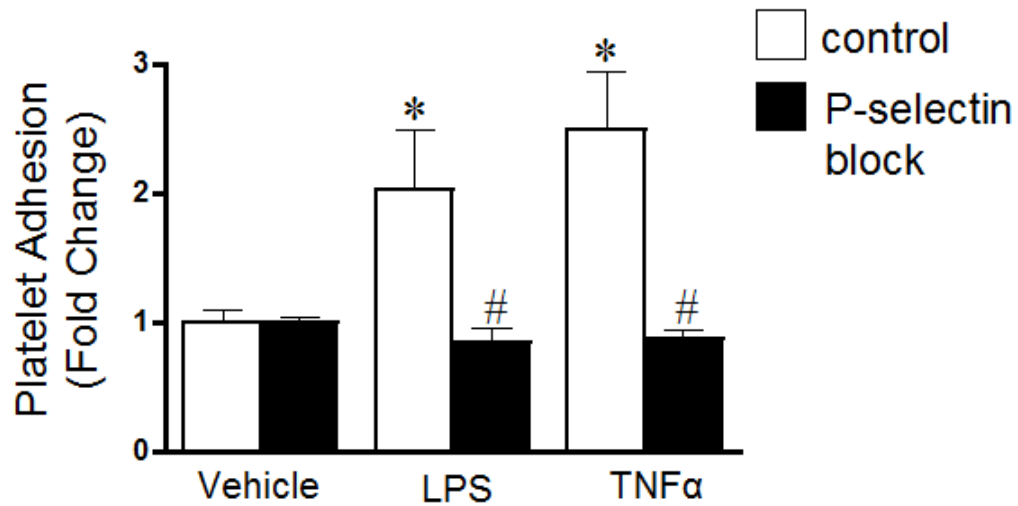


Figure 4.1. Effect of P-selectin block on LPS or TNF α treated endothelial cell-platelet adhesion. Endothelial cells treated with LPS or TNF α for 1 h were co-incubated with untreated platelets for 1 h, washed, and adherent platelets were counted by fluorescence microscopy. A separate group of cells was co-treated with ascorbate a P-selectin blocking antibody during the TNF α treatment. LPS or TNF α treatment significantly increased the endothelial cell-platelet adhesion. P-selectin block prevented this increased adhesion. (*significantly different from control untreated group, #significantly different from control group with the same treatment, $p < 0.05$, $n = 5$).

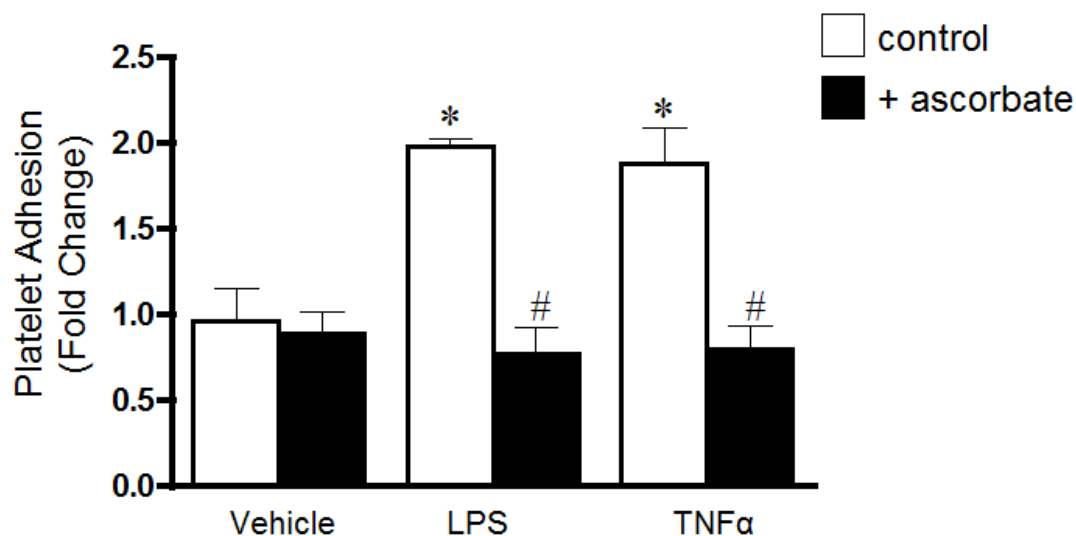


Figure 4.2. Effect of ascorbate on LPS or TNF α treated endothelial cell-platelet adhesion. Endothelial cells treated with LPS or TNF α for 1 h were co-incubated with untreated platelets for 1 h, washed, and adherent platelets were counted by fluorescence microscopy. A separate group of cells was pretreated for 4 h with ascorbate prior to LPS/TNF α treatment. LPS or TNF α treatments significantly increased the endothelial cell-platelet adhesion. Ascorbate prevented this increased adhesion. (*significantly different from control untreated group, #significantly different from control group with the same treatment, $p < 0.05$, $n = 5 - 8$).

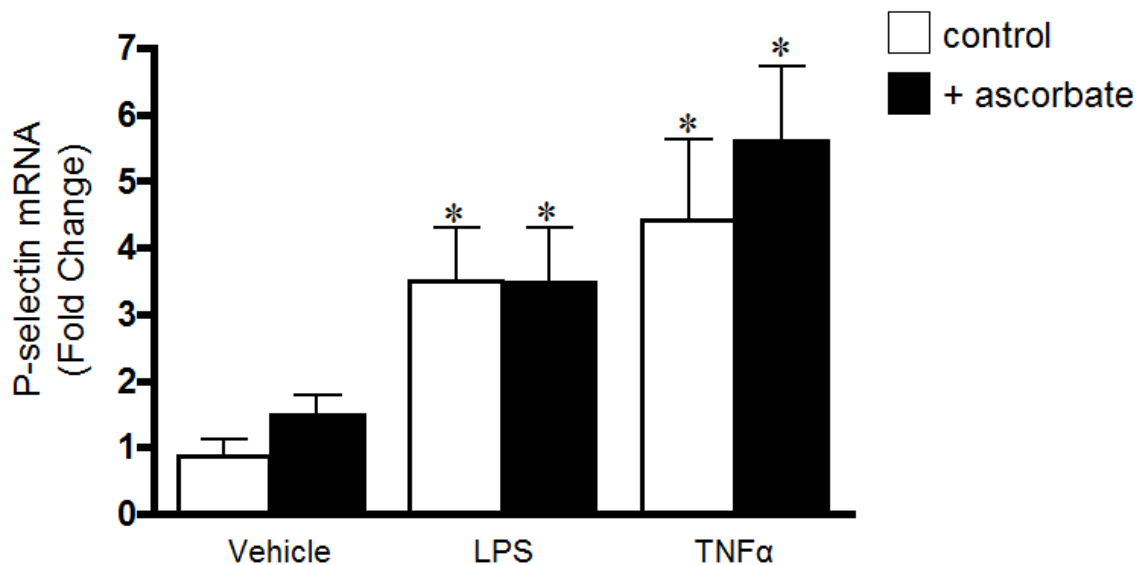


Figure 4.3. Effect of ascorbate on P-selectin mRNA expression in LPS or TNF α treated endothelial cells. Endothelial cells treated with LPS or TNF α for 1 h were assayed for P-selectin mRNA expression by real time qPCR. A separate group of cells was pretreated for 4 h with ascorbate prior to LPS/TNF α treatment. LPS or TNF α treatments significantly increased P-selectin mRNA. Ascorbate did not affect this increased expression. (*significantly different from control untreated group, $p < 0.05$, $n = 6 - 7$).

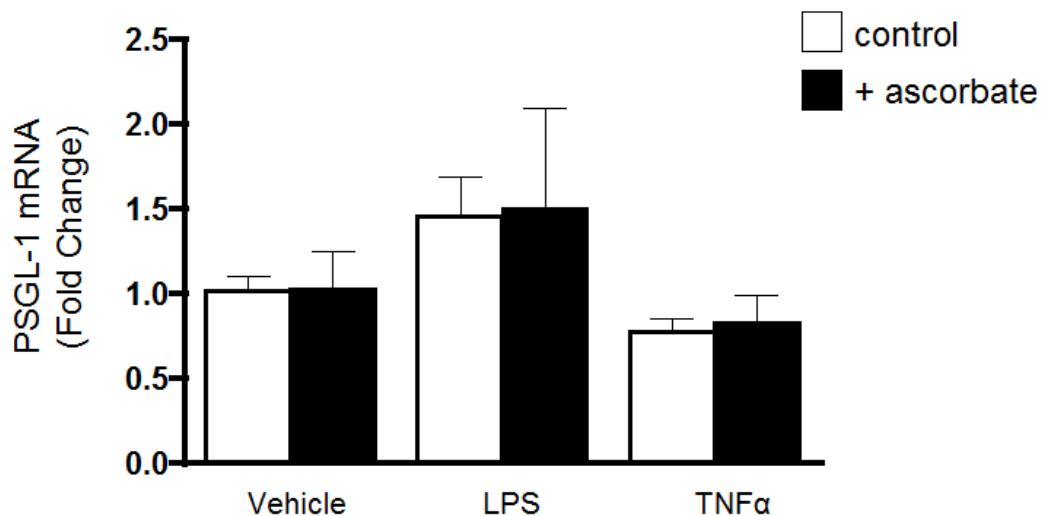


Figure 4.4. Effect of ascorbate on P-selectin glycoprotein ligand-1 (PSGL1) mRNA expression in LPS or TNF α treated endothelial cells. Endothelial cells treated with LPS or TNF α for 1 h were assayed for PSGL1 mRNA expression by real time qPCR. A separate group of cells was pretreated for 4 h with ascorbate prior to LPS/TNF α treatment. LPS and TNF α did not affect PSGL1 mRNA. (Means are not significantly different, n = 6 - 7).

4.3.3 Ascorbate reduces vWF release in LPS treated endothelial cells

LPS but not TNF α treatment increased vWF granular secretion from endothelial cells (Figure 4.5). Ascorbate pre-treatment prevented the LPS-induced vWF release. Ascorbate showed no effect in vehicle treated cells. Fold change over the vehicle treated control cells was used due to large variability in the day-to-day experimental data (range of vWF protein amount in vehicle treated control cells: 0.2 – 5.5 ng/ml).

4.3.4 Sepsis increases expression of P-selectin mRNA but not protein in muscle homogenates

Muscle homogenates from septic mice at 7 h has a 7 fold higher P-selectin mRNA compared to sham mice (Figure 4.6). However, Figure 7 shows that there was no difference in P-selectin protein content between the septic and sham mice homogenates. Ascorbate injected at 6 h did not affect either the mRNA or protein when compared to the saline treated control group (Figures 4.6, 4.7).

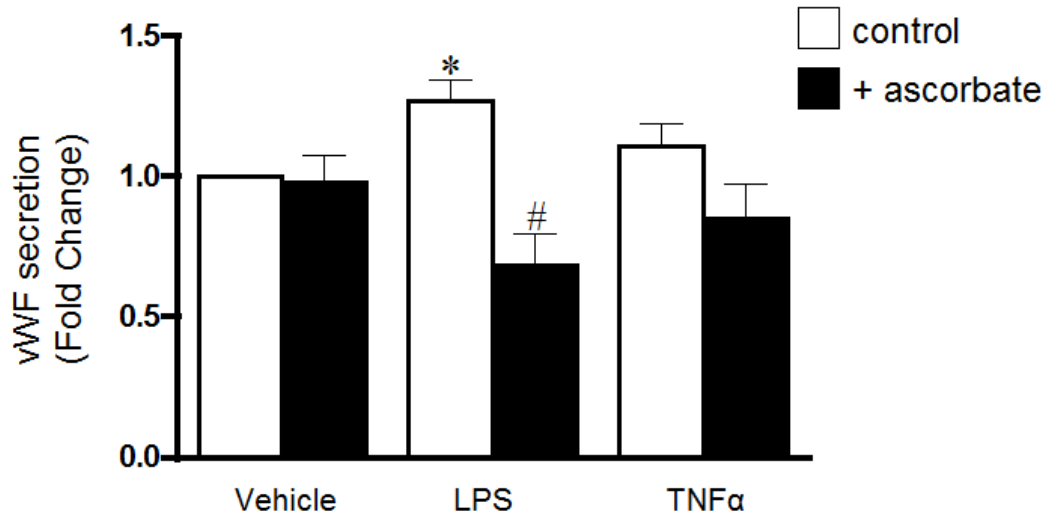


Figure 4.5. Effect of ascorbate on endothelial cell granule secretion. Media from endothelial cells treated with LPS or TNF α for 1 h was collected and assayed for von Willebrand factor (vWF) expression by ELISA. A separate group of cells was pretreated for 4 h with ascorbate prior to LPS/TNF α treatment. LPS but not TNF α increased vWF release from endothelial cells. Ascorbate prevented this increased vWF release. (*significantly different from control untreated group, $p < 0.05$, $n = 5$).

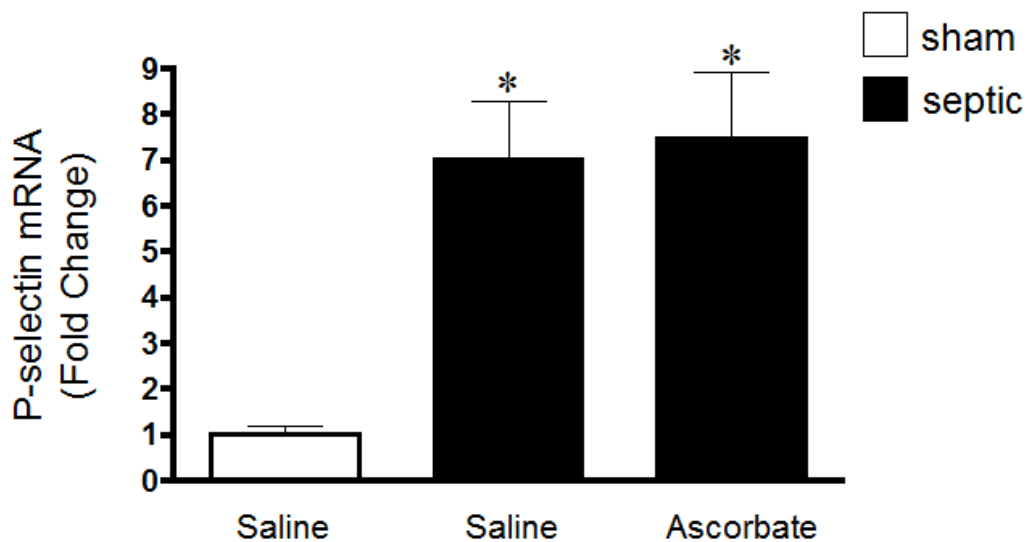


Figure 4.6. Effect of ascorbate on P-selectin mRNA expression in septic mice. At 6 h after sham injection or feces injection into peritoneum (FIP, model of sepsis), mice were given an i.v. bolus of saline or ascorbate. At 7 h post-FIP, hindlimb muscle was collected, homogenized, and analyzed for P-selectin mRNA by real time qPCR. Sepsis increased P-selectin mRNA expression. Ascorbate did not affect this increase. (* significantly different from sham, $p < 0.05$, $n = 9-13$).

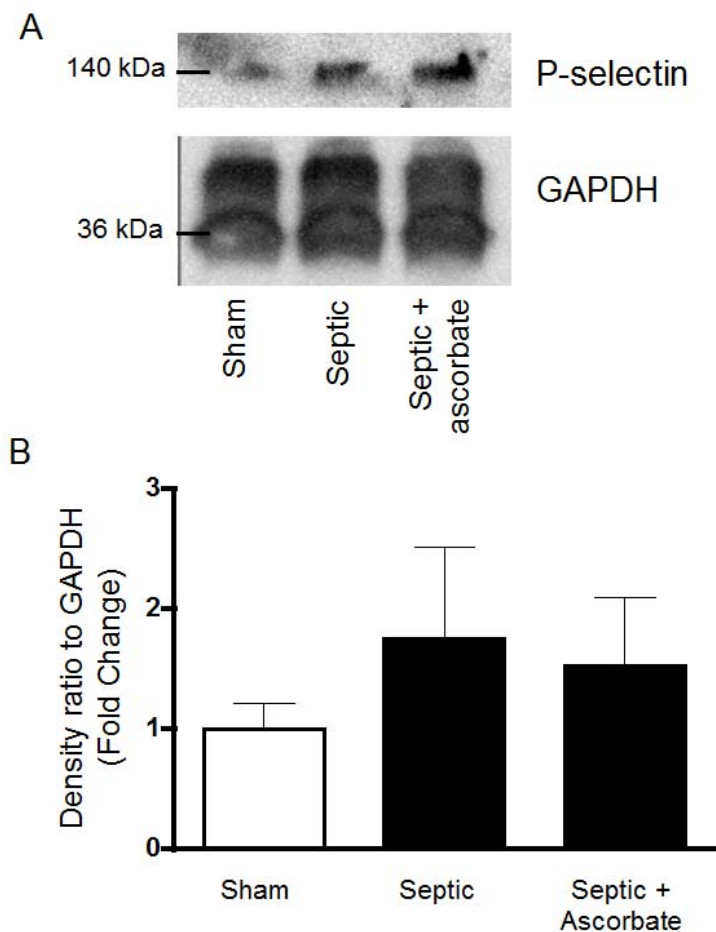


Figure 4.7. Effect of ascorbate on P-selectin protein expression in septic mice. At 6 h after sham or FIP injection, mice were given an i.v. bolus of saline or ascorbate. At 7 h post-FIP, hindlimb muscle was collected, homogenized, and analyzed for P-selectin protein by western blot. A) Shows a representative blot. B) densitometry ratio of the P-selectin:GAPDH signal. Data is displayed as the fold change when compared to the sham mice. (Means are not significantly different, $n = 6$).

4.4 DISCUSSION

In the present study we found that ascorbate reduced platelet adhesion to endothelial cells stimulated by LPS or TNF α . Ascorbate reduced vWF granular secretion from endothelial cells activated by LPS. On the other hand, ascorbate did not affect P-selectin mRNA expression in LPS or TNF α -stimulated endothelial cells and in skeletal muscle homogenates from septic mice.

Sepsis has been observed to induce platelet adhesion to the capillary wall *in vivo* (Chapter 2). Our present *in vitro* model successfully mimicked this observation. Both LPS and TNF α have previously been shown to increase platelet-endothelial cell adhesion (17, 42). In our model, this increased adhesion was P-selectin dependent as blockage of P-selectin via an antibody prevented stimulated adhesion. We interpret these observations to indicate that the P-selectin played a direct role in the observed adhesion. However, since P-selectin-PSGL1 binding can produce further signalling that also leads to adhesion involving other adhesion proteins (4), the role of P-selectin in the observed adhesion could also be indirect.

We previously reported that ascorbate reduces platelet adhesion in septic capillaries (Chapter 2). However, to our knowledge, ascorbate has not been tested in an *in vitro* assay to probe for the mechanism of this reduction. Previous reports have shown that other antioxidants namely vitamin E and quercetin, can reduce platelet-endothelial adhesion *in vitro* (11, 34). Szuwart and co-workers (34) found that the inhibitory effect of vitamin E on platelet adhesion was seen in platelet rich plasma and not in isolated platelets. Since only the endothelial cells received the treatments in the present *in vitro* model, activation of only these cells was responsible for the changes in the platelet-

endothelial adhesion. Therefore we propose that the LPS or TNF α stimulus causes an increase in P-selectin expression in the endothelial cells leading to the increased adhesion.

LPS or TNF α increased P-selectin mRNA expression in our endothelial model which is consistent with other reports (13, 33). However, treatment of the endothelial cells with ascorbate did not affect this expression. Our results disagree with a previous report that the antioxidant nebulivolol inhibits P-selectin mRNA expression on human macrovascular endothelial cells stimulated with oxidative low density lipoprotein (12). The difference could be explained by the use of a different stimuli, species, or cell type. Inflammatory mediators do not affect PSGL1 mRNA expression (9), which agrees with our present data showing no effect of TNF α or LPS on PSGL1 mRNA in our *in vitro* model.

Granular secretion measured by vWF release was used as a surrogate biomarker for P-selectin surface expression. LPS but not TNF α increased vWF release from endothelial cells (Figure 4.5). This, too, is consistent with the current literature. LPS has been shown to increase vWF release (28) as well as increase P-selectin surface expression (7) whereas TNF α failed to affect vWF secretion (31) and P-selectin surface expression (7). This suggests that LPS and TNF α increase platelet-endothelial adhesion by different mechanisms. Here we show for the first time that ascorbate can inhibit vWF release from murine microvascular endothelial cells and thus reduce P-selectin surface expression. Wang and coworkers (39) showed similar results with the antioxidant genipin on thrombin stimulated cells, NOS dependently. Likewise, other studies have shown that

antioxidants reduce P-selectin surface expression caused by thrombin (35) or hypoxia/re-oxygenation (15) in human umbilical vein endothelial cells.

In our model, ascorbate could prevent LPS-stimulated granular secretion by restoring eNOS function. Proper Weibel-Palade body exocytosis requires N-ethylmaleimide-sensitive factor (NSF) regulating membrane fusion (26). When nitrosylated by NO, NSF has reduced function and thus lowers granule secretion (26). Therefore, ascorbate could restore local NO production, nitrosylate NSF, and subsequently reduce Weibel-Palade body exocytosis including P-selectin surface expression. The effect of ascorbate on TNF α induced platelet-endothelial adhesion is unclear. TNF α did not increase vWF release and thus did not affect P-selectin surface expression on endothelial cells. However, TNF α -induced platelet adhesion was prevented by blocking endothelial P-selectin.

Although P-selectin mRNA was increased in septic murine muscle tissue, there was no change in the protein expression when assessed by immunoblot (Figure 4.7). The apparent discrepancy between mRNA and protein data could be explained by the fact that the mRNA from the muscle homogenates comes exclusively from the endothelial cells whereas the protein comes from both the endothelial cells and the platelets. Thus the platelet protein content could mask any changes in the endothelial cell protein that may occur during sepsis. Since ascorbate did not affect P-selectin expression on either the mRNA or protein level, we must conclude that the protective effect of ascorbate (i.e., reduction in adhesion) is independent of changes in the overall P-selectin levels.

4.4.1 Methodological limitations

In the present study, treatment of cultured endothelial cells with P-selectin blocking antibody (but not with control IgG) prevented platelet adhesion to these cells. Together with observed P-selectin mRNA expression in these cells, the effect of blocking strongly indicated that these cells expressed P-selectin protein. However, our inability to detect P-selectin surface protein in these cells by immunocytochemistry and flow cytometry and the total P-selectin protein by immunoblotting pointed to a limitation of the cell culture model (Figure 4.8). In the platelet adhesion assay, we counted about 5 - 20 adhering platelets in each microscopic field of view including about 180 endothelial cells. This implies that only a low percentage of endothelial cells presented P-selectin at their surface to “capture” platelets for adhesion, after excess of platelets was subjected to co-incubation with the confluent endothelial monolayer. To explain this low percentage, we speculate that the culturing conditions reduced the number of cells expressing P-selectin protein and/or the level of this expression per cell. This level could have been too low to be detected by immunocytochemistry or immunoblot. The skeletal muscle vascular bed could also have a low P-selectin expression. This low expression has been reported for liver sinusoids (8, 40). Regarding detection of P-selectin surface expression by flow cytometry, this limitation would be further worsened by the procedural steps necessary for cell preparation. Detachment of cells from dishes, suspension and fixing of cells could all negatively affect the presence of this protein at the cell surface.

In conclusion, we used an *in vitro* model of sepsis in mice to demonstrate that ascorbate (i) reduces platelet-endothelial adhesion stimulated by LPS or TNF α and (ii) lowers LPS-induced vWF granular release from endothelial cells. The ability of ascorbate to inhibit P-selectin surface expression could be an important mechanism by which ascorbate inhibits capillary plugging.

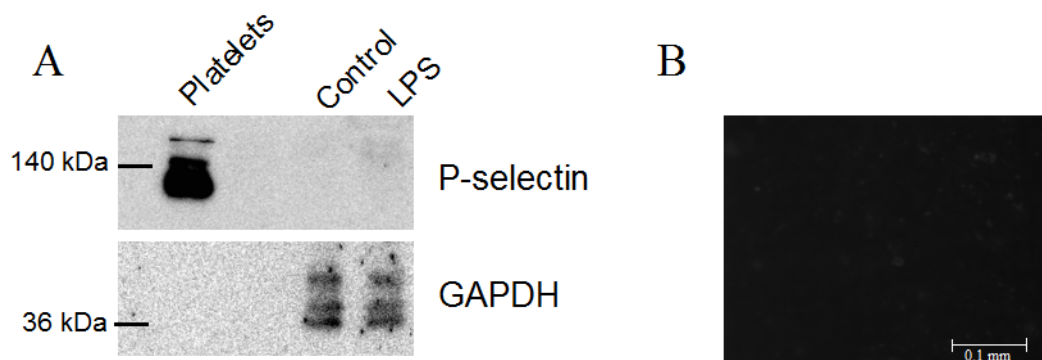


Figure 4.8. P-selectin protein expression in the endothelial cell model. A) Endothelial cells treated with LPS for 6 h were assayed for P-selectin protein expression by Western blot. Shown is a representative blot of 6 endothelial cell lines originating from 6 mice. P-selectin was not detected in the endothelial cells. Platelet lysate was used as a positive control. B) Endothelial cells treated with LPS for 1 h were assayed for surface P-selectin protein expression by immunocytochemistry. A FITC conjugated anti-P-selectin antibody was co-incubated with the cells (Clone: Wug.9 at 1:100 for 1 h). No staining was observed. Treatment of isolated platelets with the antibody was used a positive control.

4.5 REFERENCES

1. Armour J, Tyml K, Lidington D, Wilson JX. Ascorbate prevents microvascular dysfunction in the skeletal muscle of the septic rat. *J Appl Physiol* 90(3): 795-803, 2001.
2. Armstead VE, Minchenko AG, Schuhl RA, Hayward R, Nossuli TO, Lefer AM. Regulation of P-selectin expression in human endothelial cells by nitric oxide. *Am J Physiol* 273(2 Pt 2): H740-6, 1997.
3. Azevedo LC, Janiszewski M, Soriano FG, Laurindo FR. Redox mechanisms of vascular cell dysfunction in sepsis. *Endocr Metab Immune Disord Drug Targets* 6(2): 159-164, 2006.
4. Blann AD, Nadar SK, Lip GY. The adhesion molecule P-selectin and cardiovascular disease. *Eur Heart J* 24(24): 2166-2179, 2003.
5. Bolon ML, Peng T, Kidder GM, Tyml K. Lipopolysaccharide plus hypoxia and reoxygenation synergistically reduce electrical coupling between microvascular endothelial cells by dephosphorylating connexin40. *J Cell Physiol* 217(2): 350-359, 2008.
6. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* 101(6): 1644-1655, 1992.

7. Coisne C, Faveeuw C, Delplace Y, Dehouck L, Miller F, Cecchelli R, Dehouck B. Differential expression of selectins by mouse brain capillary endothelial cells in vitro in response to distinct inflammatory stimuli. *Neurosci Lett* 392(3): 216-220, 2006.
8. Couvelard A, Scoazec JY, Dauge MC, Bringuier AF, Potet F, Feldmann G. Structural and functional differentiation of sinusoidal endothelial cells during liver organogenesis in humans. *Blood* 87(11): 4568-4580, 1996.
9. da Costa Martins P, Garcia-Vallejo JJ, van Thienen JV, Fernandez-Borja M, van Gils JM, Beckers C, Horrevoets AJ, Hordijk PL, Zwaginga JJ. P-selectin glycoprotein ligand-1 is expressed on endothelial cells and mediates monocyte adhesion to activated endothelium. *Arterioscler Thromb Vasc Biol* 27(5): 1023-1029, 2007.
10. Ellis CG, Bateman RM, Sharpe MD, Sibbald WJ, Gill R. Effect of a maldistribution of microvascular blood flow on capillary O₂ extraction in sepsis. *Am J Physiol Heart Circ Physiol* 282(1): H156-64, 2002.
11. Fan PS, Gu ZL, Liang ZQ. Effect of quercetin on adhesion of platelets to microvascular endothelial cells in vitro. *Acta Pharmacol Sin* 22(9): 857-860, 2001.
12. Garbin U, Fratta Pasini A, Stranieri C, Manfro S, Mozzini C, Boccioletti V, Pasini A, Cominacini M, Evangelista S, Cominacini L. Effects of nebivolol on endothelial gene expression during oxidative stress in human umbilical vein endothelial cells. *Mediators Inflamm* 2008: 367590, 2008.

13. Gerritsen ME, Shen CP, McHugh MC, Atkinson WJ, Kiely JM, Milstone DS, Luscinskas FW, Gimbrone MA, Jr. Activation-dependent isolation and culture of murine pulmonary microvascular endothelium. *Microcirculation* 2(2): 151-163, 1995.
14. Goldman D, Bateman RM, Ellis CG. Effect of sepsis on skeletal muscle oxygen consumption and tissue oxygenation: interpreting capillary oxygen transport data using a mathematical model. *Am J Physiol Heart Circ Physiol* 287(6): H2535-44, 2004.
15. Kim CD, Kim YK, Lee SH, Hong KW. Rebamipide inhibits neutrophil adhesion to hypoxia/reoxygenation-stimulated endothelial cells via nuclear factor-kappaB-dependent pathway. *J Pharmacol Exp Ther* 294(3): 864-869, 2000.
16. Kim HJ, Lee SI, Lee DH, Smith D, Jo H, Schellhorn HE, Boo YC. Ascorbic acid synthesis due to L-gulonolactone oxidase expression enhances NO production in endothelial cells. *Biochem Biophys Res Commun* 345(4): 1657-1662, 2006.
17. Krishnamurti C, Peat RA, Cutting MA, Rothwell SW. Platelet adhesion to dengue-2 virus-infected endothelial cells. *Am J Trop Med Hyg* 66(4): 435-441, 2002.
18. Krotz F, Sohn HY, Pohl U. Reactive oxygen species: players in the platelet game. *Arterioscler Thromb Vasc Biol* 24(11): 1988-1996, 2004.
19. Kung HC, Hoyert DL, Xu J, Murphy SL. Deaths: final data for 2005. *Natl Vital Stat Rep* 56(10): 1-120, 2008.
20. Levi M, Ten Cate H. Disseminated intravascular coagulation. *N Engl J Med* 341(8): 586-592, 1999.

21. Llesuy S, Evelson P, Gonzalez-Flecha B, Peralta J, Carreras MC, Poderoso JJ, Boveris A. Oxidative stress in muscle and liver of rats with septic syndrome. *Free Radic Biol Med* 16(4): 445-451, 1994.
22. Long CL, Maull KI, Krishnan RS, Laws HL, Geiger JW, Borghesi L, Franks W, Lawson TC, Sauberlich HE. Ascorbic acid dynamics in the seriously ill and injured. *J Surg Res* 109(2): 144-148, 2003.
23. Lowenstein CJ, Morrell CN, Yamakuchi M. Regulation of Weibel-Palade body exocytosis. *Trends Cardiovasc Med* 15(8): 302-308, 2005.
24. Lowenstein CJ, Tsuda H. N-ethylmaleimide-sensitive factor: a redox sensor in exocytosis. *Biol Chem* 387(10-11): 1377-1383, 2006.
25. Martin CM, Priestap F, Fisher H, Fowler RA, Heyland DK, Keenan SP, Longo CJ, Morrison T, Bentley D, Antman N, STAR Registry Investigators. A prospective, observational registry of patients with severe sepsis: the Canadian Sepsis Treatment and Response Registry. *Crit Care Med* 37(1): 81-88, 2009.
26. Matsushita K, Morrell CN, Cambien B, Yang SX, Yamakuchi M, Bao C, Hara MR, Quick RA, Cao W, O'Rourke B, Lowenstein JM, Pevsner J, Wagner DD, Lowenstein CJ. Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. *Cell* 115(2): 139-150, 2003.

27. Mavrommatis AC, Theodoridis T, Orfanidou A, Roussos C, Christopoulou-Kokkinou V, Zakyntinos S. Coagulation system and platelets are fully activated in uncomplicated sepsis. *Crit Care Med* 28(2): 451-457, 2000.
28. McCarron RM, Doron DA, Siren AL, Feuerstein G, Heldman E, Pollard HB, Spatz M, Hallenbeck JM. Agonist-stimulated release of von Willebrand factor and procoagulant factor VIII in rats with and without risk factors for stroke. *Brain Res* 647(2): 265-272, 1994.
29. Mittermayer F, Pleiner J, Schaller G, Zorn S, Namiranian K, Kapiotis S, Bartel G, Wolfrum M, Brugel M, Thiery J, Macallister RJ, Wolzt M. Tetrahydrobiopterin corrects *Escherichia coli* endotoxin-induced endothelial dysfunction. *Am J Physiol Heart Circ Physiol* 289(4): H1752-7, 2005.
30. Naseem KM, Roberts W. Nitric oxide at a glance. *Platelets* 22(2): 148-152, 2011.
31. Paleolog EM, Crossman DC, McVey JH, Pearson JD. Differential regulation by cytokines of constitutive and stimulated secretion of von Willebrand factor from endothelial cells. *Blood* 75(3): 688-695, 1990.
32. Singer G, Urakami H, Specian RD, Stokes KY, Granger DN. Platelet recruitment in the murine hepatic microvasculature during experimental sepsis: role of neutrophils. *Microcirculation* 13(2): 89-97, 2006.

33. Slotta JE, Laschke MW, Schilling MK, Menger MD, Jeppsson B, Thorlacius H. Simvastatin attenuates hepatic sensitization to lipopolysaccharide after partial hepatectomy. *J Surg Res* 162(2): 184-192, 2010.
34. Szuwart T, Brzoska T, Luger TA, Filler T, Peuker E, Dierichs R. Vitamin E reduces platelet adhesion to human endothelial cells in vitro. *Am J Hematol* 65(1): 1-4, 2000.
35. Takano M, Meneshian A, Sheikh E, Yamakawa Y, Wilkins KB, Hopkins EA, Bulkley GB. Rapid upregulation of endothelial P-selectin expression via reactive oxygen species generation. *Am J Physiol Heart Circ Physiol* 283(5): H2054-61, 2002.
36. Tyml K. Critical role for oxidative stress, platelets, and coagulation in capillary blood flow impairment in sepsis. *Microcirculation* 18(2): 152-162, 2011.
37. Tyml K, Li F, Wilson JX. Septic impairment of capillary blood flow requires nicotinamide adenine dinucleotide phosphate oxidase but not nitric oxide synthase and is rapidly reversed by ascorbate through an endothelial nitric oxide synthase-dependent mechanism. *Crit Care Med* 36(8): 2355-2362, 2008.
38. Vincent JL. Clinical sepsis and septic shock--definition, diagnosis and management principles. *Langenbecks Arch Surg* 393(6): 817-824, 2008.
39. Wang GF, Wu SY, Rao JJ, Lu L, Xu W, Pang JX, Liu ZQ, Wu SG, Zhang JJ. Genipin inhibits endothelial exocytosis via nitric oxide in cultured human umbilical vein endothelial cells. *Acta Pharmacol Sin* 30(5): 589-596, 2009.

40. Wong J, Johnston B, Lee SS, Bullard DC, Smith CW, Beaudet AL, Kubes P. A minimal role for selectins in the recruitment of leukocytes into the inflamed liver microvasculature. *J Clin Invest* 99(11): 2782-2790, 1997.
41. Wu F, Schuster DP, Tyml K, Wilson JX. Ascorbate inhibits NADPH oxidase subunit p47phox expression in microvascular endothelial cells. *Free Radic Biol Med* 42(1): 124-131, 2007.
42. Zhang L, Gu ZL, Qin ZH, Liang ZQ. Effect of curcumin on the adhesion of platelets to brain microvascular endothelial cells in vitro. *Acta Pharmacol Sin* 29(7): 800-807, 2008.

Chapter 5

SUMMARY, DISCUSSION, and FUTURE DIRECTIONS

5.1 THESIS SUMMARY

One of the key characteristics of sepsis is the cessation of capillary blood flow that leads to tissue hypoxia and possibly multiple organ dysfunction. The mechanism behind the cessation of flow is currently unclear. A possible mechanism is physical hindrance of the flow due to the formation of microthrombi in the septic capillary. The coagulation system has been known to be involved in the pathophysiology of sepsis; a reduced platelet count in blood is a particularly telling index of the severity of sepsis. Thus, I hypothesized that increased platelet adhesion, aggregation and microthrombi formation during sepsis leads to plugging of capillaries resulting in cessation of blood flow. This hypothesis was addressed in Chapter 2.

Treatment with ascorbate prevented and rescued the capillary blood flow impairment and increased survival in our animal model of sepsis. However, the means by which ascorbate provides the beneficial effects are unknown. The effect of ascorbate is eNOS dependent, therefore locally produced NO could be involved in the protective effect of ascorbate. NO is a known platelet inhibitor and thus may modulate the formation of microthrombi in the septic capillary. I hypothesized that ascorbate lowers platelet aggregation and adhesion by decreasing P-selectin expression, thus improving capillary blood flow during sepsis. This has been addressed in Chapters 3 and 4.

In Chapter 2, I show that removal of platelets from the circulation significantly reduced the capillary blood flow impairment in septic mice. Next, I observed that platelet adhesion to the capillary wall and fibrin deposition in capillaries are increased during sepsis. Septic capillaries also showed an increased thrombogenic potential. Finally, administration of the general anticoagulant antithrombin, the platelet anti-aggregatory agent eptifibatid, and an antibody that blocks P-selectin (platelet and endothelial adhesion molecule) reduced the capillary blood flow impairment in septic mice. These data support our hypothesis that increased microthrombi formation leads to the capillary blood flow impairment.

Platelet adhesion in septic capillaries was decreased by an intravenous bolus of ascorbate administered after before the onset of sepsis or later when the cessation of capillary blood flow was already established. Also, platelet adhesion was reduced in gp91phox knockout mice suggesting that ascorbate works by its antioxidant properties. This effect, however, was not seen in eNOS knockout mice. Thus, similar to the capillary blood flow impairment, reduction in platelet adhesion by ascorbate during sepsis is eNOS dependent. Furthermore, I showed that topical application of BH₄ and the NO-donor SNAP reduced platelet adhesion in wild type septic mouse capillaries but only SNAP reduced adhesion in eNOS^{-/-} mice. Therefore, I proposed that ascorbate works by restoring eNOS function through increasing BH₄ availability (via preventing or reversing oxidative alterations to BH₄), leading to local NO production that reduces platelet adhesion.

Although ascorbate reduces platelet adhesion *in vivo*, this decrease in platelet adhesion could be a result of the other beneficial effects of ascorbate (i.e., increase blood

pressure) restoring capillary blood flow. Thus, I wanted to take a closer look at platelet adhesion and aggregation independent of flow and other *in vivo* parameters. Platelet aggregation was first examined in Chapter 3. Here, I showed that LPS, TNF α , or septic plasma do not initiate platelet aggregation nor platelet P-selectin surface expression (a marker of platelet activation). Thus, I developed our *ex vivo* model, using thrombin, ADP, and thromboxane as the stimulating agents, because the concentration of these molecules in blood is known to be increased during sepsis. I showed that ascorbate reduced platelet aggregation initiated by these factors. Furthermore, ascorbate reduced the sensitivity of platelet P-selectin surface expression induced by thrombin by effectively increasing the EC₅₀ of the dose-response curve from 0.26 to 0.40 units/ml. Ascorbate reduction of thrombin-induced aggregation or P-selectin surface expression was independent of platelet NOS activity as LNAME (non specific NOS inhibitor) treatment had no effect. We concluded that ascorbate directly inhibits platelet aggregation by reducing P-selectin surface expression. However, this reduction of platelet aggregation alone may be insufficient to improve capillary blood flow during sepsis.

Finally, I examined platelet-endothelial adhesion in Chapter 4. Primarily, I focused on the effect of ascorbate on this adhesion. To this end, I developed an *in vitro* system to model the septic platelet-endothelium interactions. Here, I show that LPS or TNF α increased platelet-endothelial adhesion similar to the increased adhesion in the septic capillary. Moreover, I demonstrated that ascorbate treatment prevented the increase in adhesion caused by LPS or TNF α , indicating that ascorbate can directly affect platelet-endothelial adhesion. To further investigate how ascorbate prevents this adhesion, we focused on the role of endothelial cell P-selectin expression. LPS or TNF α increased P-

selectin mRNA expression, however ascorbate treatment had no effect on this increase expression. By using vWF release as a marker for granule secretion from endothelial cells, I potentially showed that LPS increased granule secretion and thus P-selectin surface expression. Ascorbate treatment prevented the LPS-induced increase in vWF release suggesting a potential mechanism by which ascorbate reduces platelet adhesion caused by LPS. Finally, I showed that septic muscle tissue has increased P-selectin mRNA expression but that ascorbate treatment does not affect this increase.

In conclusion, the sepsis-induced capillary impairment is, in part, due to the formation of platelet-fibrin microthrombi. Ascorbate reduces the impairment by decreasing platelet adhesion and aggregation via a reduction in P-selectin surface expression (Figure 5.1).

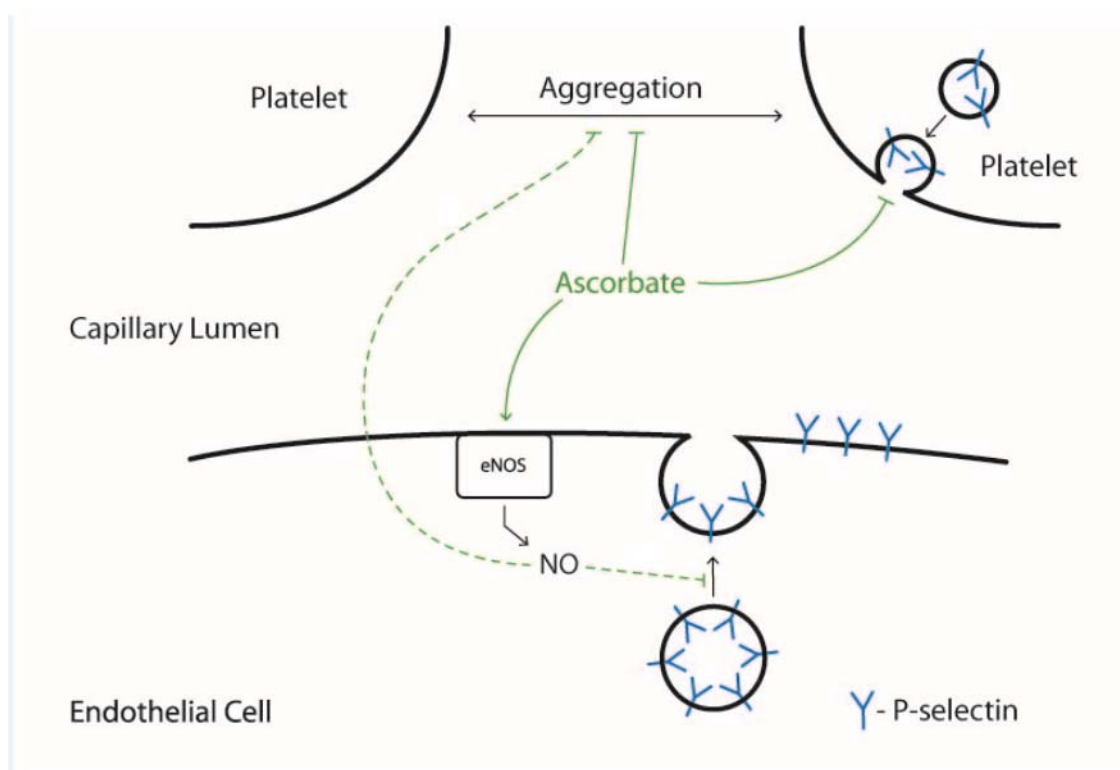


Figure 5.1. The effect of ascorbate on platelet-endothelial function in septic capillaries. Ascorbate can directly reduce platelet aggregation and P-selectin surface expression (i.e., platelet activation). Furthermore, ascorbate could restore local NO production via eNOS. The local NO may further reduce platelet aggregation and endothelial P-selectin surface expression. The summation of these effects leads to reduced platelet adhesion and aggregation in the septic capillary leading to restoration of blood flow.

5.2 GENERAL DISCUSSION

5.2.1 Comparing the *in vivo*, *ex vivo*, and *in vitro* models of sepsis

Sepsis is a complex inflammatory disease that involves numerous cell types and mediators. In addition, sepsis can be confounded by pre-existing diseases, making it very difficult to study (29). The studies presented in this thesis use *in vivo*, *ex vivo*, and *in vitro* models to investigate the effect of sepsis on capillary platelet-endothelial and platelet-platelet interactions. These models are used to gain insights on how ascorbate treatment benefits capillary flow during sepsis. Capillary impairment in the mouse extensor digitorum longus (EDL) muscle, platelet-platelet interactions of isolated platelets, and platelet interactions with mouse microvascular endothelial cells were used as *in vivo*, *ex vivo*, and *in vitro* models of sepsis, respectively. These models have both advantages and limitations.

5.2.1.1 Mouse models of sepsis

There are several animal models used to study the mechanisms governing the pathophysiology of sepsis. The goal of these models is to best mimic the pathogenesis observed in septic patients. The most commonly used models are (i) direct administration of endotoxin or viable bacteria into the animal and (ii) causing peritonitis by disrupting the barrier of the gastrointestinal tract by cecal ligation and puncture (CLP) or direct administration of fecal material into the peritoneum (FIP).

Endotoxemic models usually involve bolus injections of lipopolysaccharide (LPS), a component of the wall of Gram-negative bacteria. This model is used primarily

for its ease in standardizing the experimental conditions (22). The major criticism of this model is its lack of clinical relevance, as the LPS challenge does not have the focus of infection seen in clinical sepsis, because LPS levels are usually low in human patients (7). Furthermore, the LPS model does not account for the septic cases that involve Gram-positive bacteria (41).

The bacteremia model of sepsis involves the injection of cultures of viable bacteria (16) and thus may be more clinically relevant than the endotoxemic model. However, the injected bacteria are usually not able to replicate within the host because they are destroyed by the immune system (13). This results in an endotoxemic model rather than an infectious model causing the model to suffer from the same criticisms as the LPS model. Both of these models lead to many of the clinical characteristics of sepsis (hypotension, multiple organ dysfunction) but tend to have more acute and intense increases in inflammatory mediators than seen in septic patients (22).

Both the FIP and CLP models of sepsis are better associated with clinical sepsis. As well as having a peritoneal focus of infection and displaying the characteristic symptoms of clinical sepsis, both models produce a continuous and sustained production of cytokines similar to human patients (21, 22, 59). For the present series of investigations, the FIP model was used over the CLP because it is a more standardized model. The resulting infection due to CLP can vary greatly depending on the amount of fecal material that leaks into the peritoneum. Thus the precision in replicating the surgical procedure is essential for accurate data. The FIP model eliminates this factor by standardizing the amount of feces injected into the peritoneum. Also, the sham control animals for the FIP model receive an intraperitoneal injection of saline as opposed to a

sham surgery in the CLP model, which itself leads to the release of inflammatory cytokines and thus confound the outcome of bacterial infection. When comparing the FIP and CLP models, the major drawback for the FIP model compared to the CLP model is that it is less clinically relevant as clinical septic patients have varied degrees of infections.

5.2.1.2 Methodological limitations to the models

In Chapter 2, mouse EDL muscle was observed by intravital microscopy. Although skeletal muscle is not a critical organ, it is used to model capillary beds because the capillaries can be easily visualized microscopically when compared to other organs that move (e.g., lungs, heart). Moreover, the EDL muscle was chosen because it can be surgically exposed without disturbance to the muscle and because it has evenly distributed parallel capillaries. Also, it is possible to visualize platelets in these capillaries in real time. I used the epi-illuminated EDL muscle preparation where scattered light is reflected from the muscle surface to the microscope objective, thus limiting the optical resolution at a higher magnification. Therefore, platelets (about 1 μm diameter) needed to be labelled with a fluorescence dye to be visualized under fluorescent illumination. The labelling makes it difficult to distinguish between single platelets and small aggregates of 4 or less platelets. Furthermore, labelling of the platelets requires administering the dye just before the visualization (e.g., 6 h into sepsis when cessation of flow in capillaries has already occurred). Thus, any platelets situated in capillaries that are completely plugged (and therefore, have no flow) would not be labelled by the dye. This causes an underestimate in the reported platelet adhesion in septic capillaries.

Because ascorbate may have many effects *in vivo*, the reduction in platelet adhesion in the capillaries after ascorbate administration could be a result of these other effects (e.g., increased blood pressure to restore flow) as oppose to the direct effect ascorbate may have on platelet-endothelial interactions. To this end, we employed *ex vivo* and *in vitro* models to eliminate the complexities of the *in vivo* model. I used isolated platelets in the *ex vivo* model to measure platelet function under septic conditions. One disadvantage of this model was that LPS or TNF α treatments, which represent the initiating and inflammatory cytokine stages of sepsis, respectively, did not produce any platelet activation. Therefore, to mimic the septic condition, the stimulating agents thrombin, ADP, and thromboxane A₂ were used instead. Another limitation to the *ex vivo* model is that the conditions in the aggregation assay are pro-aggregatory (i.e., platelets aggregate spontaneously when left in the cuvette for longer than 1 - 2 h), thus diminishing the potential full inhibitory effect of ascorbate I have investigated.

The final model used is an *in vitro* model using mouse microvascular endothelial cells to investigate platelet-endothelial interactions. Although there was an increase in platelet adhesion after treatment with LPS or TNF α , the overall number of adhering platelets was very low in comparison to the total number of platelets used in the assay (i.e., ~ 0.1 % platelets adhered out of the total number of platelets applied to the endothelial monolayer). Pilot experiments with higher concentrations of LPS, TNF α , or thrombin failed to increase the platelet adhesion greater than that observed. It is proposed that culturing the endothelial cells reduces in their ability to interact with platelets. This is further evident when examining P-selectin expression in these cells. P-selectin protein was not detected in the cells using western blot, immunocytochemistry, and flow

cytometry techniques. However, P-selectin mRNA was present and blocking of P-selectin reduced platelet-endothelial adhesion (Figures 4.3, 4.1) indicating the presence of P-selectin in the cells. If P-selectin was expressed in very low amounts or only present in a small number of the cells, then this would account for the relative low adhesion seen in this model.

5.2.2 Mechanism of capillary blood flow impairment during sepsis

The impairment of capillary blood flow during sepsis was first discovered and defined in skeletal muscle almost 20 years ago (36). Since then it has been seen in other organs as well as in septic patients (4, 6). Essentially, the impairment is cessation of blood flow in approximately 50% of septic capillaries (61). The underlying pathophysiological processes that prevent the flow of blood in these septic capillaries are yet to be fully elucidated. Table 1.1 summarizes some of the possible processes that may contribute to the cessation of flow.

The blood flow in capillaries can be visualized by the intravital microscopy in terms of the movement of RBCs situated in these capillaries. Any process that prevents RBCs from this movement will contribute to the impairment seen in sepsis. This includes sepsis-induced changes in blood pressure or changes in the ability of the RBC to deform to progress through the vessel. Changes in the perfusion pressure across the capillary bed include both a decrease in arteriolar pressure at the capillary entrance as well as an increase in venular pressure (drainage pressure) at the capillary exit (2, 5, 28). Although a large enough decrease in arterial blood pressure could prevent flow in a capillary, Boczkowski *et al* (5) showed that decreases in mean arterial pressure due to haemorrhage

did not cause a dysfunction in capillary blood flow seen in LPS-treated rats. Venular pressure is also increased in sepsis (28), and could potentially inhibit flow through a capillary. However, I have observed directly with intravital microscopy a septic capillary bed supplied by a single arteriole and drained by a single venule. Here about 50 % of capillaries had stopped flow, while both the arteriole and the venule remained perfused with blood (Chapter 2). Thus, capillary plugging does not require stoppage of blood in the arteriole or the venule.

Even though blood pressure changes in the capillaries are unlikely to result in capillary blood flow impairment, they could combine with other processes in the septic microvasculature (e.g., increased wall permeability) to contribute to capillary flow stoppage. Edema and vascular leak are key features of sepsis that promote swelling of the tissue surrounding the capillaries (37). If the swelling becomes severe enough, the resulting decrease in the capillary diameter could potentially inhibit the passage of RBCs in the capillary. A similar situation could occur due to the swelling of endothelial cells, another event that occurs during sepsis (26). Blood flow could also be stopped by blocking the passage of RBCs by physical means such as adhering non-deformable RBCs, adhering WBCs, or microthrombi. RBCs have been shown to become adhesive to LPS treated endothelial cells (19) and stiffen in during sepsis (3). Leukocyte adhesion is markedly increased during sepsis (12, 45, 55). However, this event is usually measured in post-capillary venules and therefore the contribution of leukocytes to the capillary plugging is unclear. Due to the targeting of leukocytes to specific organs during sepsis (47), the role in the cessation of capillary blood flow by WBCs is organ-dependent. Roller *et al* (55) observed increase leukocyte trapping in lung capillaries in a mouse CLP

model, and Croner *et al* (12) showed increased leukocyte adhesion in liver sinusoids in a rat CLP model. On the other hand, in our skeletal muscle model I did not notice any significant increase in leukocytes in septic capillaries (Chapter 2). I showed an increase in platelet adhesion and fibrin disposition in septic capillaries in our mouse FIP model (Figure 2.1). These 2 factors combined suggest the presence of microthrombi in the capillaries that could prevent blood flow. I further showed that removal of platelets or inhibition of thrombi formation reduces the number of stopped-flow capillaries in septic mice (Chapter 2).

Overall, the stoppage of blood flow that defines the capillary blood flow impairment is most likely caused by a combination of these factors (for example, a single platelet adhering to the endothelium may not itself block flow but might reduce the capillary diameter enough that a stiffened RBC cannot fit through). The interventions that I used in Chapter 2 to reduce microthrombi formation did not reduce the capillary blood flow impairment back to sham levels. This indicates that, in addition to microthrombi formation, other mechanism(s) are contributing to the observed impairment.

5.2.3 Capillary plugging in septic skeletal muscle

As mentioned in section 5.2.2, there are 3 main cell types that can be involved in impeding capillary blood flow: leukocytes, RBCs, and platelets clumped in microthrombi. Since leukocyte involvement in the skeletal muscle is minimal, our skeletal muscle model allowed addressing the impact of only the 2 cell types (platelets and RBCs) on the capillary plugging.

5.2.3.1 Microthrombi formation

The exact size, shape, and composition of a microthrombus necessary to plug a capillary are not known. Single adhering platelets are not large enough to prevent the blood flow alone. I have observed that platelet adhesion and fibrin deposition is increased in the septic capillary and that they both usually occur in the same capillary. However due to limitations in our methodology I cannot accurately determine (i) the size (number of platelets) per aggregate, (ii) the architecture of fibrin depositions, and (iii) the relative contribution of platelets and fibrin to the microthrombi. I observed that septic conditions promote both platelet aggregation and platelet-endothelial adhesion (Chapters 3 and 4). This adhesion contributes to the microthrombi formation in the septic capillary. However, the role of fibrin in the plugging of septic capillary has not been studied.

Although fibrin has other roles, its primary function is to support clot formation and provide integrity to the thrombi (66, 68). In the septic capillaries I observed the fibrin depositions as long strands as opposed to clumps (Figure 5.2). From these observations I cannot rule out that, after fibrin is formed (by cleavage of fibrinogen), whether it remains in the plasma or it lines the vessels without participating in clot formation (thus not contributing to the plugging of the capillaries). Fibrin deposition has also been observed in capillaries of endotoxemic rat liver and kidney but not in the lung (60), further indicating that the mechanism of capillary plugging is organ specific. This study used immunohistochemistry techniques, which like our study only indicates that fibrin is present. The specific role and necessity of fibrin in the capillary plugging in sepsis will need to be further investigated to completely understand its role in capillary impairment.

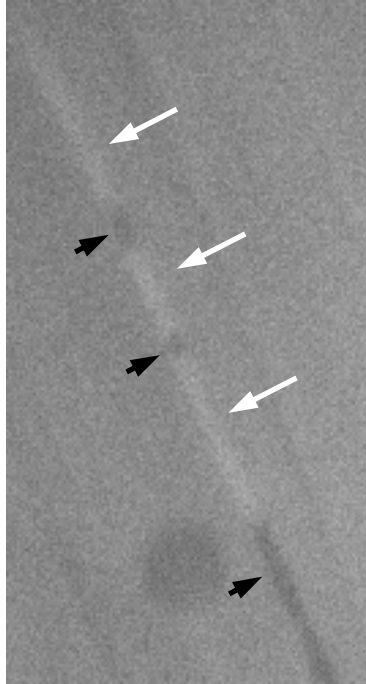


Figure 5.2. Image of fibrin plaques in septic capillaries. Fibrin plaques (white arrows) were visualized with fluorescence illumination using Alexa 488-labelled anti-fibrin antibody, injected i.v. 10 min prior visualization (antibody does not bind to fibrinogen). Plaques were seen only in capillaries with stationary red blood cells (black arrows).

5.2.3.2 RBC adhesion

In a sepsis-like state, RBCs are activated, causing red blood cell adhesion *in vitro* (18, 19). Adhering RBCs may contribute to the microthrombus formation and could potentially plug capillaries. Under septic conditions, RBCs are known to express phosphatidylserine (PS) on their surface (33). In view of the emerging evidence of endothelial cells expressing a PS binding receptor (57), this suggests a possible mechanism for RBC-EC adhesion that may contribute to the capillary plugging. Furthermore, PS will bind to thrombospondin, which will also bind to the vitronectin receptor on endothelial cells suggesting another role for PS expression on RBCs to participate in RBC-endothelium adhesion (24). I have observed RBCs in plugged capillaries; however, thus far, I was unable to determine whether they contribute to capillary stoppage or they are simply just caught behind the blockage. I conducted pilot studies in our lab using an *in vitro* approach (RBCs flowed over ECs in a parallel plate system). In these pilots, I did not visualize any obvious increase in RBC adhesion under septic conditions (LPS or TNF α treatment of ECs). Due to methodological difficulties and lack of any meaningful observations, further studies here were not pursued.

5.2.4 Ascorbate and capillary blood flow impairment during sepsis

It has been shown that an intravenous bolus of ascorbate prevents and reverses the sepsis-induced capillary blood flow impairment (2, 61, 62, Chapter 2). Although we have made progress in understanding the capillary blood flow impairment on the tissue level (i.e., oxidative stress and eNOS dysfunction contribute to the impairment) and in understanding how ascorbate inhibits impairment at this level (i.e., it reduces oxidative

stress and restores eNOS function), the potential mechanisms of how ascorbate achieves this inhibition at the cellular/molecular level are still poorly understood. In Figures 2.4 and 2.5, I show that ascorbate reduces platelet adhesion in the septic capillary, which suggests that ascorbate reduces the capillary plugging by preventing microthrombi formation. Overall, it is very likely that ascorbate reduces the capillary blood flow impairment by more than one mechanism.

Ascorbate may reduce capillary plugging by affecting platelets, RBCs, and leukocytes. Data in this thesis indicate that ascorbate decreases platelet adhesion and aggregation under septic conditions through a reduction in P-selectin surface expression. Ascorbate may also reduce RBC adhesion by decreasing PS exposure on RBCs. Ascorbate reduces PS exposure in RBCs in an apoptotic model (40). Reduced PS exposure prevents RBC adhesion by lowering its exposure to its counter receptors (described in section 5.2.3). Finally, ascorbate may reduce capillary plugging by lowering leukocyte adhesion. Ascorbic acid can reduce *in vitro* leukocyte adhesion to endothelial cells (15). Also, vitamin C can reduce *in vivo* leukocyte adhesion in diabetic and oxidized LDL models of oxidative stress (32, 38). Although the effect of ascorbate on leukocyte adhesion would only have a minor beneficial effect on capillary blood flow in skeletal muscle, this effect could be very important in other organs.

Ascorbate reduces the permeability of LPS-stimulated endothelial monolayers (25) and therefore it may reduce endothelial swelling during sepsis. There is evidence that antioxidants decrease endothelial swelling in an ischemia/reperfusion model (64). Also, antioxidants have been shown to decrease edema in lung and heart tissues in animal models of sepsis (34, 58, 69). Furthermore, the antioxidant tirilazad can reduced RBC

stiffness in an animal stress model (8). Thus, ascorbate may increase capillary diameter and RBC deformability to prevent the RBCs from getting stuck in the capillaries.

Ascorbate may influence the capillary blood flow during sepsis indirectly, since it prevents the drop in mean arterial blood pressure seen in CLP rats (62). Ascorbate improves arteriolar function by inhibiting the sepsis-reduced conducted vasoconstriction in mouse arterioles (46) and by reversing arterial hyporesponsiveness to vasoconstrictors in human patients injected with LPS (53).

5.2.5 The protective effect of ascorbate and eNOS

Our lab has shown that the protective effect of ascorbate on the sepsis-induced impairment of capillary blood flow is eNOS dependent (61). Figure 2.5 shows that ascorbate's inhibitory effect on platelet adhesion in septic capillaries is also eNOS dependent. However, using the *ex vivo* approach, the reduction in platelet aggregation by ascorbate was NOS independent suggesting that ascorbate reduces aggregation directly. This reduction in aggregation must be insufficient to improve capillary blood flow in septic capillaries without eNOS function (Chapter 3).

The NO derived from eNOS maintains vascular homeostasis by regulating vasomotor tone (51), inhibiting platelet aggregation (1), inhibiting leukocyte-endothelium adhesion (35), and inhibiting apoptosis (9). NO acts directly on guanylyl cyclase to produce cGMP that activates downstream target protein kinase G. This pathway mediates vascular smooth muscle relaxation and platelet inhibition. NO may also modulate protein function by nitrosylation of thiol residues in the amino acid sequence. One example is the nitrosylation of N-ethylmaleimide-sensitive factor (NSF), a protein critical for proper

granule docking with the plasma membrane. Nitrosylated NSF has reduced function resulting in reduced granule secretion (43).

In vivo, ascorbate prevents and reverses the oxidation of BH₄ and thus restores proper eNOS function (Figure 1.2). The local NO can then participate in the unplugging of capillaries by reducing platelet adhesion and aggregation. In the *ex vivo* model, platelet aggregation was reduced by ascorbate, independent of platelet derived NO. I proposed that endothelial derived NO may further inhibit both platelet aggregation and P-selectin surface expression (Chapter 3). Thus restoration of endothelial eNOS function may be required for ascorbate's full anti-platelet effects *in vivo*. Data in Chapter 4 suggest that ascorbate reduces platelet-endothelial adhesion by at least 2 mechanisms. One of these mechanisms could be reduced surface P-selectin protein expression on endothelial cells due to nitrosylation of NSF. It would be interesting to know whether the effect of ascorbate on LPS or TNF α -induced platelet-endothelial cell adhesion *in vitro* is NOS dependent since LPS and TNF α increase adhesion by different mechanisms (and NO could play a different role in each case).

5.2.6 Ascorbate as a treatment for sepsis

The data in the thesis suggest a major role of the coagulation system in the pathophysiology of sepsis. Specifically, Figure 2.2 shows that inhibition of coagulation by antithrombin or eptifibatid prevents or reverses the capillary blood flow impairment respectively. This indicates that targeting the coagulation pathway would be of interest for treating septic patients. However, clinical trials with anticoagulants have not been successful in improving survival (14).

Ascorbate in combination with vitamin E was shown to decrease mortality in critically ill patients (11) as well as reduce multiple organ failure in critically ill surgical patients (49). Although the above studies have indicated a beneficial affect of ascorbate, the administration of ascorbate alone has not yet being used as a therapy for septic patients.

A recent trial involving critically ill patients by Heyland *et al* (27) showed no improvement in 28-day mortality with antioxidant treatment. However, the treatment in this study was a mixture of antioxidants given orally as oppose to just ascorbate given intravenously as presented in this thesis. It is possible that reduction of platelet adhesion and restoration of capillary blood flow could have negative consequences on survival. Platelet adhesion to neutrophils is necessary for the formation of neutrophil extracellular traps that have a role in bacteria trapping (10). Restored blood flow may promote spreading of the infection. Thus, a negative effect of ascorbate treatment could be prolonging the infection.

Ascorbate may be more beneficial than other antioxidants because it is readily absorbed into microvascular endothelial cells and platelets through high affinity vitamin C transporters (67). This leads to the accumulation of high levels of intracellular ascorbate (up to 16 mM in endothelial cells and greater than 1mM in platelets (44, 67). Furthermore, excess ascorbate is quickly excreted (over 4 - 5 h) through the kidneys (50). This is beneficial because ROS are required as a defense mechanism against infections (including bactericidal effects and the up-regulation on inflammatory cytokines). Removal of ROS may be the reason that other antioxidants have not always offered protective results in septic patients (17). Moreover, mice with a major source of ROS

genetically removed (gp91phox knockouts, a subunit of NADPH oxidase) have reduced 24 h survival in a FIP model of sepsis when compared with wild type mice (unpublished observations from our lab). Thus, complete suppression and/or continuous antioxidant therapy in septic patients could be detrimental. Ascorbate being quickly absorbed or excreted would reduce the effect of this possible complication.

Septic patients have reduced plasma ascorbate levels (23). High doses of ascorbate are needed to restore plasma ascorbate levels in critically ill patients back to control (39, 49, 56). There is no evidence to suggest toxic effects of high dose ascorbate on organ function. In healthy volunteers, intravenous injections of 7.5 g, 33 mg/kg, or 2 g of ascorbate induced no pro-oxidant changes in plasma markers of oxidative stress (48), no abnormalities in blood cell counts, or liver or kidney function (42), and did not alter superoxide production by neutrophils (20), respectively.

5.2.7 Overall implications of this research

Sepsis leads to the cessation of capillary blood flow which results in tissue hypoxia and potentially multiple organ failure (36, 61). The cellular mechanisms that cause this impairment have yet to be fully determined. Furthermore, treatment of septic animals with ascorbate has shown to prevent and reverse the cessation in capillary blood flow (2, 61, 62). Again, the mechanisms underlying ascorbate's beneficial effects are not completely clear.

The research in this thesis is the first to demonstrate the participation of the coagulation system in the capillary blood flow impairment during sepsis. In particular, we show increased platelet adhesion and fibrin deposition in septic capillaries suggesting the

presence of microthrombi in the capillaries. The increase in platelet adhesion is reduced by ascorbate eNOS-dependently similar to the improvement of capillary blood flow by ascorbate. This demonstrates another mechanism by which ascorbate could improve the outcome in sepsis.

Ascorbate reduces platelet aggregation and platelet-endothelial adhesion in *ex vivo* and *in vitro* models of sepsis, respectively; by a reduction in the cell surface P-selectin protein expression. The reduced aggregation and adhesion by ascorbate leads to a decrease in the formation of microthrombi in the capillaries. Removal of microthrombi leads to the rescue of capillary blood flow, attenuating tissue hypoxia. This thesis demonstrates a mechanism of ascorbate providing a beneficial effect during sepsis and thus supports investigations of the use of ascorbate as an adjuvant treatment for septic patients.

5.3 FUTURE DIRECTIONS

5.3.1 Effect of eNOS inhibition on platelet adhesion, in vitro

As mentioned previously, the protective effects of ascorbate against the impairment of capillary blood flow and increase platelet adhesion to the capillary wall during sepsis is eNOS dependent. In Chapter 4, I show that ascorbate can reduce LPS-induced platelet-endothelial adhesion by reducing P-selectin surface expression as assayed by vWF granule expression. I propose that ascorbate acts to restore eNOS function similar to our *in vivo* model. The NO produced by restored eNOS may nitrosylate NSF, causing a reduction in granule secretion, and thus reduce surface P-selectin protein expression (43).

Furthermore, ascorbate is expected to reduce TNF α -induced platelet-endothelial adhesion by a different mechanism since TNF α does not cause an increase in granule secretion. Investigating the effect of NOS inhibition in the *in vitro* model of LPS-induced platelet-endothelial adhesion should clarify the mechanism of ascorbate's inhibitory effect.

5.3.2 Mechanism of ascorbate inhibition of TNF α -induced platelet adhesion, *in vitro*

TNF α caused an increase in platelet-endothelial adhesion in our *in vitro* model. However, this increase was not due to increased P-selectin protein expression on the endothelial cell surface. Blockade of P-selectin prevented this increase in adhesion indicating the increased adhesion still involved P-selectin. Ascorbate reduced the platelet adhesion through a mechanism that did not include reduction in granule secretion. It has been suggested that ascorbate could affect the protein kinase C (PKC) pathway in platelets (Chapter 3), but this pathway has not been examined in endothelial cells. TNF α is known to increase the activity of the transcription factor nuclear factor κ B (NF κ B) through a PKC pathway (54). NF κ B up-regulates inflammatory cytokines and numerous genes that regulate cell adhesion including P-selectin (52), E-selectin (65), vascular cell adhesion molecule (VCAM)-1 (30), and intercellular adhesion molecule (ICAM)-1 (63). Moreover, PKC has been shown to modulate the function of platelet-endothelial cell adhesion molecule (PECAM)-1 (31), which has the potential to facilitate the adhesion of platelets to the endothelial cells in our model. The role of PKC in TNF α -induced platelet adhesion in our *in vitro* model could be examined by pharmacological inhibition of PKC

by calphostin C. PKC activity assays in platelets and endothelial cells could be used to determine if ascorbate alters PKC function.

5.4 REFERENCES

1. Alheid U, Frolich JC, Forstermann U. Endothelium-derived relaxing factor from cultured human endothelial cells inhibits aggregation of human platelets. *Thromb Res* 47(5): 561-571, 1987.
2. Armour J, Tyml K, Lidington D, Wilson JX. Ascorbate prevents microvascular dysfunction in the skeletal muscle of the septic rat. *J Appl Physiol* 90(3): 795-803, 2001.
3. Bateman RM, Jagger JE, Sharpe MD, Ellsworth ML, Mehta S, Ellis CG. Erythrocyte deformability is a nitric oxide-mediated factor in decreased capillary density during sepsis. *Am J Physiol Heart Circ Physiol* 280(6): H2848-56, 2001.
4. Bateman RM, Sharpe MD, Ellis CG. Bench-to-bedside review: microvascular dysfunction in sepsis--hemodynamics, oxygen transport, and nitric oxide. *Crit Care* 7(5): 359-373, 2003.
5. Boczkowski J, Vicaut E, Aubier M. In vivo effects of Escherichia coli endotoxemia on diaphragmatic microcirculation in rats. *J Appl Physiol* 72(6): 2219-2224, 1992.
6. Boerma EC, van der Voort PH, Spronk PE, Ince C. Relationship between sublingual and intestinal microcirculatory perfusion in patients with abdominal sepsis. *Crit Care Med* 35(4): 1055-1060, 2007.
7. Casey LC, Balk RA, Bone RC. Plasma cytokine and endotoxin levels correlate with survival in patients with the sepsis syndrome. *Ann Intern Med* 119(8): 771-778, 1993.

8. Chmiel B, Grabowska-Bochenek R, Piskorska D. Tirilazad mesylate improve water immersion stress induced decrease in erythrocyte deformability in the rats. *Med Sci Monit* 7(1): 38-41, 2001.
9. Chung HT, Pae HO, Choi BM, Billiar TR, Kim YM. Nitric oxide as a bioregulator of apoptosis. *Biochem Biophys Res Commun* 282(5): 1075-1079, 2001.
10. Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, Patel KD, Chakrabarti S, McAvoy E, Sinclair GD, Keys EM, Allen-Vercoe E, Devinney R, Doig CJ, Green FH, Kubes P. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med* 13(4): 463-469, 2007.
11. Crimi E, Liguori A, Condorelli M, Cioffi M, Astuto M, Bontempo P, Pignalosa O, Vietri MT, Molinari AM, Sica V, Della Corte F, Napoli C. The beneficial effects of antioxidant supplementation in enteral feeding in critically ill patients: a prospective, randomized, double-blind, placebo-controlled trial. *Anesth Analg* 99(3): 857-63, table of contents, 2004.
12. Croner RS, Hoerer E, Kulu Y, Hackert T, Gebhard MM, Herfarth C, Klar E. Hepatic platelet and leukocyte adherence during endotoxemia. *Crit Care* 10(1): R15, 2006.
13. Cross AS, Opal SM, Sadoff JC, Gemski P. Choice of bacteria in animal models of sepsis. *Infect Immun* 61(7): 2741-2747, 1993.

14. De Backer D, Donadello K, Taccone FS, Ospina-Tascon G, Salgado D, Vincent JL. Microcirculatory alterations: potential mechanisms and implications for therapy. *Ann Intensive Care* 1: 27, 2011.
15. De la Fuente M, Victor VM. Ascorbic acid and N-acetylcysteine improve in vitro the function of lymphocytes from mice with endotoxin-induced oxidative stress. *Free Radic Res* 35(1): 73-84, 2001.
16. Deitch EA. Animal models of sepsis and shock: a review and lessons learned. *Shock* 9(1): 1-11, 1998.
17. Domenighetti G, Suter PM, Schaller MD, Ritz R, Perret C. Treatment with N-acetylcysteine during acute respiratory distress syndrome: a randomized, double-blind, placebo-controlled clinical study. *J Crit Care* 12(4): 177-182, 1997.
18. Eichelbronner O, Sibbald WJ, Chin-Yee IH. Intermittent flow increases endotoxin-induced adhesion of human erythrocytes to vascular endothelial cells. *Intensive Care Med* 29(5): 709-714, 2003.
19. Eichelbronner O, Sielenkamper A, Cepinskas G, Sibbald WJ, Chin-Yee IH. Endotoxin promotes adhesion of human erythrocytes to human vascular endothelial cells under conditions of flow. *Crit Care Med* 28(6): 1865-1870, 2000.
20. Ellis GR, Anderson RA, Lang D, Blackman DJ, Morris RH, Morris-Thurgood J, McDowell IF, Jackson SK, Lewis MJ, Frenneaux MP. Neutrophil superoxide anion--generating capacity, endothelial function and oxidative stress in chronic heart failure:

effects of short- and long-term vitamin C therapy. *J Am Coll Cardiol* 36(5): 1474-1482, 2000.

21. Feihl F, Waeber B, Liaudet L. Is nitric oxide overproduction the target of choice for the management of septic shock?. *Pharmacol Ther* 91(3): 179-213, 2001.

22. Freise H, Bruckner UB, Spiegel HU. Animal models of sepsis. *J Invest Surg* 14(4): 195-212, 2001.

23. Galley HF, Davies MJ, Webster NR. Ascorbyl radical formation in patients with sepsis: effect of ascorbate loading. *Free Radic Biol Med* 20(1): 139-143, 1996.

24. Gayen Betal S, Setty BN. Phosphatidylserine-positive erythrocytes bind to immobilized and soluble thrombospondin-1 via its heparin-binding domain. *Transl Res* 152(4): 165-177, 2008.

25. Han M, Pendem S, Teh SL, Sukumaran DK, Wu F, Wilson JX. Ascorbate protects endothelial barrier function during septic insult: Role of protein phosphatase type 2A. *Free Radic Biol Med* 48(1): 128-135, 2010.

26. Hauptmann S, Klosterhalfen B, Weis J, Poche R, Mittermayer C, Kirkpatrick CJ. Morphology of cardiac muscle in septic shock. Observations with a porcine septic shock model. *Virchows Arch* 426(5): 487-491, 1995.

27. Heyland D, Muscedere J, Wischmeyer PE, Cook D, Jones G, Albert M, Elke G, Berger MM, Day AG, Canadian Critical Care Trials Group. A randomized trial of

glutamine and antioxidants in critically ill patients. *N Engl J Med* 368(16): 1489-1497, 2013.

28. Hinshaw LB. Sepsis/septic shock: participation of the microcirculation: an abbreviated review. *Crit Care Med* 24(6): 1072-1078, 1996.

29. Hubbard WJ, Choudhry M, Schwacha MG, Kerby JD, Rue LW, 3rd, Bland KI, Chaudry IH. Cecal ligation and puncture. *Shock* 24 Suppl 1: 52-57, 2005.

30. Iademarco MF, McQuillan JJ, Rosen GD, Dean DC. Characterization of the promoter for vascular cell adhesion molecule-1 (VCAM-1). *J Biol Chem* 267(23): 16323-16329, 1992.

31. Ilan N, Cheung L, Pinter E, Madri JA. Platelet-endothelial cell adhesion molecule-1 (CD31), a scaffolding molecule for selected catenin family members whose binding is mediated by different tyrosine and serine/threonine phosphorylation. *J Biol Chem* 275(28): 21435-21443, 2000.

32. Jariyapongskul A, Patumraj S, Yamaguchi S, Niimi H. The effect of long-term supplementation of vitamin C on leukocyte adhesion to the cerebral endothelium in STZ-induced diabetic rats. *Clin Hemorheol Microcirc* 27(1): 67-76, 2002.

33. Kempe DS, Akel A, Lang PA, Hermle T, Biswas R, Muresanu J, Friedrich B, Dreischer P, Wolz C, Schumacher U, Peschel A, Gotz F, Doring G, Wieder T, Gulbins E, Lang F. Suicidal erythrocyte death in sepsis. *J Mol Med (Berl)* 85(3): 273-281, 2007.

34. Koga H, Hagiwara S, Inomata M, Kono Y, Oyama Y, Kai S, Nishida T, Noguchi T. The new vitamin E derivative, ETS-GS, protects against cecal ligation and puncture-induced systemic inflammation in rats. *Inflammation* 35(2): 545-553, 2012.
35. Kubes P, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A* 88(11): 4651-4655, 1991.
36. Lam C, Tyml K, Martin C, Sibbald W. Microvascular perfusion is impaired in a rat model of normotensive sepsis. *J Clin Invest* 94(5): 2077-2083, 1994.
37. Lee WL, Liles WC. Endothelial activation, dysfunction and permeability during severe infections. *Curr Opin Hematol* 18(3): 191-196, 2011.
38. Lehr HA, Frei B, Olofsson AM, Carew TE, Arfors KE. Protection from oxidized LDL-induced leukocyte adhesion to microvascular and macrovascular endothelium in vivo by vitamin C but not by vitamin E. *Circulation* 91(5): 1525-1532, 1995.
39. Long CL, Maull KI, Krishnan RS, Laws HL, Geiger JW, Borghesi L, Franks W, Lawson TC, Sauberlich HE. Ascorbic acid dynamics in the seriously ill and injured. *J Surg Res* 109(2): 144-148, 2003.
40. Mahmud H, Qadri SM, Foller M, Lang F. Inhibition of suicidal erythrocyte death by vitamin C. *Nutrition* 26(6): 671-676, 2010.
41. Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* 348(16): 1546-1554, 2003.

42. Matsuda T, Tanaka H, Yuasa H, Forrest R, Matsuda H, Hanumadass M, Reyes H. The effects of high-dose vitamin C therapy on postburn lipid peroxidation. *J Burn Care Rehabil* 14(6): 624-629, 1993.
43. Matsushita K, Morrell CN, Cambien B, Yang SX, Yamakuchi M, Bao C, Hara MR, Quick RA, Cao W, O'Rourke B, Lowenstein JM, Pevsner J, Wagner DD, Lowenstein CJ. Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. *Cell* 115(2): 139-150, 2003.
44. McCulloch RK, Vandongen R. Measurement of ascorbic acid in platelets and its relationship to polymorphonuclear leukocyte levels. *Clin Chim Acta* 213(1-3): 15-22, 1992.
45. McCuskey RS, Urbaschek R, Urbaschek B. The microcirculation during endotoxemia. *Cardiovasc Res* 32(4): 752-763, 1996.
46. McKinnon RL, Lidington D, Tysl K. Ascorbate inhibits reduced arteriolar conducted vasoconstriction in septic mouse cremaster muscle. *Microcirculation* 14(7): 697-707, 2007.
47. Mizgerd JP. Molecular mechanisms of neutrophil recruitment elicited by bacteria in the lungs. *Semin Immunol* 14(2): 123-132, 2002.
48. Muhlhofer A, Mrosek S, Schlegel B, Trommer W, Rozario F, Bohles H, Schremmer D, Zoller WG, Biesalski HK. High-dose intravenous vitamin C is not associated with an increase of pro-oxidative biomarkers. *Eur J Clin Nutr* 58(8): 1151-1158, 2004.

49. Nathens AB, Neff MJ, Jurkovich GJ, Klotz P, Farver K, Ruzinski JT, Radella F, Garcia I, Maier RV. Randomized, prospective trial of antioxidant supplementation in critically ill surgical patients. *Ann Surg* 236(6): 814-822, 2002.
50. Padayatty SJ, Sun H, Wang Y, Riordan HD, Hewitt SM, Katz A, Wesley RA, Levine M. Vitamin C pharmacokinetics: implications for oral and intravenous use. *Ann Intern Med* 140(7): 533-537, 2004.
51. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327(6122): 524-526, 1987.
52. Pan J, McEver RP. Regulation of the human P-selectin promoter by Bcl-3 and specific homodimeric members of the NF-kappa B/Rel family. *J Biol Chem* 270(39): 23077-23083, 1995.
53. Pleiner J, Mittermayer F, Schaller G, Marsik C, MacAllister RJ, Wolzt M. Inflammation-induced vasoconstrictor hyporeactivity is caused by oxidative stress. *J Am Coll Cardiol* 42(9): 1656-1662, 2003.
54. Rahman A, Bando M, Kefer J, Anwar KN, Malik AB. Protein kinase C-activated oxidant generation in endothelial cells signals intercellular adhesion molecule-1 gene transcription. *Mol Pharmacol* 55(3): 575-583, 1999.
55. Roller J, Wang Y, Rahman M, Schramm R, Laschke MW, Menger MD, Jeppsson B, Thorlacius H. Direct in vivo observations of P-selectin glycoprotein ligand-1-mediated

leukocyte-endothelial cell interactions in the pulmonary microvasculature in abdominal sepsis in mice. *Inflamm Res*, 2012.

56. Schorah CJ, Downing C, Piripitsi A, Gallivan L, Al-Hazaa AH, Sanderson MJ, Bodenham A. Total vitamin C, ascorbic acid, and dehydroascorbic acid concentrations in plasma of critically ill patients. *Am J Clin Nutr* 63(5): 760-765, 1996.

57. Setty BN, Betal SG. Microvascular endothelial cells express a phosphatidylserine receptor: a functionally active receptor for phosphatidylserine-positive erythrocytes. *Blood* 111(2): 905-914, 2008.

58. Smeding L, Leong-Poi H, Hu P, Shan Y, Haitzma JJ, Horvath E, Furmli S, Masoom H, Kuiper JW, Slutsky AS, Parker TG, Plotz FB, dos Santos CC. Salutary effect of resveratrol on sepsis-induced myocardial depression. *Crit Care Med* 40(6): 1896-1907, 2012.

59. Stamme C, Bundschuh DS, Hartung T, Gebert U, Wollin L, Nusing R, Wendel A, Uhlig S. Temporal sequence of pulmonary and systemic inflammatory responses to graded polymicrobial peritonitis in mice. *Infect Immun* 67(11): 5642-5650, 1999.

60. Terada Y, Eguchi Y, Nosaka S, Toba T, Nakamura T, Shimizu Y. Capillary endothelial thrombomodulin expression and fibrin deposition in rats with continuous and bolus lipopolysaccharide administration. *Lab Invest* 83(8): 1165-1173, 2003.

61. Tyml K, Li F, Wilson JX. Septic impairment of capillary blood flow requires nicotinamide adenine dinucleotide phosphate oxidase but not nitric oxide synthase and is

rapidly reversed by ascorbate through an endothelial nitric oxide synthase-dependent mechanism. *Crit Care Med* 36(8): 2355-2362, 2008.

62. Tyml K, Li F, Wilson JX. Delayed ascorbate bolus protects against maldistribution of microvascular blood flow in septic rat skeletal muscle. *Crit Care Med* 33(8): 1823-1828, 2005.

63. van de Stolpe A, Caldenhoven E, Stade BG, Koenderman L, Raaijmakers JA, Johnson JP, van der Saag PT. 12-O-tetradecanoylphorbol-13-acetate- and tumor necrosis factor alpha-mediated induction of intercellular adhesion molecule-1 is inhibited by dexamethasone. Functional analysis of the human intercellular adhesion molecular-1 promoter. *J Biol Chem* 269(8): 6185-6192, 1994.

64. Ward BJ, Scoote M. Antioxidants attenuate posts ischemic endothelial cell swelling and luminal membrane blebbing in cardiac capillaries. *Microvasc Res* 53(2): 179-186, 1997.

65. Whelan J, Ghersa P, Hooft van Huijsduijnen R, Gray J, Chandra G, Talabot F, DeLamarter JF. An NF kappa B-like factor is essential but not sufficient for cytokine induction of endothelial leukocyte adhesion molecule 1 (ELAM-1) gene transcription. *Nucleic Acids Res* 19(10): 2645-2653, 1991.

66. Whittaker P, Przyklenk K. Fibrin architecture in clots: a quantitative polarized light microscopy analysis. *Blood Cells Mol Dis* 42(1): 51-56, 2009.

67. Wilson JX, Dixon SJ, Yu J, Nees S, Tymi K. Ascorbate uptake by microvascular endothelial cells of rat skeletal muscle. *Microcirculation* 3(2): 211-221, 1996.
68. Wolberg AS. Determinants of fibrin formation, structure, and function. *Curr Opin Hematol* 19(5): 349-356, 2012.
69. Xiao X, Yang M, Sun D, Sun S. Curcumin protects against sepsis-induced acute lung injury in rats. *J Surg Res* 176(1): e31-9, 2012.

Appendices

Appendix 1: Copyright approval for previously published work

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS	
	Feb 22, 2013
<hr/>	
<p>This is a License Agreement between Dan Secor ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.</p>	
<p>All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.</p>	
License Number	3094210511922
License date	Feb 22, 2013
Licensed content publisher	John Wiley and Sons
Licensed content publication	Microcirculation
Licensed content title	Ascorbate reduces mouse platelet aggregation and surface P-selectin expression in an ex vivo model of sepsis
Licensed copyright line	© 2013 John Wiley & Sons Ltd
Licensed content author	Dan Secor, Scott Swarbreck, Christopher G. Ellis, Michael D. Sharpe, Karel Tymi
Licensed content date	Feb 13, 2013
Start page	n/a
End page	n/a
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Total	0.00 USD

**SPRINGER LICENSE
TERMS AND CONDITIONS**

Feb 26, 2013

This is a License Agreement between Dan Secor ("You") and Springer ("Springer") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3096520288898
License date	Feb 26, 2013
Licensed content publisher	Springer
Licensed content publication	Intensive Care Medicine
Licensed content title	Impaired microvascular perfusion in sepsis requires activated coagulation and P-selectin-mediated platelet adhesion in capillaries
Licensed content author	Dan Secor
Licensed content date	Jan 1, 2010
Volume number	36
Issue number	11
Type of Use	Thesis/Dissertation
Portion	Full text
Number of copies	6
Author of this Springer article	Yes and you are the sole author of the new work
Order reference number	
Title of your thesis / dissertation	Mechanism of ascorbate protection against sepsis-induced capillary blood flow impairment
Expected completion date	Mar 2013
Estimated size(pages)	165
Total	0.00 USD
Terms and Conditions	

Appendix 2: Ethics approval for animal use

AUP Number: 2011-062

PI Name: Tymi, Karel

AUP Title: Microvascular Dysfunction In Sepsis

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Microvascular Dysfunction In Sepsis" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2011-062::1

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

VITA

Dan Secor

- Place of Birth:** Cambridge, Ontario, Canada
- Date of Birth:** February 3, 1984
- Education:** The University of Western Ontario
London, Ontario, Canada
2008-2013 Ph.D. Pharmacology
- The University of Western Ontario
London, Ontario, Canada
2003-2007 B.M.Sc. (Honours) Physiology and Pharmacology
- Awards:** 2011 **Queen Elizabeth II graduate scholarship in science and technology**
- 2010 **Ontario graduate scholarship in science and technology**
- 2009 **Zweifach Student Award**
Awarded by the Microcirculatory Society based on submitted abstract
- Related Work Experience:** Teaching assistant for Pharmacology 3580Y
(Pharmacology Laboratory) at UWO
2008 – 2013

REFERREED PUBLICATIONS

Secor D, Swarbreck S, Ellis CG, Sharpe MD, Tyml K. Ascorbate reduces mouse platelet aggregation and surface P-selectin expression in an ex vivo model of sepsis. *Microcirculation*, 2013.

Secor D, Li F, Ellis CG, Sharpe MD, Gross PL, Wilson JX and Tyml K. Impaired microvascular perfusion in sepsis requires activated coagulation and P-selectin-mediated platelet adhesion in capillaries. *Intensive Care Med.* 36: 11: 1928-1934, 2010.

MANUSCRIPTS SUBMITTED FOR PUBLICATION

Secor D, Swarbreck S, Ellis CG, Sharpe MD, Tyml K. Ascorbate reduces P-selectin mediated platelet-endothelial adhesion in an in vitro model of sepsis via reduced endothelial granule secretion. Submitted to *Microcirculation*, 2013.

Swarbreck S, **Secor D**, Li F, Ellis CG, Sharpe MD, Gross PL, Wilson JX and Tysl K
Effect of ascorbate on fibrinolytic factors in septic mouse skeletal muscle:
disparity between microcirculatory and systemic blood assessment. Submitted to
Microcirculation, 2013.

PUBLISHED ABSTRACTS

Secor D, Li F, Ellis CG, Sharpe MD, Wilson JX and Tysl K. Septic impairment of
capillary blood flow requires activated coagulation pathway and is reversed by ascorbate
through eNOS-dependent dislodging of platelets in capillaries. FASEB J 23:593.6