MYOCARDIAL DYSFUNCTION FOLLOWING RUPTURED ABDOMINAL AORTIC ANEURYSM REPAIR: THE ROLE OF TUMOUR NECROSIS FACTOR-α

By

Rohan Shahani, B.Sc.

A thesis submitted in conformity with the requirements for the degree of Master of Science,
Institute of Medical Science,
University of Toronto

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Rupture and repair of an abdominal aortic aneurysm, a combination of hemorrhagic shock and lower torso ischemia, is associated with a 50-70% mortality. Since myocardial dysfunction may contribute to the high mortality rate following aneurysm repair, the purpose of this thesis was to determine whether ruptured abdominal aortic aneurysm repair results in cardiac dysfunction, mediated by tumour necrosis factor-α (TNF-α). We modeled aortic rupture and repair in the rat by inducing hemorrhagic shock to a mean blood pressure of 50 mmHg for 1 hour followed by supramesenteric clamping of the aorta for 45 minutes. After 90 minutes of reperfusion, cardiac contractile function was assessed using an isolated heart perfusion apparatus. Myocardial TNF-α levels, myocardial energy stores (ATP and CP), neutrophil sequestration, markers of oxidant stress (F₂-isoprostanes) and necrosis were measured. Cardiac function in the combined shock and clamp rats was significantly depressed compared to sham operated control rats, but was similar to that noted in animals subjected to shock alone. Myocardial TNF-α concentrations increased 10-fold in the combined shock and clamp rats compared to shams while there was no difference in myocardial ATP, CP, F₂-isoprostanes and necrosis. Administration of a neutralizing anti-TNF-α antibody prior to shock improved cardiac function by 50% in the combined shock and clamp rats. Thus, Hemorrhagic shock is the primary insult inducing cardiac dysfunction in this model of ruptured abdominal aortic aneurysm repair and no synergistic effect between shock and aortic clamping was noted. An improvement in cardiac contractile function following immunoneutralization of TNF-α indicates that TNF-α mediates a significant portion of the myocardial dysfunction in this model.
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“It is not how hard you push along the way. It is having something in you to finish.”

Michael Jordan
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AAA, abdominal aortic aneurysm repair
ADP, adenosine diphosphate
ATP, adenosine triphosphate
CAT, catalase
CHF, congestive heart failure
CK, creatine kinase
CP, creatine phosphate
+dP/dt Max., rate of left ventricle contractility
-dP/dt Max., rate of left ventricular relaxation
dBP, diastolic blood pressure
EAAA, elective abdominal aortic aneurysm repair
EDP, end diastolic pressure
H₂O₂, hydrogen peroxide.
HOCl⁻, hypochlorous acid
HS, hemorrhagic shock
HS/R, hemorrhagic shock and resuscitation
I/R, ischemia/reperfusion
Il-1 β, interleukin-1 β
KHB, Krebs-Henseleit Bicarbonate
LPS, lipopolysaccharide, endotoxin
MAP Kinase, mitogen activated protein kinase
MAP, mean arterial pressure
MODS, multiple organ dysfunction syndrome
MPO, myeloperoxidase
MWC, myocardial water content
NADH, nicotinamide adenine dinucleotide
NADPH, nicotinamide adenine dinucleotide phosphate
NF-κB, nuclear factor-kappa B
NO, nitric oxide
O₂⁻, superoxide radical
P₅, high energy phosphate
PMN, polymorphonuclear leukocyte, neutrophil
PSP, peak systolic pressure
RAAA, ruptured abdominal aortic aneurysm
ROS, reactive oxygen species
S+C, combination of hemorrhagic shock and supramesenteric aortic clamping
SMA, superior mesenteric artery
sBP, systolic blood pressure
SOD, superoxide dismutase
TNF-α, tumour necrosis factor-α
TNFR1, TNF-α Receptor Type 1 (p55)
TNFR2, TNF-α Receptor Type 2 (p75)
X/XO, xanthine/xanthine oxidase
XD, xanthine dehydrogenase
XO, xanthine oxidase
CHAPTER 1

INTRODUCTION
INTRODUCTION

Clinical Problem

Rupture of an abdominal aortic aneurysm (RAAA) remains a major clinical problem despite improvements in the success of elective aneurysm repair. RAAA is the thirteenth leading cause of mortality in males over the age of 65 (1). While the number of elective abdominal aortic aneurysm (EAAA) repairs have increased in North America over the past decade, the number of RAAA cases have not significantly reduced (2-4). Frequently, the initial presentation occurs at the time of rupture, as aneurysms often remain asymptomatic and undiagnosed.

The survival rate after RAAA repair has not fallen over the last decade despite a drop in the mortality following EAAA repair. The mortality rate following RAAA repair is estimated to be between 50% and 70% depending upon the centre (5), while mortality following EAAA repair has declined to 3-5% (6). The overall mortality rate due to the rupture of an abdominal aortic aneurysm has been reported to be as high as 90% (if pre-operative death is incorporated) (7). The improvement in survival following EAAA repair is thought to be a result of advances in all aspects of peri-operative AAA management. Despite application of these advances to RAAA repair procedures, improvements in mortality and morbidity have not been noted.

The principle difference between elective and ruptured AAA repair is a variable period of hypotension secondary to hemorrhagic shock (HS). All patients undergoing elective or ruptured AAA repair experience aortic occlusion, reconstruction and subsequent reperfusion. However, the low mortality rate seen following EAAA repair
indicates that aortic occlusion and reperfusion is well tolerated. The combination of the two injuries (HS and aortic occlusion) results in a profoundly greater injury than either of the injuries alone.

The causes of mortality after ruptured AAA repair differs depending upon the timing of death after repair. Early deaths (within 48 hours of repair) occur secondary to irreversible shock, myocardial infarction and/or failure, acidosis and bleeding with coagulopathy (8). Several authors suggest that between 40-50% of the postoperative morbidity secondary to RAAA repair is related to myocardial dysfunction. Late deaths (greater than 48 hours after repair) are associated with the development of multiple organ dysfunction syndrome (MODS) (8).

In examining the predictive factors of mortality following RAAA repair, Halpern et al. showed that a pre-operative systolic blood pressure of less than 90 mmHg, hemoglobin levels less than 10g/dl and a creatinine greater than 2mg/dl are strongly correlated with mortality (9). As well, intra-operative systolic blood pressure of less than 90 mmHg and blood loss greater than six litres were found to be strong predictive factors for mortality. These indicators are thought to be a function of the severity of HS, and thus suggest that the degree of HS is strongly correlated with mortality.

In a search for the mechanisms that mediate organ dysfunction following RAAA repair, several studies have begun to implicate inflammatory mediators as major players in the progression of the disorder. It has recently been demonstrated that the rupture of an AAA results in the priming of phagocytes even before the onset of the operative procedure (10). Phagocytes from EAAA patients were not primed prior to the operative

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procedure, but became primed one to two days post-operatively. Upon stimulation, phagocytes from RAAA patients were found to produce Reactive Oxygen Species (ROS) in an exaggerated fashion, which was measured by whole blood chemiluminescence (10). In another study, plasma cytokines were measured in both RAAA and EAAA patients. Upon admission, the level of interleukin-1 β (IL-1 β) and tumour necrosis factor-α (TNF-α) were elevated in the RAAA group over the EAAA group (11). These studies indicate that hemorrhagic shock acts as an initiating stimulus and the addition of a second injury result in an enhanced inflammatory response. Rupture and repair of an abdominal aortic aneurysm is a combination of two sequential ischemia reperfusion events, hemorrhagic shock and lower torso ischemia. It appears that the combination of the two injuries results in a synergistic systemic organ injury.

In order to examine the mechanisms responsible for mediating the remote organ injury following RAAA repair, a rat model of hemorrhagic shock and lower torso ischemia was developed in our laboratory (12). Male Sprague-Dawley rats were randomized into one of three groups: one hour of HS followed by one hour of infra-renal aortic clamping; two hours of HS followed by one hour of infra-renal aortic clamping; or one hour of HS followed by one hour of supramesenteric aortic clamping. In this model, hemorrhage represents the event of aortic rupture in a RAAA patient. Hemorrhage was induced by the withdrawal of blood through the carotid artery and was maintained at a mean arterial blood pressure of 50 mmHg. Following 60 minutes of HS and a period of aortic occlusion, each group underwent a reperfusion period of five hours. Lung permeability (measured by radiolabeled $^{125}$I albumin flux) increased in the HS and supramesenteric aortic occlusion group compared to the sham, HS alone plus aortic

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clamp alone control groups. The combination of HS and infra-renal clamp did not result in a significant increase in lung permeability. Subsequently, increases in lung, liver and bowel neutrophil sequestration and oxidant stress have been noted following the combination of one hour of hemorrhage and 45 minutes of supramesenteric aortic clamping (13). The use of an anti-neutrophil intervention, directed towards the surface-binding molecule of the neutrophil, CD18, reduced bowel neutrophil sequestration and permeability in addition to reducing lung permeability and pulmonary oxidative stress. However, neutrophil sequestration in the lung was unaffected. These studies demonstrate that both local and remote organs become significantly dysfunctional following RAAA repair.

Optimal myocardial function may be required to withstand the sequential insults of hypotension, aortic clamping, declamping and the hyperdynamic state required for post operative recovery. Myocardial function may be depressed secondary to the combination of ischemia/reperfusion (I/R) associated with rupture and repair of an AAA, or myocardial infarction. Depressed cardiac function may exacerbate the systemic ischemic injury secondary to continued mismatch between oxygen supply and demand, a form of cardiogenic shock. This sequence of interrelated events (a reduction in cardiac function inducing further ischemic organ injury, which may further exacerbate the cardiac dysfunction) may contribute to the rapid progression to death that many ruptured AAA patients experience. However, to date, the impact of RAAA repair on cardiac contractile function has not been assessed. It is critical to determine the mechanisms mediating the development of depressed cardiac function following RAAA repair, as this

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will facilitate the development of therapeutic strategies to reduce the progression of the disorder from myocardial depression through to MODS.

It is necessary to discuss the role of each of the components of RAAA repair (HS and lower torso ischemia) in the development of cardiac dysfunction, along with other models of remote organ injury. These can then be compared and contrasted with classical models of cardiac dysfunction; namely myocardial ischemia/reperfusion and cardiac failure.

Remote Organ Injury

Following I/R of various organs (in particular, the intestine and hindlimb), it was noted that organs remote from the ischemic tissue displayed a significant degree of dysfunction. Models of intestinal I/R have been found to result in significant pulmonary, myocardial and hepatic dysfunction (14-16). Circulating mediators originating from the site of I/R have been implicated in inducing this dysfunction. These mediators include neutrophils (PMNs), oxidants (including superoxide, \( \text{H}_2\text{O}_2, \text{HOCl}^- \) and \( \text{OH}^- \)), complement factors and cytokines (17-21).

Over 50% of the total morbidity and mortality seen following RAAA repair results from remote organ failure. Rupture and repair of an AAA includes two independent, temporally associated ischemic events: hemorrhagic shock and lower torso ischemia. The combination of these two ischemic events has been shown to induce a greater organ injury than the sum of the two individual injuries. Both periods of ischemic injury are unavoidable; thus, determining the mechanisms by which remote organ

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dysfunction develops following hemorrhagic shock and lower torso ischemia will allow for optimal strategies to improve the outcome of this disorder.

Depressed myocardial contractile function has been noted in various disease states remote to the heart such as hemorrhagic shock, gut ischemia and surface burn injury as well as in models of myocardial ischemia/reperfusion (acute myocardial infarction and global ischemia) and congestive heart failure.

**Hemorrhagic Shock**

Hemorrhagic shock (HS) occurs when more than 40 percent of the circulating blood volume is lost, and is a leading cause of death in patients with sustained gastrointestinal or anticoagulant induced bleeding, major trauma or rupture of an aortic aneurysm. The myocardial contractile dysfunction that develops after HS may contribute to the high mortality associated with this condition. Following resuscitation from HS, persistent myocardial dysfunction may lead to ongoing hypoperfusion of vital organs, including the heart, brain and kidneys. Systemic hypoperfusion of many organ systems may play a role in the etiology of multi-organ failure following HS, which is a frequent prodrome of death in patients who have experienced major blood loss. The pathophysiologic mechanisms that cause myocardial dysfunction following HS and resuscitation (HS/R) have not been thoroughly investigated.

Death may occur secondary to severe, irreversible shock alone; however, less severe hemorrhagic shock results in various clinical outcomes including multiple organ failure. It has been proposed that hemorrhage acts as a hypoxic cellular priming stimulus, resulting in an exaggerated inflammatory response to a second stimulus (12,22). During

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RAAA repair, occlusion of the aorta is required to facilitate reconstruction. Thus, a second insult occurs which may exacerbate the HS induced injury, resulting in the significant organ dysfunction seen following rupture and repair of an AAA.

Upon the induction of hemorrhagic shock, various compensatory mechanisms are invoked to ensure appropriate perfusion of vital organs. These include peripheral vasoconstriction and a reduction in fluid excretion (23). These mechanisms are controlled at various physiological levels. Neuroendocrine activation is the first response to hypovolemia in an effort to preserve blood flow to the heart and brain (24). The degree of neuroendocrine response is proportional to the degree, magnitude and rate of decrease in circulating blood volume (25). Subsequently, various hormonal pathways become activated which result in the release of hormones such as cortisol, angiotensin II, glucagon, vasopressin and adrenocorticotropic hormone (ACTH). These are all intended to induce a hypermetabolic state to protect organ systems from ischemic damage (26-30).

The combination of neuroendocrine responses and adequate fluid resuscitation is often sufficient to compensate for acute hypovolemia. However, persistent and uncontrolled loss of circulating blood volume overshadows the normal physiologic responses and results in a downward spiraling phenomenon known as decompensated shock. The cycle includes prolonged hypoperfusion of the myocardium due to low cardiac output and a continued decline in perfusion to systemic organs (22). This cycle continues until a transition from decompensated shock to irreversible shock occurs. Profound capillary leak often occurs during irreversible shock and thus, even upon massive resuscitation, normal blood pressure cannot be maintained (22).
In RAAA patients who arrive at the hospital alive and survive aortic reconstruction, it is recognized that the severity of HS is correlated with survival. A mean blood pressure of less than 90 mmHg was found to be strongly correlated with mortality in patients suffering an RAAA (9). Many of these patients may have experienced a significant amount of decompensated shock whereby the heart, brain and other systemic organs undergo pronounced hypoperfusion. Upon resuscitation from this ischemic event, as is often seen following reperfusion of ischemic tissues, a profound systemic inflammatory response develops which may initiate and potentiate the multiple organ dysfunction seen following ruptured abdominal aortic aneurysm repair.

**Cardiac Dysfunction in Hemorrhagic Shock**

The reduced coronary perfusion that occurs during hemorrhagic shock may result in a significant hypoxic or ischemic area of the myocardium. As a result, it has been postulated that HS results in a significant depression in cardiac contractile function. Horton *et al.* demonstrated that HS leads to a rapid depression of cardiac contractility (31). In a guinea pig model of HS, mean blood pressure was lowered to 32.8 mmHg for 2 hours, after which cardiac function was assessed using an isolated heart preparation. Shocked hearts developed less ventricular pressure, had less contractility (+dP/dt) and relaxation (-dP/dt), and had a depressed response to increased extracellular calcium levels. Pacing was also unable to return cardiac function to normal in shocked hearts. These observations confirm that HS results in myocardial contractile dysfunction. However, the molecular mechanisms responsible for the HS/R induced deterioration in myocardial dysfunction have not been defined.

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Several authors have attempted to determine the mechanistic basis of the cardiac dysfunction seen following HS. Horton and Mitchell utilized a dog model of hemorrhagic shock for two hours, after which it was determined that left ventricular systolic dimensions were altered resulting in a reduced left ventricular stroke volume and ejection fraction (32,33). A reduction in cardiac output in this model was found to result in an elevation in myocardial edema combined with an increase in tissue sodium levels, which was reversed by blocking cardiomyocyte Ca$^{2+}$ entry (34).

Kapoor and Prasaad also used a dog model of HS to implicate neutrophils (PMNs) as mediators of the cardiac dysfunction seen following HS. Following two hours of HS and two hours of resuscitation, cardiac function was significantly depressed with a concomitant increase in plasma creatine kinase (CK) and CK-MB. Resuscitation with an anti-PMN immune serum following the two-hour period of HS significantly ameliorated the dysfunction noted (35). In an alternate study utilizing the same model, Kapoor et al. showed that pre-treatment with the antioxidant, superoxide dismutase (SOD) linked to polyethylene glycol (PEG-SOD) resulted in a marked improvement in cardiac function with a significant reduction in plasma CK and CK-MB levels (36). However, Horton found that infusing the combination of SOD and Catalase (CAT) as an antioxidant therapy during hemorrhage or resuscitation had very little effect on the depression of cardiac contractile function or the alterations in myocardial oxygen metabolism seen following HS/R (37).
While studies attempting to delineate the mechanisms mediating cardiac dysfunction following HS/R are underway, it is important to realize that significant species specific and HS protocol specific differences exist, which lead to a significant disparity in results. Vedder et al. described hemorrhagic shock as a “whole body” I/R injury” (38). In a rabbit model of HS and resuscitation (HS/R), significant injury to the liver, lungs, stomach and intestine were noted upon necropsy along with a 100% five-day mortality. Significant histological changes occurred following HS/R in this model. There was significant PMN infiltration into many organ systems and employing a monoclonal antibody to the PMN glycoprotein adhesion receptor, CD18, resulted in a significant reduction in organ injury and five-day mortality. In contrast, Demling et al. showed that following two hours of hemorrhage to a mean arterial pressure of 50 mmHg and 24 hours of resuscitation with either blood or crystalloid in a sheep model, no significant permeability of the alveolar barrier developed (39). Similar findings have been noted at the clinical level, where uncomplicated hemorrhage has been found to induce only a minor degree of organ dysfunction (40). Thus, the mechanisms that mediate cardiac dysfunction following HS are poorly defined.

Recently, it has been recognized that cytokines, including tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), have negative inotropic activities. The role of cytokines, in particular TNF-α, in cardiac dysfunction will be discussed later. However, it is important to note that in a study of HS/R, 20 minutes of HS to a mean blood pressure of 35 mmHg followed by 20 minutes of resuscitation resulted in a 10-fold increase in myocardial TNF-α levels. Therefore, TNF-α may also be responsible for the depression in cardiac function noted following HS/R.

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Irrespective of the mechanistic basis, cardiac dysfunction secondary to hemorrhagic shock likely represents a common event, which is under recognized in clinical practice. The role that depressed cardiac contractile function plays in contributing to the mortality associated with HS/R has not been thoroughly evaluated. The reduction in cardiac contractile function may be self-perpetuating: it induces a further decline in cardiac contractility, contributing to a high rate of mortality. This may lead to a decline in systemic organ perfusion and ultimately contribute to multiple organ dysfunction.

**Lower Torso and Intestinal Ischemia/Reperfusion**

Patients undergoing aortic reconstruction following rupture of an abdominal aortic aneurysm experience both lower torso and intestinal ischemia depending upon the level of clamp application. Individually, lower torso and intestinal I/R have been found to result in local intestinal, as well as remote organ injuries. Mucosal permeability, excessive leak of fluid into the intestinal lumen, edema and necrosis characterize local ischemic injury (41,42), while pulmonary and hepatic dysfunction have also been noted following intestinal and hindlimb I/R (15,43,44).

Several mediators of the local and remote organ injury following intestinal ischemia have been implicated, including neutrophils, oxidant stress and cytokines. In a model of mesenteric I/R, a significant degree of protein leak and PMN sequestration was noted in the intestinal mucosa (45). The use of an antibody directed towards the surface binding molecule of the PMN, CD18, reduced the PMN sequestration and mucosal permeability in the intestine. Neutrophils have also been shown to play a significant role in the pulmonary injury that develops following intestinal I/R (14). 120 minutes of

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intestinal ischemia and 90 minutes of reperfusion were found to result in a significant increase in pulmonary microvascular permeability and PMN sequestration. Pre-treatment with a monoclonal antibody to P-selectin (a molecule involved in neutrophil rolling on the endothelial surface) significantly reduced the lung permeability without altering the degree of neutrophil sequestration. This indicates that the remote organ injury seen following intestinal I/R results from the interaction of various mediators.

Following reperfusion of the ischemic intestine, significant amounts of reactive oxygen species (ROS) are produced. The intestine possesses a large quantity of the enzyme xanthine dehydrogenase (XD). Upon catalytic conversion, XD can be converted to xanthine oxidase (XO). During resting conditions, approximately 90% of the enzyme exists in the XD isoform. Under ischemic conditions, a mass conversion of XD to XO occurs. XO oxidizes either hypoxanthine or xanthine to superoxide (O₂⁻) in the presence of molecular oxygen (O₂). Adenosine Triphosphate (ATP) breakdown during ischemia yields both hypoxanthine and xanthine as end products, although no O₂ is present. Upon reperfusion, the return of O₂ provides the remaining substrate to result in a flux of ROS production. O₂⁻ can then be further converted to more potent oxidative substances such as hydrogen peroxide (H₂O₂), hypohalous acids (such as HOCl⁻) and hydroxy radicals (OH⁻), which can then oxidize lipid membranes to result in organ injury (46).

Lower torso I/R and intestinal I/R share similar mechanisms of injury: in both, a significant degree of PMN induced injury, oxidative stress and cytokine induced injury have been noted locally in ischemic skeletal muscle, as well as systemically (47,48). In a rabbit model of hindlimb I/R, four hours of ischemia followed by four hours of

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reperfusion was found to induce a significant degree of microvascular permeability in the contralateral soleus muscle as well as in the lung (49). Additionally, a significant elevation in neutrophil sequestration was noted. The use of the anti-inflammatory cytokine, interleukin-10 (IL-10) was found to significantly reduce the permeability noted in both the soleus muscle and in the lung. However, the reduction in injury noted in the lung occurred irrespective of neutrophil activity, as pulmonary neutrophil sequestration was not reduced. The increased circulating TNF-α level noted in the hindlimb I/R animals was reduced following IL-10 administration, suggesting that the remote organ injury noted in this model may be cytokine dependent. Pre-treatment with free radical scavengers was also found to significantly reduce the injury seen following lower torso I/R (41). Four hours of hindlimb ischemia and 60 minutes of reperfusion resulted in a significant degree of skeletal muscle microvascular permeability. Pretreatment with various free radical scavengers (with varying mechanisms of activity) reduced the permeability noted, indicating a role for reactive oxygen species. In particular, xanthine oxidase was found to be the primary source of the oxidants derived in this model. These studies indicate that the injury, either local or remote, noted after lower torso I/R includes many potential mediators that are closely linked.

**Cardiac Dysfunction in Intestinal I/R**

Intestinal ischemia has been found to induce a significant reduction in pulmonary and hepatic function. However, the impact of mesenteric I/R on myocardial contractile function was unknown, until Horton et al. found that occlusion of the superior mesenteric artery (SMA) and its collaterals for 20 minutes followed by two hours of reperfusion resulted in a significant depression of cardiac contractile function (16). The dysfunction
appeared to be transient as it began at 2 hours of reperfusion and persisted for up to 16 hours of reperfusion. After this, cardiac function began to return to baseline. This dysfunction was reversed by pretreatment with allopurinol, an inhibitor of the xanthine/xanthine oxidase (X/XO) pathway. This indicates that the X/XO pathway was involved in mediating the dysfunction following intestinal I/R.

In searching for the mechanism mediating the cardiac dysfunction following intestinal I/R, Horton measured the degree of lipid peroxidation in the myocardium. Increased levels of malondialdehyde were found in the myocardium following 20 minutes of intestinal ischemia and two hours of reperfusion (19,50). The use of the antioxidant combination superoxide dismutase (SOD) and catalase (CAT), which converts superoxide \( \text{O}_2^- \) to molecular oxygen \( \text{O}_2 \) and water, significantly ameliorated the cardiac dysfunction seen following mesenteric I/R (51). Thus, lipid peroxidation appears to play an important role in the cardiac dysfunction seen following intestinal I/R.

**Thermal (Burn) Injury**

Thermal or burn injury represents a different type of tissue insult from those mentioned previously. However, the presence and mechanistic basis of myocardial dysfunction following thermal injury has been well described. Adams et al. made the initial observation of cardiac dysfunction in an isolated, whole heart preparation following burn injury (52). Following a 47% surface burn injury, left ventricular pressure, \(+\) and \(-\) dP/dt Max. were significantly depressed versus sham operated controls. In addition, a reduction in left-ventricular compliance occurred, as indicated by a shift to the left of the ventricular compliance curve of the burn injured rats.

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Horton and colleagues have gone on to elegantly characterize the mechanisms mediating the depression in cardiac contractile function seen following thermal injury. Horton has shown, as was seen following intestinal ischemia, that neutrophil (PMN), oxidant and cytokine activities are closely linked with the cardiac dysfunction associated with thermal injury, although, no significant changes in myocardial energy stores were noted upon burn shock (53). Administration of a monoclonal antibody (R6.5) to intracellular adhesion molecule-1 (ICAM-1), an endothelial cell surface adhesion molecule used by leukocytes for firm adhesion, was found to improve cardiac function in a rabbit model of 30% surface burn injury (54). Initially, cardiac function was depressed in both the anti-ICAM-1 antibody treated and control antibody treated groups. However, upon increasing preload (increasing left ventricular volume), cardiac function in the antibody treated group returned to sham operated levels. While treatment with an antibody to ICAM-1 improved cardiac function, the degree of PMN sequestration remained unchanged. In an alternate study, Horton isolated PMNs from plasma of rabbits undergoing thermal injury and incubated them with myocytes from control or burned rabbits (55). Between 120 and 180 minutes of incubation with either burn plasma or burn PMNs, myocyte viability was significantly depressed and creatine kinase levels in the supernatant was significantly increased. These studies indicate that PMN adhesion to the myocardial endothelial surface followed by transmigration into the extravascular space and ultimate adhesion to the myocytes plays an important role in the pathogenesis of myocardial dysfunction following thermal injury.

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Reactive oxygen species (ROS) have also been implicated in postburn cardiac dysfunction from studies showing increased serum concentrations of lipid peroxides in burn patients (56-58), and increased plasma and cardiac tissue malondialdehyde (MDA) levels in models of surface burn injury (59). In initial studies, the free radical scavenger, superoxide dismutase (SOD) bound to either polyethylene glycol (PEG-SOD) or Ficoll (Ficoll-SOD) was unable to reverse the burn-induced cardiac dysfunction (60). However, in a later study, the combination of PEG-SOD and PEG-catalase (PEG-CAT) resulted in a significant improvement in cardiac function in a model of burn injury (61). Finally, Horton attempted to establish a causative relationship between xanthine oxidase (XO) activity and PMN activity in mediating the myocardial dysfunction seen following thermal injury (62). Allopurinol, a XO inhibitor, and tungsten, which prevents xanthine dehydrogenase (XD) conversion to XO, were given enterally prior to burn injury to determine the role of XO in the postburn cardiac dysfunction. As well, vinblastine, a PMN depleting agent, was given to rats undergoing thermal injury. All treatments significantly improved cardiac function compared to burn control animals but function remained significantly depressed compared the sham operated controls. These findings led to the conclusion that XO derived oxidants act to recruit PMNs to ischemic tissue, and it is the cytotoxic activity of the PMNs that result in the depressed cardiac function noted following thermal injury. Thus, the cardiac dysfunction seen following thermal injury is controlled at several levels.

While PMNs and ROS appear to play a role in the cardiac dysfunction noted following thermal injury, tumour necrosis factor-α (TNF-α), a cytokine with negative inotropic properties, was shown to be responsible for a majority of the postburn cardiac dysfunction.
dysfunction. In approximately one-third of adult burn victims, it was found that serum TNF-α was significantly increased (63). Thus, Giroir et al. studied the role that TNF-α plays in mediating the cardiac dysfunction seen following thermal injury (64). Following a 43% surface burn injury, guinea pigs were treated with a recombinant human p80 (75-80 kDa) TNF receptor type 2 (TNFR2) linked to a murine immunoglobulin heavy chain domain (rhTNFR:Fc). Cardiac contractile function was subsequently assessed. Inhibition of TNF-α activity following thermal injury was found to significantly improve cardiac function back to sham burn levels, thus indicating that TNF-α is responsible for a significant portion of the depression of cardiac contractile function noted following surface burn injury.

These models of remote organ injury have both common and differing features to models of myocardial ischemia and reperfusion and congestive heart failure, which are the best-characterized models of cardiac contractile dysfunction.

**Myocardial Ischemia/Reperfusion (Myocardial Infarction)**

Myocardial ischemia is a term for events that lead to a critical reduction in O₂ supply to the myocardium; that is, an impeded or interrupted supply of blood flow (65). Myocardial ischemia typically develops secondary to coronary artery occlusion, following atherosclerotic plaque rupture, coronary thrombosis or coronary artery spasm (66). Thus, ischemic heart disease is often referred to as coronary heart disease or atherosclerotic heart disease (67). Whichever name is used, the result of an ischemic myocardium is diminished cardiac contractile function. It has been noted that contractile force showed an initial decline within 60 seconds of the onset of myocardial ischemia in
an isolated heart model (65). This reduction in myocardial function is characterized by reduced ejection fraction, rate of contractility and ultimately, cardiac output (67).

Models of myocardial ischemia/reperfusion have been developed to determine the mechanisms that mediate the cardiac dysfunction seen upon myocardial I/R. These models include coronary artery ligation and left ventricular wall cryo-injury. The primary result of prolonged coronary obstruction is necrosis of the affected tissue (68). It has been found that the myocardium undergoes necrosis in a "wavelike" pattern from the subendocardium to the subepicardium (69). The loss of functional myocardial tissue is the primary mechanism for the decline in cardiac function, as the myocardium no longer has the ultrastructure to maintain adequate pump function (67). The leak of proteins and enzymes from the ischemic tissue can be measured systemically as markers of necrosis, including creatine kinase (CK) and its isozyme, CK-MB (66).

Another characteristic feature of the ischemic myocardium before the onset of necrosis is a reduction in energy stores (70). Under normal conditions, ATP is broken down to release high energy phosphates to maintain cellular function. Through aerobic metabolism, ADP and AMP are recycled to produce new ATP molecules in order to maintain the energy supply. However, a lack of oxygen prevents the regeneration of ATP. Thus, ADP and AMP are further metabolized to release energy and ultimately, hypoxanthine and xanthine are produced, at the expense of ATP stores. As well, Creatine Phosphate (CP), an additional energy store found in muscle tissue, is sacrificed to release energy. In the resting, aerobic rat myocardium, the concentration of ATP is approximately 25 μmoles/g dry weight of heart and the concentration of CP is 34
μ moles/g dry weight. However, in the ischemic myocardium, the concentration falls to approximately 20 and 8 μ moles/g dry weight of ATP and CP, respectively (71). Several authors have noted that approximately 10-20% of the myocardial energy store is depleted within the first 60 seconds of myocardial ischemia (65). Due to the lack of energy stores in the myocardium, the ability of the myocardium to contract significantly reduces.

The most notable histologic feature of the ischemic myocardium is a significant infiltration of neutrophils (PMNs) in the interstitial space of the myocardium (72). The first sign of PMN sequestration in the myocardium was found to occur within one hour of the onset of reperfusion and reached a maximum 24 hours later (73,74). Cytokine release by the ischemic myocardium has been suggested to act as the chemoattractant factor for PMN migration from the circulation to the interstitium (74). Several authors have implicated PMNs in the cytotoxic injury seen as a result of ischemic heart disease (75). PMNs contain various proteolytic enzymes such as elastase and collagenase intended to fight against attacking foreign antigens. As well, PMNs have the unique ability to produce a burst of reactive oxygen species (ROS) which also help against invading organisms. During myocardial ischemia, PMNs become sequestered in the interstitial spaces of the myocardium (76). Upon reperfusion, more PMNs get sequestered and become activated. This results in the release of proteolytic enzymes and reactive oxygen species (74). These enzymes subsequently attack the myocytes, resulting in a further loss of viable myocardial mass (77). Inhibition of neutrophil adhesion to the coronary endothelium (by treatment with antibodies to adhesion molecules such as CD-18, P-Selectin or ICAM-1) or blockade of the cytotoxic actions of PMNs (by treatment with novel molecules directed towards PMN adhesion or serine protease inhibitors) have

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proven successful in reducing the injury and dysfunction seen in the myocardium following ischemia and reperfusion (78-82).

Along with PMNs, ROS have been implicated in the injury noted following myocardial I/R (83). As mentioned previously, the Xanthine-Xanthine Oxidase (X/XO) pathway is a potent generator of ROS. The onset of ischemia in the myocardium results in a depletion of myocardial energy stores and a conversion of the enzyme Xanthine Dehydrogenase (XD) to Xanthine Oxidase (XO) in the coronary endothelium. With the return of oxygen during reperfusion, the metabolites of ATP degradation, namely hypoxanthine and xanthine, are converted to superoxide, which can be further catalyzed to hydrogen peroxide and hypohalous acids (46). These potent oxidative substances can also induce a significant degree of myocardial injury. The use of various antioxidant agents such as superoxide dismutase and catalase, or N-acetylcysteine (NAC) in models of myocardial I/R have been found to significantly improve cardiac function and reduce reperfusion injury (84-86).

Recently, it has become increasingly evident that cytokines play a role in the cardiac dysfunction resulting from myocardial I/R. The role of cytokines, especially tumour necrosis factor-α (TNF-α), in generalized cardiac dysfunction will be discussed in future sections. However, it is important to note that the myocardium is a TNF-α generating organ. Upon endotoxin administration, cardiomyocytes were found to be responsible for up to 50% of the TNF-α generated in the myocardium, as indicated by immunolocalization (87). TNF-α is also generated in the heart following myocardial I/R. Isolated perfused rat hearts were arrested with warm cardioplegia and following one hour

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of global ischemia and 30 minutes of reperfusion, a significant increase in TNF-α in the coronary effluent was noted, which correlated with post-ischemic deterioration in peak systolic pressure (88). Following coronary artery by-pass surgery, during which the ischemic myocardium is allowed to reperfuse, plasma levels of pro-inflammatory cytokines, including TNF-α and interleukin-1β (IL-1β) were found to be significantly elevated (89). As well, in myocardial biopsies taken from patients undergoing cardiopulmonary by-pass surgery, significant amounts of TNF-α were localized to the cardiomyocytes (90).

Thus, it can be seen that myocardial damage secondary to remote organ injury possesses many characteristics that are similar to those seen following myocardial I/R. PMN activity, oxidant stress and pro-inflammatory cytokines all appear to be intimately involved in the injury and dysfunction of the heart. However, a loss of viable myocardium and energy stores noted following myocardial I/R is not seen following remote organ injury. This suggests that there may be differing mechanisms mediating the cardiac dysfunction in remote organ injury versus the ischemic and reperfused myocardium.

**Congestive Heart Failure**

Congestive heart failure (CHF) has been defined as “A pathophysiological state in which an abnormality of cardiac function is responsible for the failure of the heart to pump blood at rate commensurable with the requirements of the metabolizing tissues.” (Eugene Braunwald, 1980) (91). The failing heart is often associated with reduced cardiac contractile function, which results in an inability to meet the metabolic demands
of various organ systems (92). As a result of the poor cardiac output seen in CHF, many clinical manifestations arise, including cardiac hypertrophy and dilatation (cardiomegaly), pulmonary hypertension, pulmonary and peripheral edema, poor exercise capacity and cardiac cachexia. However, the basis for these symptoms are not well understood.

Studies into the molecular basis of CHF have begun to implicate various cytokines, especially tumour necrosis factor-α (TNF-α) as potential mediators of the progression of the disease (93). Patients in late stage CHF have been found to have significantly elevated levels of plasma TNF-α over patients with mild heart failure (94). Cytokines are low molecular weight proteins (usually 15 to 30 kilodaltons (kDa)) which are responsible for cell-to-cell communication (95). Many types of cells can produce these molecules; primarily, immunoinflammatory cells such as macrophages and neutrophils (95). Recently, cardiomyocytes have also been shown to be a source of cytokine production (96). Evidence suggests that myocardial TNF-α has the ability to act in a paracrine as well as an autocrine fashion to induce many of the symptoms seen in CHF. The link between TNF-α and CHF is not well understood, although increasing evidence has begun to implicate TNF-α as a mediator of the progression of congestive heart failure and other cardiac pathologies.

**Pathophysiology of Congestive Heart Failure**

The mechanisms by which CHF develops and persists remain unclear. It is suggested that initiating pathological stimuli including hemodynamic overloading (pressure or volume overload), myocardial infarction, inflammation (primary myocardial
infection such as myocarditis), valvular disease or genetic predisposition can result in myocardial hypoperfusion and arterial underfilling, thus inducing the initial stages of heart failure (97,98). The hypoperfusion of the myocardium leads to the activation of compensatory mechanisms, including the induction of the sympathetic nervous system and the renin-angiotensin system, resulting in the expression of neurohormones, catecholamines and proinflammatory cytokines (98). These mechanisms are intended to maintain cardiac output in response to poor perfusion. Sympathetic stimulation leads to an increase in circulating levels of catecholamines such as norepinephrine and renin-angiotensin activation results in release of angiotensin II. Along with increases in circulating cytokines, norepinephrine and angiotensin II result in increased heart rate, cardiac contractility, blood pressure and blood volume (preload), all of which all increase cardiac output (98).

Long-term exposure to factors intended to improve cardiac function compromise the ability of the heart to maintain perfusion (leading to cardiac decompensation) to all essential organ systems (99). Increases in circulating cytokine concentrations are intended to improve cardiac output. However, over time, cytokines play a role in the progression of CHF. While increases in angiotensin II and norepinephrine play a role in the progression of CHF, cytokines, especially tumour necrosis factor-α, have the potential to mediate a majority of the pathophysiology and thus, the progression of CHF. Activation of the renin-angiotensin system and the sympathetic nervous system influence the heart independently of hemodynamic alterations (98). However, depression of cardiac function is the driving force perpetuating CHF. TNF-α has been shown to induce cardiac dysfunction, apoptosis and myocardial structural alterations (hypertrophy), all of which

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are thought to influence cardiac contractile function in CHF. Also, TNF-α has been found to cause alterations in gene expression and a reduction in β-adrenergic receptor density in the heart, as well as cause pulmonary edema, increases in circulating atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and muscle wasting, which are all characteristic of CHF (97).

**Tumour Necrosis Factor-α**

Tumour Necrosis Factor-α (TNF-α) or Cachectin is a 17-kilodalton (kDa) molecule, which was originally described by Carswell et al. as a protein with anti-tumour capabilities (100). However, it has been shown that TNF-α has pleotropic capabilities; that is, it has many target cells and can perform various functions (95). Some of these functions include lymphocyte and neutrophil activation, induction of other cytokines such as interleukin-1 (IL-1), interleukin-4 (IL-4) and induction of its own production (positive feedback) (101).

The circulating concentration of TNF-α in the bloodstream influences the role it plays in cellular signaling and function. In low concentrations (≤10^{-10} mol/L), TNF-α functions in its normal fashion of immune signaling. Circulating soluble TNF-α receptors (TNFR) control the concentration of TNF-α in the circulation (102). These include the 55-kDa TNF-α receptor 1 (TNFR1) and the 75-kDa TNF-α receptor 2 (TNFR2). Both of these receptors are cleaved from cellular membranes and ensure that the concentration of TNF-α remains at a level to facilitate the immune functions that TNF-α serve. It is thought that TNF-α plays a protective role in the heart by reducing the demand for O₂ by

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the myocardium. The reduced O₂ demand results in an increased energy supply for contraction. Mice were unable to survive in a model of myocardial infarction, where the TNF-α gene was knocked out. Thus, TNF-α has beneficial effects in the myocardium (103). However, when the concentration of TNF-α reaches higher than 10⁻⁸ mol/L, there appears to be a "spill-over" effect. The total available soluble TNF-α receptors become saturated, thus leaving excess circulating TNF-α. The excess TNF-α can bind to myocyte or other cell-surface receptors in a paracrine fashion to initiate signaling cascades. These cascades can ultimately result in cellular alterations, including a reduction in myocyte shortening and the induction of myocyte apoptosis. The binding of TNF-α to membrane-bound receptors also induces a further up-regulation of TNF-α receptor production (104). Excess TNF-α has also been shown to induce metabolic wasting (cachexia), microvascular coagulation and hypotension (105).

Infusion of physiological concentrations of TNF-α into rats via an implanted osmotic pump induced significant long-term cardiac dysfunction similar to that noted in heart failure patients (106). Along with inducing a time-dependent depression of cardiac contractile function, TNF-α was shown to cause remodeling of the left ventricular cavity, which is also characteristic of CHF. This was reversed by immunoneutralization of TNF-α. Beyond left-ventricular dysfunction, increased levels of TNF-α have been shown to induce various clinical manifestations of cardiac decompensation such as cardiomyopathy (107), pulmonary edema (108-111), and ventricular remodeling (112-114). As well, TNF-α has been linked to fibrosis and scar tissue formation (115), which are hallmarks of CHF. In addition, TNF-α has been shown to induce the production of

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other cytokines such as interleukin-1 (IL-1) and interleukin-4 (IL-4) (95). IL-1 has been shown to provoke a heart failure phenotype, which includes the down-regulation of the Ca²⁺-ATPase gene, the up-regulation of ANP production and uncoupling of the β-adrenoreceptor from adenylate cyclase (116-118). Thus, TNF-α may mediate the pathophysiology of many cardiac related disorders.

**Mechanism of TNF-α Synthesis in the Myocardium**

The mechanism of TNF-α activation is not well understood; however, several mechanisms have been proposed. Endotoxin-induced macrophage production of TNF-α is the best-characterized model (Fig. 1). Endotoxin (LPS) administration has been found to result in production of TNF-α by binding of LPS to the Lipopolysaccharide Binding Protein (LBP), which then activates the CD14 receptor on the macrophage cell surface (119). Binding to the CD14 receptor results in an intracellular cascade, which ends in the production and release of TNF-α. It has been proposed that a group of stress activated protein kinases (SAP Kinase) are activated upon binding of LPS to the receptor. This group of kinases includes the c-Jun N-terminal Kinase (JNK), the Extracellular Regulated Kinase (ERK) and the Mitogen Activated Protein Kinase (MAP Kinase) families of protein kinases. In particular, a 38-kDa MAP Kinase (p38 MAP Kinase) is the major regulating kinase in the TNF-α activation pathway (120-124). p38 MAP Kinase, the mammalian homologue of the Hog-1 gene product in yeast and a member of the MAP Kinase subfamily, is also called a CSBP (cytokine-suppressive anti-inflammatory drug binding protein) (123). p38 MAP Kinase is activated by diverse stimuli including myocardial ischemia and exposure to cytokines, endotoxin and hydrogen peroxide.

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Figure 1. Pathway of TNF-α synthesis and secretion following Lipopolysaccharide (LPS) stimulation of macrophages. Adopted from Meldrum, 1998 (104).

p38 MAP Kinase is activated by phosphorylation. This activated state can be detected by immunoblot analysis with an anti phospho-p38 MAP Kinase antibody or by

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in-gel kinase assay using specific target substrates (MAPKAPK2). The involvement of p38 MAP Kinase in the cascade of events that results in TNF-α production has been demonstrated using the pyridinyl-imidazole compounds (SB 203580). These compounds fit into the highly specific binding pocket of ATP within the p38 MAP Kinase structure, and have been used to define the role of p38 MAP Kinase in a variety of pathological conditions (126-128). Studies demonstrated that global ischemia of the rat heart followed by reperfusion resulted in a significant activation of p38 MAP Kinase (125). Subsequently, it was found that specific inhibition of p38 MAP Kinase with SB203580 prevented myocardial TNF-α production in the human heart following myocardial I/R, indicating that p38 MAP Kinase activation is required for TNF-α synthesis in the myocardium (129). Through a series of upstream phosphorylation steps, p38 MAP Kinase is phosphorylated, resulting in its activation. Activation of p38 MAP Kinase results in further phosphorylation of downstream mediators of the pathway.

Nuclear Factor kappa B (NF-κB) is a transcription factor known to result in increased TNF-α transcription. NF-κB is composed of a group of functional subunits (c-rel, NF-κB1, RelA, NF-κB2 and RelB) and an inhibitory subunit, IκB (130). Phosphorylation of IκB by p38 MAP Kinase results in its dissociation from the complex and its ultimate degradation, thus removing its inhibitory influence on the complex (131-133). This allows for the translocation of NF-κB to the nucleus. NF-κB binds to the promoter region of the TNF-α gene to increase the transcription of TNF-α mRNA (134). NF-κB is known to activate gene transcription sites of numerous genes responsible for the acute inflammatory response including intracellular adhesion molecule-1 (ICAM-1),

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cytokines such as TNF-α and IL-1β and chemokines including CINC and MIP-2 (135,136). NF-κB can be activated and induced to migrate to the nucleus in response to many stimuli including cytokines (TNF-α, IL-1β), oxidant stress and LPS (137-139). NF-κB activation has been shown to be prevented by administration of superoxide dismutase, inhibitors of Xanthine Oxidase (XO) and α-adrenergic antagonists (140-142).

Following TNF-α mRNA transcription, translation proceeds to produce a 25-kDa TNF-α precursor protein (Pro-TNF-α) (143-145). Post-translational modification includes myristoylation (addition of a lipid moiety to facilitate insertion into the membrane) and proteolytic cleavage by TNF-α Converting Enzyme (TACE). This results in the release of the secreted form of TNF-α (17-kDa) to circulate as a 51-kDa trimer (144,146).

TNF-α synthesis may be activated by other stimulants. Acute pressure overload (116) and oxidant stress (147) have been shown to result in TNF-α production. Passive stretching of isolated papillary muscles results in increased TNF-α mRNA production followed by an increase in de Novo TNF-α protein expression. Increasing the perfusion pressure into isolated feline hearts, again, resulted in increased TNF-α mRNA and protein expression. Studies have also shown that hemodynamic pressure overload in vivo results in increased TNF-α production. Hydrogen peroxide (H₂O₂) infusion into the heart has also been shown to stimulate TNF-α production and result in the depression of cardiac contractile function (147). Pre-treatment with a soluble TNF-α binding protein (sTNFBP) improved cardiac function and the use of a pyridinyl-imidazole compound

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(SB203580), a specific inhibitor of p38 MAP Kinase, prevented the production of TNF-α and blocked the reduction in cardiac function. This suggests that oxidant-induced TNF-α production occurs by a p38 MAP Kinase pathway similar to that seen in LPS challenge and results in depressed cardiac contractile function.

**TNF-α Induced Myocardial Dysfunction**

TNF-α has recently been recognized to play a major mechanistic role in the depression of cardiac function associated with myocardial ischemia, viral myocarditis, cardiac allograft rejection, acute pressure overload, endotoxin exposure and thermal injury (64,87,104). TNF-α has been shown to have the ability to induce cardiac dysfunction both in an isolated cardiomyocyte system as well as in a whole heart preparation (148-150).

Incubation of isolated rat papillary muscles with various concentrations of TNF-α resulted in a dose-dependent reduction in muscle tension (148). These effects occurred within 2-3 minutes of exposure and the maximal effect was noted at 5 minutes. Following a 30-minute wash out period, the tension generated returned to baseline. Additional studies have shown that isolated cardiomyocyte systems respond similarly to increasing concentrations of TNF-α (151,152). Incubation of isolated cardiomyocytes with TNF-α concentrations greater than 100 U/ml were shown to result in a significant depression of contractile function in a concentration- and time-dependent manner (153). Also, administration of TNF-α to crystalloid buffer perfusing feline isolated hearts resulted in a dose-dependent reduction in developed pressure and +dP/dt, an effect that could be reversed by a 30-minute washout period (152).

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Although the TNF-α mediated cardiac dysfunction seen in these models could be reversed following a 30-minute washout period, myocytes and resident macrophages continue to be exposed to factors that stimulate TNF-α production (oxidant and hemodynamic stress) in models of myocardial I/R and in models of remote organ injury. Cardiomyocytes, as well as macrophages, have been shown to be potential sources of TNF-α production in the myocardium. As much as 50% of the total TNF-α found in the myocardium upon endotoxin administration was localized to the cardiomyocytes (87). TNF-α is also known to potentiate its own production by positive feedback mechanisms, thus increasing the circulating concentration of TNF-α. Prolonged exposure to physiologic concentrations of TNF-α (80-100 U/mL) has been shown to result in a time-dependent development of cardiac dysfunction (106). Further to this controversy, administration of TNF-α (40 μg/kg) over 1 hour to chronically instrumented dogs resulted in an initial improvement on cardiac function (154). By 25 hours post-infusion, all parameters of cardiac function were depressed, accompanied by LV dilatation, which suggests the possibility of a third phase of TNF-α induced cardiac dysfunction. Therefore, TNF-α may continue to be produced in the hearts of animals undergoing ischemic events.

**Mechanisms of TNF-α Induced Cardiac Dysfunction**

The cellular basis of TNF-α mediated myocardial dysfunction is proposed to be biphasic (Fig. 2) (154). The first phase of activity involves sphingolipid metabolism,
which occurs within minutes (155). TNF-α mediated metabolism of sphingomyelin results in the release of ceramide and sphingosine (Fig. 3). Sphingomyelin belongs to a group of complex lipids (sphingolipids) which have the following basic structure: a polar head group, a fatty acid side chain and a molecule of sphingosine or one of its derivatives. The polar head group is either phosphoethanolamine (PE) or phosphocholine (PC). Removal of PC or PE from a sphingomyelin, via the enzyme sphingomyelinase, leaves ceramide. Ceramide is composed of sphingosine (an amino alcohol) that has a

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**Sphingomyelin**

![Sphingomyelin Structure](image)

**Sphingomyelinase**  
Sphingomyelin → Ceramide + PE or PC → Sphingosine + Free Fatty Acid

**Ceramidase**

Figure 3. Structure of Sphingomyelin and cleavage pathway from Sphingomyelin through Ceramide yielding Sphingosine.

long chain saturated or mono-unsaturated fatty acid connected to its amino group by an amide linkage. Sphingosine is separated from the fatty acid by the enzyme ceramidase.

In feline myocytes, sphingomyelin hydrolysis by TNF-α-activated sphingomyelinase, resulted in a rapid increase in free sphingosine levels. The binding of TNF-α to the p55 TNF receptor (TNFR1) initiates the hydrolysis of sphingomyelin.

Germaine to studies mentioned previously, incubation of cardiomyocytes with sphingosine resulted in a depression in contractility similar to that seen following TNF-α incubation. Inhibition of TNF-α induced sphingosine production by n-oleoylethanolamine (NOE) blunted the increase in free sphingosine concentration by 75% and prevented the TNF-α-induced reduction in cell shortening (155). Sphingosine has been linked to the inhibition of ryanodine receptors on the surface of myocytes, thus influencing Ca^{2+} transients, leading to Ca^{2+} dyshomeostasis in myocytes (156,157).

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Deregulation of Ca\(^{2+}\) handling due to increased free sphingosine concentrations may be responsible for the dysfunction seen due to TNF-\(\alpha\) administration. Ca\(^{2+}\) plays an important role in cardiac action potential development. During the plateau phase, Ca\(^{2+}\) influx occurs via the voltage gated L-type Ca\(^{2+}\) channel. This small influx of Ca\(^{2+}\) triggers a much greater release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) ryanodine receptor Ca\(^{2+}\) release channels. Cytosolic Ca\(^{2+}\) levels rise to allow for binding to troponin C, activating the contraction process. Relaxation occurs when the SR actively sequesters Ca\(^{2+}\) using the Ca\(^{2+}\)-ATPase (68). TNF-\(\alpha\) administration decreased the peak intensity of fluorescence brightness of cardiomyocytes loaded with fluo-3, suggesting that TNF-\(\alpha\) decreased the intracellular Ca\(^{2+}\) release during contraction. These alterations were independent of alterations in cross bridge activation. Further work by the same authors demonstrated that application of D-sphingosine resulted in similar alterations in fluorescence brightness as the application of TNF-\(\alpha\) to cardiomyocytes. These data support the concept that TNF-\(\alpha\) induces alterations in calcium homeostasis, which may mediate cardiac contractile dysfunction. In a model of thermal injury, an altered time course of the calcium transient was identified (158). The thermal injury was prevented with a rhTNFR:Fc, however no studies of the Ca\(^{2+}\) transient have been performed (64).

The second phase of TNF-\(\alpha\) mediated cardiac dysfunction is thought to occur through nitric oxide (NO) induction (148,153). Several hours of TNF-\(\alpha\) exposure to cardiomyocytes has been shown to induce NO synthesis by the transcription of the inducible nitric oxide synthase (iNOS) (152). Basal levels of NO have been shown to be protective to the myocardium under conditions of ischemic stress. Normally, NO is

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produced by the constitutive form of nitric oxide synthase (cNOS). However, when TNF-α levels are increased, iNOS is activated and rapid increases in circulating NO levels occur. This changes the role of NO from protective to destructive (159). The combination of TNF-α and IL-1 infused together in a working rat heart resulted in an early increase (15 minutes) in peak systolic pressure and cardiac output (150). After 1 or 2 hours of perfusion with these cytokines, cardiac work declined. Inhibitors of nitric oxide (L-NAME) prevented the decline in cardiac work. Cytokine-induced activation of iNOS and the ultimate production of NO have been linked to the reduced responsiveness of β-adrenergic receptors to inotropic agents, thus reducing the ability of the heart to contract (160). Like sphingosine, NO mediates a significant portion of the TNF-α induced cardiac dysfunction. Both sphingosine and NO may play an important role in the development and maintenance of the cardiac dysfunction seen in various cardiac disorders.

**TNF-α Induced Cardiomyocyte Apoptosis**

Depression of left ventricular function has also been proposed to be the result of a loss of viable cardiomyocytes. Apoptosis or programmed cell death, a potential third phase of TNF-α-induced cardiac dysfunction, may mediate the depression in cardiac contractile function over a prolonged period of time (161). Apoptosis is a cellular event where cells are lost with the development of double stranded nicks in their DNA. For hemopoietic cells, this is a constitutive process. However, this process can be activated by the immune system leading to premature death of cells of other types. Unlike necrotic cell death where the cell membrane is breached, the membrane remains intact excluding vital dyes and does not release intracellular enzymes or other potentially toxic products
during apoptosis. Apoptosis of cardiomyocytes has been observed in right ventricular dysplasia, after ischemia and in chronic heart failure (130,162). Recent studies have shown that both hypertrophy and passive myocardial stretch, which occur during chamber dilation, can stimulate myocyte apoptosis (163,164). Continuous ischemia for two and a quarter hours alone or 45 minutes of ischemia and a minimum of one hour of reperfusion resulted in the development of apoptosis (165). These stresses have also been shown to induce TNF-α production. In examining TNF-α as the potential link to apoptosis, Krown et al. showed that TNF-α induces apoptosis in cardiomyocytes by increases in free sphingosine concentration (166). Sphingosine has also been linked to TNF-α mediated cardiac dysfunction. Therefore, TNF-α may mediate left-ventricular dysfunction by directly inducing the production of cardiodepressant substances (sphingosine and NO) as well as inducing the loss of ventricular mass by apoptosis.

**The Role of TNF-α in the Myocardium Following Remote Organ Injury**

Over the past decade, it has become apparent that TNF-α plays a role in the myocardial dysfunction seen following remote organ injury. Several authors have investigated the role of TNF-α in the myocardium in hemorrhagic shock, intestinal ischemia/reperfusion (I/R), thermal injury and septic shock. TNF-α appears to play a part in the myocardial dysfunction in a variety of injuries remote to the heart.

Hemorrhagic shock is known to result in a significant depression of cardiac contractile function (31). However, the mechanistic basis of cardiac dysfunction following hemorrhage and resuscitation, still remains unclear. A recent investigation has demonstrated that TNF-α is elevated in the rat myocardium following 20 minutes of HS

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to a mean blood pressure of 35 mmHg and 20 minutes of resuscitation (167). A gel shift mobility assay identified translocation of the transcription factor, NF-κB to the nucleus, suggesting that this is responsible for the production of TNF-α in the myocardium (167). This is the initial demonstration that HS induces endogenous myocardial synthesis of TNF-α. This is important, as TNF-α produced both locally and systemically is known to depress myocardial function. However, the role that TNF-α plays in the myocardial dysfunction noted following hemorrhagic shock and resuscitation is unknown.

In an alternate study of hemorrhagic shock, it was found that ten minutes of hemorrhage followed by ten minutes of resuscitation resulted in a translocation of NF-κB to the nucleus. This translocation was inhibited by pretreatment with the α₁-adrenoreceptor antagonist, prazosin hydrochloride (168). It was found that the activation of NF-κB following hemorrhage did not result in de Novo protein synthesis (142). This indicates that the induction of NF-κB translocation to the nucleus upon hemorrhage occurs through an α₁-adrenergic pathway, thus adding a new mechanism by which NF-κB may be activated to induce TNF-α synthesis.

The intestine has been found to be a pro-inflammatory cytokine-generating organ following mesenteric I/R (169). These cytokines, especially TNF-α, have been implicated as mediators of the multiple organ dysfunction that develops following intestinal I/R (21,170,171). TNF-α has been shown to possess chemotactic properties. Thus, TNF-α induces PMN sequestration into the interstitial spaces of the tissue, which then undergo respiratory burst to induce oxidant injury (172). The role of TNF-α in mediating the myocardial dysfunction seen following intestinal I/R was undefined. Yao et al.

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demonstrated a mechanistic role for TNF-α in the hemodynamic depression resulting from intestinal I/R (173). Pretreatment of rats with a monoclonal antibody to TNF-α significantly improved the mean arterial pressure, cardiac index and stroke volume following 75 minutes of superior mesenteric artery occlusion and six hours of reperfusion. Thus, TNF-α plays a significant role in mediating the cardiac dysfunction seen following intestinal I/R.

Surface burn injury has also been shown to induce a profound depression of cardiac contractile function. Thermal injury to 43% of body surface area induced maximal cardiac dysfunction 24 hours after the burn. These studies indicate that ischemic and inflammatory events remote from the heart can have a significant depressive impact upon heart function. In this model of burn injury, a soluble recombinant human receptor conjugated to the Fc portion of an immunoglobulin (rhTNFR:Fc) was used successfully to significantly reduce cardiac dysfunction (64). Thus, exogenously administered agents that neutralize TNF-α are capable of reaching the site of TNF-α activity to prevent its cardiodepressive effects. Although this study did not differentiate between endogenous cardiac and systemic TNF-α production, the neutralizing activity of the rhTNFR:Fc was successful.

In the early 1980s, a circulating myocardial depressant factor was identified in patients during the acute phase of septic shock (174). A recent follow-up study demonstrated that serum from septic patients depressed in vitro cardiomyocyte shortening, which was reversed by neutralization of both TNF-α and IL-1β (175). Septic shock is recognized to induce profound depression of cardiac contractile function.
Lipopolysaccharide (LPS) or endotoxin has frequently been used to model the effects of septic shock in the laboratory setting. Endotoxin administration has been shown to significantly depress cardiac contractile function by the induction of cytokine production, PMN cytotoxicity and oxidant damage (96,176-179). TNF-α, along with interleukin-1β (IL-1β), has been shown to play an integral role mediating the cardiac dysfunction seen following septic shock (180). Endotoxin administration in an isolated heart model resulted in TNF-α mRNA expression within 30 minutes and TNF-α protein was detected 90 minutes after administration (87). In this model, myocytes and resident cardiac macrophages produced equal amounts of TNF-α. In an alternate study, immunoneutralization of TNF-α with a monoclonal antibody was found to significantly improve cardiac function towards control levels in a rat model of sepsis (181). Similar studies have been attempted in clinical trials. Vincent et al. employed a monoclonal antibody to TNF-α in humans suffering from septic shock (182). Neutralization of TNF-α resulted in a mild, yet significant improvement in left ventricular stroke work index and arterial oxygenation. However, the use of interventions directed towards TNF-α still remain controversial, as several trials have proven unsuccessful in reducing the degree of mortality (183). Thus, while TNF-α appears to play a significant role in mediating cardiac dysfunction seen in many remote organ injuries, the pathophysiological basis of TNF-α induced cardiac dysfunction has not been well defined.

A review of cardiac muscle physiology and cardiac contractile function is beneficial to understanding how the combination of hemorrhage and aortic occlusion can influence cardiac contractility.
**Overview of Cardiac Muscle Physiology**

The ultrastructure of the cardiomyocyte facilitates contraction of the myocardium. Thus, understanding the ultrastructural design of the cardiomyocyte will allow for insight into the contractile mechanism of the myocardium. Cardiomyocytes compose a significant portion of the myofiber. Microscopically, the cardiomyocyte appears as a striated structure. These striations result from the interaction several proteins, which include myosin thick filaments, actin thin filaments and a group of regulatory proteins (Fig. 4) (184). The sarcomere is comprised of the several regions including the A-band (region of actin thin filament and myosin thick filament interaction), the I-band (region of actin thin filament alone) and the H-zone (region of myosin thick filament alone) all bounded within the Z-lines where the actin thin filaments attach. Each of the filaments and their accessory protein are involved in the contractile process of the myocyte.

The myosin thick filament is composed of individual myosin units, which have a rod-like tail component and a double globular head component. The myosin units are arranged laterally with the heads anti-parallel to each other. The heads project outward to enable the actin-stimulated ATP-ase located in the head to be in contact with the actin thin filament. Upon cross-linking of the myosin thick filament and the actin thin filament, the actin-dependent ATP-ase hydrolyzes ATP to ADP and P, to facilitate the myocyte cell shortening (68,185).

The actin thin filament is a double helical structure of F-actin (polymerized G-actin molecules) (186). Associated with the actin thin filament are four proteins, which are responsible for the control of Ca^{2+} regulation and actin-myosin coupling.

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Tropomyosin is a filamentous molecule that is responsible for regulating the interaction between actin and myosin. Each strand of tropomyosin sits within the groove between the two F-actin chains (68). Tropomyosin cooperates with a group of proteins known as the troponin complex to control the Ca\(^{2+}\) and contraction. The troponin

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complex is composed of three proteins; troponin T, troponin I and troponin C. Troponin T is responsible for the binding of tropomyosin to the actin filament. Troponin I directly inhibits the interaction of actin and myosin by adding a steric hindrance to the actin filament. Troponin C, unlike the other two members of the complex, is not involved in preventing the myosin thick filament from binding to the actin thin filament but instead, facilitates the binding of myosin to actin. Troponin C contains Ca\(^{2+}\) binding sites, which act as sensory mechanisms for the contractile apparatus. Upon an increase in cytosolic Ca\(^{2+}\) concentrations, the troponin hindrance and ultimately, the tropomyosin filament is removed to allow for the actin binding sites to be made available for the actin-dependent ATP-ase in the heads of the myosin thick filament (68).

The contractile phase of the myocyte is initiated upon the binding of an ATP molecule to myosin heads. This results in a reduction in the affinity of the myosin head for the actin thin filament, thereby causing the muscle to relax. The ATP-ase activity of the myosin head results in hydrolysis of the ATP molecule to ADP and P\(_i\), resulting in an energized state for the myosin molecule. This powers the myosin molecule to pivot upon its hinge into an activated conformation, a step known as the “power stroke” (186). As a result, the myosin head moves to a position perpendicular to the actin filament.

Concurrently, the intracellular Ca\(^{2+}\) concentration rises and binds to the troponin C binding sites. This results in a conformational change of the tropomyosin-troponin complex to expose the actin binding sites, at which time, the P\(_i\) molecule is released from the myosin head (68,186). This facilitates the binding of the myosin thick filament to the actin thin filament. Once attached to the actin molecule, the remaining ADP molecule is released, enabling the myosin head to return to its original position. The pivot of the

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myosin head moves the actin filament relative to the fixed myosin position resulting in muscle contraction (68,186). Binding of another ATP molecule to the myosin head initiates the next contraction phase by inducing the release of the myosin head from the actin chain. Thus, as long as the intracellular Ca\textsuperscript{2+} and ATP concentrations remain sufficiently high, the myosin-actin bond will continue to cycle resulting in muscle contraction (68,186).

**Starling’s Principle of the Heart**

The force of contraction generated by the contractile proteins in the myocardium is relative to the number of actin-myosin interactions. To attain optimal myocardial contraction, every myosin head must be in contact with an actin molecule (68). The volume of blood in the ventricle has been shown to influence the degree of myosin-actin interactions. Otto Frank, in 1895, first described a relationship between ventricular blood volume and the generated pressure or force of the ventricle, which was also described by Ernest Starling in 1914 (68). This has come to be known as the Frank-Starling Relationship or the Starling’s Principle of the Heart, which is a manifestation of the length-tension principle noted for skeletal muscle.

This principle states that the strength of ventricular contraction (stroke volume) varies directly with the end-diastolic volume (preload) (Fig. 5) (68). Increases in ventricular volume facilitate the interaction of more myosin and actin molecules, thereby increasing the force of contraction. Today, this principle has expanded applications to include force-length relationships. This property can be simulated by the insertion of a balloon into the left ventricle. Inflation of the balloon with an incompressible fluid, such
as saline (increased length) results in an increase in generated pressure (increased force) (68).

Alterations in ventricular volume have also been shown to influence changes in systolic blood pressure (sBP) (systemic pressure generated during the cardiac contraction phase) and diastolic blood pressure (dBP) (systemic pressure generated during the cardiac relaxation phase) (185). As the ventricular volume increases, the sBP rises (68).

**Figure 5.** Frank-Starling’s Pressure-Volume Relationship (Starling’s Law of the Heart). Systolic blood pressure (sBP) rises as the number of actin-myosin bridges increases and then falls as the number of bridges decreases. As the sBP falls, the diastolic blood pressure (dBP) rises. Adopted from Fox, 1996 (185).
However, once the ventricular volume increases beyond the maximal number of myosin-actin interactions (2.0 μm), the force of contraction begins to decline. Therefore, the volume-pressure curve of sBP rises, reaches a pinnacle and then falls. Concurrently, as the sBP curve falls, the dBP curve rises. The rise in dBP is the result of a negative-feedback mechanism, intended to prevent over-filling of the ventricle, thereby reducing the maximal ventricular volume (68). The end result is a reduction in compliance (stiffening) of the left ventricle.

Alterations to the contractile mechanisms of the myocardium have often been shown to result in alterations to the Starling principle of the heart. Often, upon injury to the myocardium, the contractile proteins are influenced, thereby negatively impacting on the heart’s ability to contract. This leads to the development of cardiac contractile dysfunction.

There are two forms of cardiac contractile dysfunction; systolic and diastolic dysfunction (187). The first is defined as the inability of the ventricle to shorten against an increasing load (188,189). According to the Frank-Starling length-tension principle, upon increasing stretch (length) of muscle fibres, the muscle, in turn, contracts (tension) with greater force (185). Upon increasing preload, a healthy ventricle generates greater force with which to maintain a normal ejection fraction. However, in systolic dysfunction, the left ventricle loses its ability to contract normally against increases in preload or afterload and therefore, is unable to eject a normal stroke volume (98). Dilation of the left ventricle is a compensatory mechanism, which develops upon systolic dysfunction in order to increase preload. The left-ventricular function curve generated in

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the systolic failure patients becomes significantly flatter (Fig. 6c) than the curve for normal patients (Fig. 6a).

Diastolic dysfunction is defined as the impairment in left ventricular filling at near normal or mildly elevated left atrial and ventricular pressures (189,190). This results in a decrease in ventricular compliance. As a result, the end-diastolic pressure curve for patients suffering from diastolic heart failure shifts to the left and up (Fig. 6b) compared with normal compliance curves (Fig. 6a). The onset of diastolic dysfunction is due to structural alterations or inappropriate Ca\(^{2+}\) removal within the heart, which prevent appropriate ventricular relaxation and filling, such as ventricular hypertrophy (191).

Figure 6. Characteristics of Systolic and Diastolic cardiac contractile dysfunction. A. Normal contractile function. B. Diastolic contractile dysfunction. C. Systolic contractile dysfunction. Adopted from Harrison's Principles of Internal Medicine, 1998 (221).
**Measurements of Cardiac Contractile Performance**

The development of cardiac contractile dysfunction must be quantified to determine the degree of injury to the heart as well as the type of dysfunction that has developed. Several methods of quantifying the cardiac contractile dysfunction have been developed over the past century, each having its own specific purpose. These include the Langendorff perfusion model and the Working Heart perfusion model.

**Langendorff Perfusion Model**

The Langendorff perfusion apparatus was first described by Oscar Langendorff in 1895 (192). The model was the first to be developed in order to facilitate measurements of cardiac mechanical activity (193). The principle of this model is to perfuse the myocardium in a retrograde fashion with either whole blood or other oxygenated fluid through a cannula inserted into the ascending aorta (192). Retrograde perfusion through the aorta keeps the aortic valve closed thereby forcing the perfusate through the coronary arteries, into the coronary sinus through to the right atrium (193).

Today, saline-based solutions, including Krebs-Henseleit Buffer (KHB), Tyrode or Locke solutions bubbled with carbogen (95% O$_2$-5% CO$_2$) are most commonly used as the perfusate in this model (193). The basic arrangements for the hardware required to maintain appropriate coronary perfusion can be seen in Figure 7. The perfusate is pumped (A) through an in-line filter (B) into a temperature maintained reservoir (C). Perfusate then moves into a bubble trap set at a particular height (D) to perfuse the heart at a constant pressure (125 cm of H$_2$O) through a cannula inserted in the ascending aorta (E). A device inserted into the left ventricle (F) (often a latex-balloon) facilitates the measure
Figure 4. Langendorff Perfusion Apparatus. Krebs-Henseleit Buffer (KHB) is pumped by a circular pump (A) through an in-line filter (B) into a temperature maintained buffer reservoir (C). KHB passes through a bubble trap (D) set at 125 cm of H₂O perfusing the heart through an Aortic Cannula (E). A water bath maintains the buffer at 37°C throughout the entire apparatus through water-jacketed tubing. A ventricular balloon (F) senses contractile force, which is converted to an electric signal by a transducer (G). The trace is recorded by a chart recorder (H).

of cardiac contractile performance. The force of contraction is converted into a wave form signal by a transducer (G), which is then displayed on a chart recorder (H) (193).
Gottleib and Magnus first described the balloon method of quantifying cardiac mechanics in 1904 (193). The balloon, filled with an incompressible fluid such as saline, is connected to a mechano-electric pressure transducer, which can convert the force of contraction into an electric pulse to be quantified (193). The use of an intraventricular balloon adds a potential source of preload to the heart. According to the Starling principle of the heart, increasing preload results in an increase in contractile force. Therefore, as the volume within the balloon is increased, the alterations in cardiac contractile function can be determined, and the effect of various interventions can be stratified.

**Working Heart Perfusion Model**

As opposed to the retrograde perfusion of the Langendorff model, the working heart model involves anterograde perfusion of the heart through the left atrium and out through the aorta. In the Langendorff perfusion apparatus, the aorta is cannulated to facilitate perfusion of the coronary arteries. The Working Heart model includes cannulation of the aorta as well as the left atrium (through the pulmonary vein). This results in the filling of the left ventricle. Upon systole, the left ventricle pumps the volume of blood out through the aortic cannula.

Beyond differences in the mode of perfusion in the Working Heart model, the preload (the perfusion pressure of the left ventricle) and the afterload (the pressure seen beyond the aortic valve) can be adjusted, while only the preload can be altered in the Langendorff model. The ability to control both the preload and afterload in the Working Heart model allows for the measurement of cardiac output through the aortic cannula, while the Langendorff model allows for measurements of isovolumic pressure (193).
The Langendorff perfusion apparatus represents an artificial model of the working heart, but it is useful in defining the Frank-Starling’s relationship (length-tension principle) and the Law of Laplace (the wall tension at any given pressure is increased as the radius of the ventricle increases) of the heart (68). Alterations in the contractile properties of the heart can indicate when there are basic structural or underlying biochemical alterations to the heart. The Working Heart model enables one to study the physiologic parameters of the heart. Since the heart is not isovolumic, as in the Langendorff model, the heart ejects at varying preload and afterload. Thus, measures of work, and the work of the heart during each beat (stroke work), can be measured along with cardiac output and ejection fraction. This then represents a method to measure how the heart may work \textit{in vivo} in an \textit{ex vivo} setting (68).

In an effort to characterize the potential cardiac dysfunction in this study of RAAA repair, the Langendorff perfusion apparatus was utilized. This method is best suited for this study, as the presence or absence of cardiac dysfunction following RAAA repair has yet to be determined. This model enables one to define a Starling’s relationship for hearts from control animals and animals undergoing RAAA repair in order to differentiate the ability of the heart to contract and relax. The Langendorff perfusion apparatus will also provide insight into the mechanistic basis of the cardiac dysfunction that develops in this model of RAAA repair.

\textbf{Summary}

Ruptured abdominal aortic aneurysm (RAAA) repair includes two ischemic events, hemorrhagic shock and intestinal or lower torso ischemia. The synergy of the two
injuries has been found to result in a significant depression of organ function (including respiratory, hepatic and intestinal). The pathophysiologic bases of the Multiple Organ Dysfunction noted following RAAA repair have been studied. However, the role of cardiac contractile dysfunction and the mechanisms by which it develops in patients undergoing RAAA repair remains unknown.

The preceding review discussed many of the areas relevant to RAAA repair; that is, hemorrhagic shock and intestinal/lower torso ischemia and their impact on cardiac contractile function, as well as other pertinent models of myocardial dysfunction, both local and remote. A significant portion of the literature suggests that the organ dysfunction seen following I/R injury is heterogeneous and involves the activity of many cytotoxic agents, including PMNs and ROS. However, the role of cytokines as mediators of the organ injury has not been fully examined.

It is becoming more apparent that cytokines, especially Tumour Necrosis Factor-\(\alpha\) (TNF-\(\alpha\)), are playing a pivotal role in the depression of cardiac contractile function in various pathological states. TNF-\(\alpha\) has been found to induce a direct depression of contractile function of the heart and cardiomyocytes are known to be potent generators of TNF-\(\alpha\). Following hemorrhage, thermal injury and septic shock, TNF-\(\alpha\) has been found in the myocardium and neutralization of TNF-\(\alpha\) in models of intestinal I/R, surface burn injury as sepsis have implicated TNF-\(\alpha\) in the myocardial dysfunction that develops.

Taken together, these studies indicate that ischemic and inflammatory events remote from the heart can have a significant depressive impact upon cardiac contractile function, and suggest that cytokines may be involved in this process. This depression in

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cardiac function may compromise the ability of patients to survive severe insults such as sepsis or remote organ ischemia following RAAA repair. However, to date, no studies have investigated myocardial performance and the role of TNF-α following the sequential ischemia-reperfusion events, which comprise rupture and repair of an AAA.

Introduction
CHAPTER 2

HYPOTHESIS, COROLLARY

AND

OVERVIEW OF EXPERIMENTS
HYPOTHESIS

We hypothesize that the sequential ischemic events of aortic rupture and repair (hemorrhagic shock and aortic occlusion) act synergistically to induce depressed myocardial contractile function.

COROLLARY

To determine the mechanisms that mediate the depression of myocardial contractile function seen in a model of ruptured abdominal aortic aneurysm repair.

OVERVIEW OF EXPERIMENTS

Cardiac contractile function was assessed in hearts from rats undergoing a model of RAAA repair. An isolated, Langendorff perfusion model was utilized for measurement of myocardial function.

Various mediators known to induce depressed cardiac function in models of myocardial ischemia-reperfusion were assessed in this model of RAAA repair. These included myocardial TNF-α levels, energy stores (ATP and CP), myocardial neutrophil sequestration, oxidant stress and necrosis.

Neutralization of TNF-α was performed to assess the role that TNF-α plays in the depressed cardiac contractile function in this model of RAAA repair.
CHAPTER 3

MATERIALS

AND

METHODS
**Materials and Methods**

**Surgical Procedure**

A rat model of RAAA repair designed in our laboratory was employed. Briefly, adult male Sprague-Dawley rats weighing 350 to 400 g (Charles River, Wilmonton, MA) were allowed to acclimatize for five days given water and rat chow *ad libitum*. All experiments were carried out in accordance with the requirements of the “Animals for Research Act” of the province of Ontario, and the regulations of the Toronto Hospital Animal Care Committee. Rats were pretreated intramuscularly with atropine sulfate (25 µg/kg) and anaesthetized intraperitoneally with sodium pentobarbital (50 mg/kg). Catheters (22 gauge) were placed in the tail vein and carotid artery. Venous access was used for administration of supplemental anaesthetic, return of withdrawn blood and fluid resuscitation (Lactated Ringer’s solution), while the carotid artery was utilized for measurement of mean arterial blood pressure (Hewlett Packard model 78304A, Palo Alto, CA) and removal of blood for the induction of hemorrhagic shock. A tracheostomy (14 gauge catheter) and laparotomy were performed. The abdominal aorta was isolated between the celiac axis and the superior mesenteric artery and immediately proximal to the aortic bifurcation (Fig. 8). The abdomen was then closed and the animal allowed to stabilize for 30 minutes.

**Experimental Groups**

Rats were divided into four groups (Fig. 9). The first group consisted of sham operated control rats (which underwent the surgical preparation). The second group (hemorrhagic shock alone group) underwent shock alone. Following 30 minutes of
Figure 8. Rat model of RAAA repair. The model includes an arterial and venous line, a tracheostomy and a laparotomy where the aorta is isolated proximal to the superior mesenteric artery and the aortic bifurcation.

stabilization and 30 minutes of baseline measurements, blood was withdrawn from the arterial line into pre-heparinized syringes to maintain a mean arterial pressure of 50 mmHg for a period of 60 minutes. Blood was maintained at room temperature and rocked

Materials and Methods
Figure 9. In vivo surgical and experimental protocol. Following the surgical preparation, 30 minutes of stabilization and 30 minutes of pre-shock baseline measurements, animals experienced 60 minutes of hemorrhage to a MAP of 50 mmHg, 45 minutes of SMA clamping and 90 minutes of reperfusion. ↑ represents return of ½ withdrawn blood volume. ↑ represents the point when the heart is excised for assessment of left ventricular contractile function.

throughout the experiment. Following this period, half the shed blood volume was returned and the second half returned 45 minutes later. Then the animals underwent 90 minutes of monitoring (reperfusion). In the third group (clamp alone group), a Scoville Lewis intracranial atraumatic microvascular clip was applied to the abdominal aorta proximal to the superior mesenteric artery and a second clip applied proximal to the aortic bifurcation. Following clamp removal, animals were monitored for 90 minutes.

The final group (Shock and Clamp; S+C) underwent a sequential period of hemorrhagic shock (mean arterial pressure of 50 mmHg for 60 minutes) and supramesenteric aortic clamping (45 minutes) followed by 90 minutes of monitoring. Both sham operated control and clamp alone animals received equivalent volumes of heparin at the appropriate time points. All animals were supplemented with Lactated Ringer’s solution to maintain a mean arterial pressure (MAP) of 100 mmHg during the monitoring period.

Model Development

The rat model of RAAA repair has been used previously in our laboratory to study the effects of varying periods of hemorrhage and levels of aortic clamping on lung permeability (12). We have previously used sodium pentobarbital for general anesthesia

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(13). After careful assessment of various anesthetic regimens, we determined that pentobarbital provided the most effective and reproducible method to ensure deep anesthesia. While sodium pentobarbital is recognized to have cardiodepressive activities (194), we ensured that each subject received an equal amount of anesthetic throughout the protocol.

In order to reduce operator variability, efforts had to be undertaken to ensure that similar injuries that were noted previously were maintained. Lung and liver neutrophil sequestration (as quantified by a myeloperoxidase assay) and lung lipid oxidative stress (as quantified by F₂-isoprostanes) have been used in our laboratory as markers of injury.

![Figure 10. Myeloperoxidase content in the lung. □ represents Sham Operated controls and ■ represents combined S+C group. All values are expressed as Mean ± SEM. *p<0.0002 vs. Sham Operated control group.](image)

**Materials and Methods**
Thus, similar analyses were performed to act as an internal control. Increased lung and liver MPO were noted in the S+C group compared to the sham operated control group, similar to that reported previously (Fig. 10 and Fig. 11). Lung F$_2$-isoprostanes also increased significantly following the combination of hemorrhagic shock and lower torso ischemia (Fig. 12).

![Graph showing liver MPO levels in Sham and S+C groups.](image)

**Figure 11.** Myeloperoxidase content in the liver. □ represents Sham Operated controls and ■ represents combined S+C group. All values are expressed as Mean ± SEM. *p=0.009 vs. Sham Operated control group.

Additionally, efforts to standardize the degree of hemorrhage have proven difficult. Several methods have been employed previously by various investigators, including hemorrhage to predefined volumes or blood pressures. Both methods contain

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inherent shortcomings; however, no described method has proven universally successful in rodent models of HS. Thus, we continue to hemorrhage rats to a defined mean blood pressure of 50 mmHg. It is recognized that there is a rate-dependent response to HS, in that rapid removal of blood results in activation of the adrenocortical axis and subsequent feedback mechanisms to induce an elevation of mean blood pressure. A second source of variability associated with this model of HS and lower torso ischemia is that there appears to be a narrow window outside which too little hemorrhage results in no injury, while too much hemorrhage results in a significant mortality. Thus, a rigid protocol for hemorrhage was developed and employed in these studies to ensure uniform severity of injury without inducing irreversible, fatal shock. This included passively withdrawing

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blood to induce hemorrhage to a MAP of 50 mmHg and continuing to passively withdraw blood to ensure that the MAP remained within a pre-defined range (46-54 mmHg).

Assessment of Left Ventricular Performance

Following the 90-minute monitoring period, heparin (200 IU) was infused and the hearts were rapidly excised and placed in 4°C Krebs-Henseleit Bicarbonate (KHB) buffer. The KHB buffer used in this study is similar to that previously reported with isolated heart muscle preparations (195). The solution contained: (mM) NaCl 118, KCl 4.7, NaHCO₃ 21, CaCl₂ 1.25, MgSO₄ 1.2, KH₂PO₄ 1.2 and glucose 11. All solutions were prepared daily with deionized water and bubbled with 95% O₂-5% CO₂. The pH of the solution was 7.4 and the temperature was maintained at 37°C. The ascending aorta was cannulated with an 18 gauge cannula that was subsequently connected through glass tubing to a KHB buffer reservoir for perfusion of the coronary circulation at a constant pressure of 120 cm of water. Intraventricular pressure was measured with a saline-filled latex balloon attached to a polyethylene tube and threaded into the left ventricular chamber through the left auricle. Left ventricular pressure was measured with a mini pressure transducer (Gould Electronics, Valley View, Ohio) attached to the balloon cannula. Left ventricular + and - dP/dt maximum (Max.) values were obtained using an electronic differentiator (Gould Electronics, Model 13-4615-17, Valley View, Ohio) and recorded using a Windo-Graph chart recording system (Gould Electronics). Following 20 minutes of stabilization, coronary effluent was collected over a two-minute period to determine coronary flow rates, as previously described (196).

Materials and Methods
Model Development

In an attempt to measure cardiac contractile function in hearts from rats undergoing sham operation, hemorrhagic shock alone, aortic clamp alone or the combination of hemorrhagic shock and aortic clamp (S+C), appropriate controls had to be established. These include ensuring appropriate perfusion buffers and left ventricular balloon sizes. Initially, experiments were performed using a balloon that did not appropriately fill the ventricular cavity. The results obtained from these experiments did not accurately reflect the cardiac contractile function in these hearts. Thus, several

![Figure 13](image)

**Figure 13.** Isolated balloon pressure in response to increasing balloon volume. ◊ represents 0.5 cm length balloon. ● represents 1.0 cm length balloon. ▽ represents 1.5 cm length balloon and ■ represents 2.0 cm length balloon. The flat portion of the curve of either the 1.5cm length balloon or the 2.0cm length balloons fall within end-diastolic pressure range of the rat heart.

Materials and Methods
balloons of varying sizes were prepared and the pressure generated by the isolated balloon was measured. The pressure for each balloon is depicted in Figure 13. Several authors have previously reported that the range of left ventricular volume should fall within the flat range of the isolated balloon pressure curve (60). Thus, the 1.5 cm balloon was selected as it closely represented the volume of the ventricular cavity of the rats used in these studies.

**Left Ventricular Function Curves**

A Starling relationship for the different groups was determined by plotting left ventricular peak systolic pressure (PSP), $+\text{dP/dt Max.}$ (a measure of contractility) and $-\text{dP/dt Max.}$ (a measure of relaxation) against the physical parameters of increasing left ventricular volume and increasing end-diastolic pressure (Fig. 14). As a second method of determining cardiac contractile function independent of alterations in ventricular volume, we stimulated the isolated heart with the β-adrenergic agonist, isoproterenol,

![Figure 14. Isolated Heart Ex Vivo Cardiac Performance Assessment Protocol. Following a 20-minute stabilization period, three independent measures of cardiac performance are undertaken. Measurement 1 includes incrementally increasing the left ventricular volume and recording the resultant contractile function. Measurement 2 includes incrementally increasing the left ventricular end-diastolic pressure and recording the resultant contractile function. Measurement 3 includes recording cardiac contractile function in response to increasing isoproterenol concentration. $\downarrow$ represents a 5 minute stabilization period following each measurement period. $\uparrow$ represents removal of the myocardium for myocardial water content.](image-url)
through a side-port, as previously described (197). Increasing concentrations of isoproterenol were utilized and it was noted that maximal cardiac stimulation occurred at the concentration of 50 ng/ml.

The relationship between left ventricular capacity and balloon volume was determined by plotting the pressure-volume relationship of the isolated balloon. All experiments were performed on the flat portion of the balloon pressure-volume curve.

**Myocardial Water Content**

Following coronary perfusion, hearts were removed, the myocardium isolated and the right ventricle weighed. The hearts were flash frozen and dried for 24 hours (Lyph-Lock 6 Lyophilizer, Labconco, Kansas City, MO) and dry weights were taken for determination of myocardial water content (MWC).

\[
MWC = \frac{(Wet \ Weight - Dry \ Weight)}{Wet \ Weight}
\]

**Biochemical Analysis of the Myocardium**

A second series of experiments were undertaken in order to analyze the myocardium for TNF-α and energy metabolites, neutrophil sequestration and markers of lipid peroxidation in this model of RAAA repair.

Rats underwent either sham operation, shock alone, aortic clamping alone or the combination of shock and aortic clamping (six per group). The hearts were collected for biochemical analyses following the 90-minute monitoring period (hearts did not undergo

**Materials and Methods**
functional analysis) by *in situ freeze clamping* (198), were flushed free of residual plasma and flash frozen in liquid N₂. Hearts were stored at -80 °C until analysis.

**Myocardial TNF-α Quantification**

Frozen biopsies were homogenized according to the method of Torre-Amione *et al.* (199). Samples were suspended in phosphate buffered saline (PBS) containing phenylmethylsulfonyl fluoride (PMSF, 1.49 mM), leupeptin (475.6 μM) and aprotinin (0.31 μM). The homogenates were centrifuged for 20 minutes at 60,000 rpm. The pellet was solublized according to the method of Stauber *et al.* (200), by resuspension in an equal volume of PBS containing PMSF (1 mM), aprotinin (50 μl) and 1% Triton X-100. Following 1 hour of incubation at 4°C, the solublized protein was centrifuged for 20 minutes at 60,000 rpm. The supernatant was analyzed in duplicate using the Cytoscreen rat TNF-α ELISA (Enzyme-Linked Immuno Adsorbent Assay) kit (Biosource International, Camarillo, CA). This assay is linear between 0 and 1000 pg/ml. TNF-α levels were standardized to total soluble protein content, determined using a commercial protein assay (Pierce Chemical Co., Rockford, IL).

**Myocardial Energy Stores**

Determination of myocardial adenosine triphosphate (ATP) and creatine phosphate (CP) was adopted from Harris *et al.* (201). Each sample was freeze dried for 24 hours and stored at -80 °C (Lyph-Lock 6 Lyophilizer, Labconco, Kansas City, MO). Upon analysis, dried muscle tissue was separated from any remaining blood or connective tissue and homogenized in 0.5 M perchloric acid (PCA) with 1 mM ethylenediamine tetraacetic acid (EDTA). Samples were centrifuged at 3000 rpm and the

*Materials and Methods*
supernatant collected and neutralized with 2 M KOH and 0.5 M PCA. 20 μl of neutralized substrate was added to 2 ml of sample buffer (including 1 M Tris HCl, pH 8.1, 0.1 M MgCl₂, 50 mM dithiothreitol (DTT), 50 mM NADP, 10 mM glucose and 10 μL glucose-6-phosphate in a final volume of 50 ml). Enzymatic reactions utilizing hexokinase (HK) and creatine phosphokinase (CPK) were performed and NADPH production was measured by fluorometric analysis (COBAS FARA, Roche Diagnostics Systems Inc., Nutley, NJ) at an excitation wavelength of 340 nm and an emission wavelength of 470 nm. Each unit of NADPH produced represented 1 unit of ATP or CP.

**Myocardial Myeloperoxidase Assay**

Tissue neutrophil sequestration can be quantified by determining tissue myeloperoxidase (MPO) content. MPO is an enzyme that constitutes approximately 5% of the dry weight of neutrophils and resides in intracellular granules. MPO acts to convert H₂O₂ in the presence of a halogen ion (primarily chloride ion) to a hypohalous acid and water (Fig. 15). All myeloid cells (neutrophils, eosinophils and monocytes) contain MPO, however, eosinophils and monocytes contribute a very small amount of MPO to the total activity as they represent only a small fraction of the circulating granulocytes. Thus, MPO has become a recognized method of quantifying neutrophil sequestration in the coronary vasculature and tissue under inflammatory conditions. Studies have shown that tissue MPO content closely correlates to the PMN infiltration into tissues, as determined either histologically or by Coulter counting (202).

**Materials and Methods**
Figure 15. The myeloperoxidase dependent formation of hypochlorous acid from hydrogen peroxide.

Neutrophil sequestration in the heart was quantified by the presence of MPO by the method of Mullane et al. (203). This assay uses the principle of MPO catalytic activity to quantify neutrophil sequestration. Tissue samples were homogenized with a blade homogenizer (Polytron, Brinkman, Westbury, NY) in ice cold phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide and 5 mM EDTA. The homogenate was then sonicated (Vibra Cell Sonicator, Sonics and Materials Inc., Danbury, CT) and centrifuged at 12,000 rpm. 100 µL of supernatant was added to 2.9 ml of assay buffer containing Na$_2$PO$_4$, 0.3% H$_2$O$_2$ and 0.1% o-dianisidine hydrochloride in a final volume of 50 ml. The H$_2$O$_2$ dependent oxidation of o-dianisidine hydrochloride was used as an index of myeloperoxidase activity. One unit of myeloperoxidase is defined as the amount of myeloperoxidase required to degrade 1 µmole of H$_2$O$_2$ per minute at 25°C. Myeloperoxidase values were standardized to tissue protein content.

**F$_2$-Isoprostane Quantification**

Recently, a novel class of stable lipid peroxidation products known as F$_2$-isoprostanes have been characterized and have been shown to provide a reliable index of lipid peroxidation *in vivo* in various models (204-206). F$_2$-isoprostanes are formed *in situ* on phospholipids predominantly by non-enzymatic, free radical mediated oxidation of membrane bound arachidonic acid (207). These esterified F$_2$-isoprostanes are a stable product and thus allow tissue specific quantification of lipid peroxidation.

**Materials and Methods**
Phospholipase A2 has been implicated in the cleavage of F₂-isoprostanes from the arachidonic acid backbone releasing the products into body fluid, including plasma and urine (Fig. 16).

The extent of membrane lipid peroxidation was estimated using the production of myocardial F₂-isoprostanes. Frozen biopsies were thawed and homogenized with a blade homogenizer in phosphate buffered saline. Tritiated prostaglandin F₂α (PGF₂α) was added to each sample in order to determine recoveries following extraction. Protein was extracted by ethanol precipitation, centrifuged and discarded. Fatty acid side chains,
including F₂-isoprostanes were cleaved from their glycerol backbone by alkaline hydrolysis. The samples were then acidified to a pH below 4 with HCl and loaded on to an activated C₁₈ reverse phase SPE cartridge (Sep-Pak Column, Waters Inc. Mississauga, Ont.). The column was washed with ultrapure water and hexane, the F₂-isoprostanes eluted with ethyl acetate containing 1% methanol and the eluent evaporated to dryness under nitrogen and stored at -80°C. Samples were reconstituted and analyzed in duplicate utilizing a commercially available 8-epi F₂-isoprostane enzyme immunoassay (Cayman Chemical Co., Ann Arbour, MI). Extraction efficiency from the purification step was analyzed by scintillation counting of each sample and averaged 70%. F₂-isoprostane values were standardized to tissue protein content.

Myocardial Necrosis

A group of animals (2 sham and 2 shock) underwent the surgical procedure and then were subjected to 60 minutes of hemorrhagic shock to a mean blood pressure of 50 mmHg. Half the withdrawn blood was returned following the hemorrhagic shock period and the second half returned 45 minutes later. Following the return of withdrawn blood, venous and arterial cannulae were removed and the animals were allowed to wake. After 24 hours, the heart was excised by midline sternotomy and sectioned transversely (20% total mass per section) and incubated with 1.25% triphenyl tetrazolium red (TTC) stain (Toronto General Hospital Pharmacy) for 15 minutes at 37°C. Normal cardiac muscle are stained by tetrazolium salts when redox systems including NAD/NADH, NADP/NADPH, flavoprotein and cytochrome B reduce the salts through the action of tissue diaphorases and dehydrogenases. A lack of staining occurs when these enzyme pathways are inactivated following cell death.

Materials and Methods
**TNF-α Neutralization**

In an effort to determine the role of TNF-α in the myocardial dysfunction seen following HS and lower torso ischemia, a neutralizing antibody to TNF-α was employed, which has previously been utilized to reduce pulmonary microvascular injury following intestinal ischemia (21). A third series of 16 S+C animals were randomized into two groups. The first received a Polyclonal Rabbit Anti-Mouse TNF-α Neutralizing Antibody (600 μL/kg of IP-400; Genzyme Diagnostics, Cambridge, MA) 5 minutes prior to the onset of hemorrhagic shock by intravenous administration. The second group received a control Rabbit IgG molecule (500 μL/kg, Zymed Laboratories, San Francisco, CA). Following completion of the experimental protocol, hearts were excised and left ventricular function was assessed as described above.

The dosage of the anti-TNF-α antibody was calculated by determining the number of units of TNF-α in the myocardium of rats undergoing the combination of hemorrhage and aortic occlusion. Krown et al. have previously reported that 68 pg/mL of TNF-α is equivalent to 3,500 Units/mL of tissue (166). This antibody has been described to neutralize 1000 Units of TNF-α per μL of solution. Thus, after determining that the concentration of TNF-α in the myocardium in the S+C rats was approximately 6000 pg/mL of heart tissue, it was calculated that the appropriate dose of neutralizing antibody would be 300 μL per animal (600μL/kg). The concentration of the isotype control antibody used was determined by quantifying the protein content of anti-TNF-α antibody and isotype control and giving an equivalent amount of the isotype control antibody (250μL per animal; 500μL/kg).

**Materials and Methods**
Statistical Analysis

Statistical analysis was performed using SPSS statistical software (SPSS Inc., Chicago, IL). All values are expressed as mean ± standard error of the mean (SEM). A probability of less than 0.05 was considered significant.

Analysis of In Vivo Response

Differences in withdrawn blood volumes were compared using an ordinary (student's) t-test. Changes in mean arterial blood pressure, differences in resuscitation volumes required, blood gases and pH levels were analyzed using a one-way analyses of variance (ANOVA) followed by Student Newman Keuls post-hoc test for multiple pairwise comparisons.

Analysis of Left Ventricular Performance

Myocardial function in response to increasing left ventricular volume, left ventricular end-diastolic pressure and isoproterenol stimulation were compared using a one-way analyses of variance (ANOVA) followed by Student Newman Keuls post-hoc test for multiple pairwise comparisons at each individual volume, pressure or isoproterenol concentration point, as previously described (52).

Analysis of Myocardial Biochemistry

Myocardial TNF-α levels, ATP, CP, MPO and F₂-isoprostane levels in the four groups were compared using a one-way analyses of variance (ANOVA) followed by Student Newman Keuls post-hoc test for multiple pairwise comparisons.

Materials and Methods
CHAPTER 4

RESULTS
RESULTS

In Vivo Response of Experimental Groups

The *in vivo* responses to hemorrhagic shock and aortic clamping were measured including fluid resuscitation requirements and blood gases to validate the severity of the model prior to assessment of cardiac contractile function. The alterations in mean blood pressure (MAP) during the experimental protocol are shown in Figure 17.

![Graph showing mean arterial pressure (MAP) through experimental protocol.](image)

Figure 17. Mean arterial pressure (MAP) through experimental protocol. ● represents Sham Operated controls. ■ represents SMA Clamp alone. ▲ represents Hemorrhagic Shock alone and ○ represents combined S+C group. All values are expressed as Mean ± SEM. *p*<0.05 vs. S+C group at the same time point.
The volume of blood removed to maintain a mean arterial pressure of 50 mmHg in both the shock alone group (20.9 ± 1.3 ml/kg) and the combined S+C group (19.1 ± 0.9 ml/kg) during the shock period were equivalent (Table 1). After application of the aortic clamp in the S+C group, MAP rose but was significantly lower than that of the clamp alone group. To maintain the mean arterial pressure at the 100 mmHg target during the reperfusion (monitoring) period, the S+C animals required significantly larger volumes of supplemental fluid compared to sham operated controls, shock alone animals and clamp alone animals (p<0.001; ANOVA) (Fig. 18).

Figure 18. Resuscitation volume required in each group. □ represents Sham Operated Controls. ■ represents SMA Clamp alone. ✶ represents Hemorrhagic Shock alone and ■ represents Combined S+C group. All values are expressed as Mean ± SEM. †p<0.05 vs. Sham Operated controls, Clamp alone and Shock alone. §p<0.05 vs. Sham Operated controls and Shock alone.

Results
Immediately prior to the completion of the experimental protocol, arterial blood gases were performed and no significant differences were noted in the pO₂, pCO₂, O₂ saturation and pH between groups (Table 1). The significant increase in fluid requirements suggested that a substantial injury resulted from the induction of hemorrhagic shock followed by lower torso ischemia.

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=12)</th>
<th>Clamp (n=8)</th>
<th>Shock (n=8)</th>
<th>S+C (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Volume (ml/kg)</td>
<td>-</td>
<td>-</td>
<td>20.9 ± 1.3</td>
<td>19.1 ± 0.9</td>
</tr>
<tr>
<td>pO₂ (mmHg)</td>
<td>94.12 ± 8.78</td>
<td>100.42 ± 5.09</td>
<td>91.15 ± 2.09</td>
<td>97.69 ± 7.25</td>
</tr>
<tr>
<td>PCO₂ (mmHg)</td>
<td>24.08 ± 2.13</td>
<td>19.05 ± 1.40</td>
<td>20.51 ± 2.09</td>
<td>21.89 ± 1.38</td>
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<td>HCO₃ (mM/liter)</td>
<td>18.50 ± 1.30</td>
<td>14.64 ± 0.65</td>
<td>15.95 ± 1.65</td>
<td>14.46 ± 0.85</td>
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<tr>
<td>O₂ Saturation (%)</td>
<td>96.725 ± 0.83</td>
<td>97.84 ± 0.25</td>
<td>96.44 ± 0.45</td>
<td>97.46 ± 0.49</td>
</tr>
<tr>
<td>pH</td>
<td>7.487 ± 0.012</td>
<td>7.453 ± 0.015</td>
<td>7.468 ± 0.007</td>
<td>7.461 ± 0.012</td>
</tr>
</tbody>
</table>

Table 1. In vivo metabolic response to hemorrhagic shock and intestinal ischemia. All values are expressed as Mean ± SEM.

Assessment of Left Ventricular Performance

Myocardial function was subsequently assessed by recording peak systolic pressure (PSP), contractility (+dP/dt Max.) and relaxation (-dP/dt Max.) in a total of 36 animals (12 Shams, 8 Shock controls, 8 Clamp controls and 8 S+C) on a Langendorff perfusion apparatus. No difference in end diastolic pressure was noted between groups as ventricular volume (preload) was increased (Fig. 19).

Results
Figure 19. Left ventricular end-diastolic pressure (LVEDP) upon increasing left ventricular volumes. ● represents Sham Operated controls. ■ represents SMA Clamp alone. ▲ represents Hemorrhagic Shock alone and ○ represents combined S+C group. All values are expressed as Mean ± SEM.

Significant myocardial systolic dysfunction was noted after hemorrhagic shock alone, lower torso ischemia alone (clamp alone group) and in the combined shock and clamp group (S+C) as indicated by the downward shift of the function curves (Fig. 20). An intermediate dysfunction was noted following lower torso ischemia compared to sham operated controls. Hearts from the shock alone and combined S+C animals demonstrated a significant cardiac dysfunction compared to sham operated controls. In the clamp alone, shock alone and combined S+C groups, the initial PSP (Fig. 20a), +dP/dt Max. (Fig. 20b) and -dP/dt Max. (Fig. 20c) were significantly depressed by approximately 35% compared to sham operated animals (p<0.001; ANOVA). The PSP and both + and - dP/dt Max. in the shock alone and the S+C groups remained depressed
by approximately 40% as preload was increased. However, the clamp alone group responded differently. Upon increasing preload, improved PSP and +dP/dt Max., which approached sham operated control levels, were noted.

Figure 20. Cardiac Function at increasing left ventricular volume. A. Peak Systolic Pressure. B. +dP/dt Max. C. -dP/dt Max. ● represents Sham Operated controls. ■ represents SMA Clamp alone. ▲ represents Hemorrhagic Shock alone and ▲ represents combined S+C group. All values are expressed as Mean ± SEM. *p<0.05 vs. Clamp alone, Shock alone and S+C groups. p<0.05 vs. Shock alone and S+C groups.

Plots of peak systolic pressure, + and -dP/dt Max. against increasing left ventricular end-diastolic pressure (Fig. 21) displayed similar results as those in Figure 20. Hearts from clamp control animals, shock control animals and from S+C animals all experienced significantly lower initial PSP (Fig. 21a), lower contractility (Fig. 21b) and relaxation (Fig. 21c). While all three parameters remained depressed in the hearts from shock control animals and S+C animals upon increasing left ventricular end-diastolic

Results
pressure, PSP and +dP/dt Max. rose in hearts from clamp control animals to sham operated control levels (Fig. 20 and 21).

![Graphs showing cardiac function at increasing left ventricular diastolic pressures. A. Peak Systolic Pressure (PSP). B. +dP/dt Max. C. -dP/dt Max. ○ represents Sham Operated controls. ■ represents SMA Clamp alone. ▲ represents Hemorrhagic Shock alone and ● represents combined S+C group. All values are expressed as Mean ± SEM. *p<0.05 vs. Clamp alone, Shock alone and S+C groups. †p<0.05 vs. Shock alone and S+C groups.](image)

*Figure 21. Cardiac function at increasing left ventricular diastolic pressures. A. Peak Systolic Pressure (PSP). B. +dP/dt Max. C. -dP/dt Max. ○ represents Sham Operated controls. ■ represents SMA Clamp alone. ▲ represents Hemorrhagic Shock alone and ● represents combined S+C group. All values are expressed as Mean ± SEM. *p<0.05 vs. Clamp alone, Shock alone and S+C groups. †p<0.05 vs. Shock alone and S+C groups.*

We stimulated the isolated heart with isoproterenol (a β-adrenergic agonist) as a preload-independent control. Initially, the PSP of the clamp alone, shock alone and S+C group was significantly depressed compared to sham operated controls (p<0.05 vs. sham operated control at 0 ng/mL) (Fig. 22). Upon isoproterenol stimulation, PSP in the clamp alone group rose to 100% of sham stimulated levels (sham operated control at 50 ng/ml of isoproterenol). However, cardiac function in the shock alone and S+C groups returned

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to only 80% of sham stimulated levels. Thus, while isoproterenol stimulation significantly improved PSP in the clamp alone group, cardiac contractile function remained significantly depressed in both the shock alone and S+C groups compared to sham operated control and clamp alone hearts (p<0.001). Increases in +dP/dt Max. were similar to those noted in the PSP for each of the treatments (data not shown).

Figure 22. Assessment of Left Ventricular Function in response to isoproterenol stimulation. Peak Systolic Pressure (PSP) as a percentage of Sham Operated control group at either 0 or 50 ng/mL. ■ Clamp alone group. □ Hemorrhagic Shock alone group and ▪ S+C group. All values are expressed as Mean ± SEM. *p<0.05 vs. Clamp alone, Shock alone and S+C groups. †p<0.05 vs. Shock alone and S+C groups.
We attempted to determine if altered heart rate or general myocardial damage as indicated by increased tissue fluid content (edema) were responsible for inducing the observed cardiac dysfunction. Coronary flow rates increased from 14.0 ml/min in the sham operated control group to 15.1 ml/min in the clamp alone group, 15.9 ml/min in the shock alone group and to 18.8 ml/min in the combined S+C group. Heart rate was similar in all four groups over the course of cardiac contractile performance analysis. This model of RAAA repair did not induce myocardial edema, as the right ventricular water content remained was unchanged in all four groups (p=0.5, ANOVA) (Table 2).

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=12)</th>
<th>Clamp (n=8)</th>
<th>Shock (n=8)</th>
<th>S+C (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSP (mmHg)</td>
<td>147.58 ± 3.65&quot;</td>
<td>138.13 ± 7.00</td>
<td>123.63 ± 5.35</td>
<td>125.50 ± 6.30</td>
</tr>
<tr>
<td>+dP/dt Max. (mmHg/s)</td>
<td>2170.42 ± 112.92&quot;</td>
<td>1843.75 ± 89.86&quot;</td>
<td>1507.50 ± 60.38</td>
<td>1508.75 ± 65.48</td>
</tr>
<tr>
<td>-dP/dt Max. (mmHg/s)</td>
<td>1062.50 ± 41.91&quot;</td>
<td>858.13 ± 27.29</td>
<td>912.50 ± 67.81</td>
<td>861.88 ± 38.89</td>
</tr>
<tr>
<td>Coronary Flow (mL/min)</td>
<td>13.96 ± 0.24</td>
<td>15.06 ± 0.20&quot;</td>
<td>15.88 ± 0.38&quot;</td>
<td>18.75 ± 0.49&quot;</td>
</tr>
<tr>
<td>Heart Rate (beats/min)</td>
<td>285.00 ± 10.77</td>
<td>292.50 ± 13.75</td>
<td>296.25 ± 3.75</td>
<td>285.00 ± 15.00</td>
</tr>
<tr>
<td>Myocardial Water Content (% dry weight)</td>
<td>84.16 ± 0.29</td>
<td>85.20 ± 0.20</td>
<td>84.11 ± 0.34</td>
<td>84.31 ± 0.35</td>
</tr>
</tbody>
</table>

Table 2. Ex vivo cardiac contractile performance and tissue edema in response to hemorrhagic shock and intestinal ischemia. Functional parameters measured at intravenricular pressure of 5 mmHg. All values are expressed as Mean ± SEM. * p<0.05 vs. Sham & Shock. "p<0.05 vs. all. "p<0.05 vs. Shock & S+C. "p<0.05 vs. Sham.

**Myocardial TNF-α Concentration**

We attempted to implicate a specific mediator of the observed cardiac dysfunction by comparing myocardial TNF-α levels in this model of RAAA repair with the degree
of cardiac dysfunction noted (Fig. 23). Myocardial TNF-α levels increased three-fold from the sham operated controls (22.2 pg/mg of soluble protein) to the clamp alone hearts (71.7 pg/mg). Hemorrhagic shock resulted in a two-fold increase of myocardial TNF-α levels (145.2 pg/mg) compared to hearts from clamp alone animals and a six-fold increase compared to sham operated controls (p<0.05 vs. sham and clamp alone groups). The combination of hemorrhagic shock and aortic clamping (S+C) resulted in an increase in myocardial TNF-α levels to 222.2 pg/mg that was significantly greater than the myocardial TNF-α levels seen in the shock alone and clamp alone animals (p<0.001 vs. sham, clamp alone and shock alone groups).

Figure 23. Concentration of TNF-α in the myocardium. ■ represents Sham Operated controls. □ represents SMA Clamp alone. □ represents Hemorrhagic Shock alone and □ represents combined S+C group. All values are expressed as Mean ± SEM. *p<0.05 vs. Sham Operated controls, Clamp alone and Shock alone. †p<0.05 vs. Sham Operated controls and Clamp alone. ‡p<0.05 vs. Sham Operated controls.
Therefore, an intermediate level of myocardial TNF-α seen in the clamp alone animals was associated with an intermediate degree of cardiac contractile dysfunction. The larger increase in myocardial TNF-α seen in the shock alone and the combined S+C groups resulted in extensive cardiac dysfunction.

*Myocardial Energy Stores*

Myocardial energy stores remained unchanged in the four groups (Fig. 24 and

![Figure 24](image)

**Figure 24.** Concentration of ATP in the myocardium. □ represents Sham Operated controls. ■ represents SMA Clamp alone. ● represents Hemorrhagic Shock alone and ■ represents combined S+C group. All values are expressed as Mean ± SEM.
Myocardial ATP levels in the sham operated control hearts (33.62 ± 3.71 μmoles/g dry weight) was similar to that seen in the aortic clamp alone group (33.76 ± 3.33), hemorrhagic shock alone group (33.38 ± 2.57) and the combined S+C group (33.36 ± 0.82) (Fig. 24). Creatine Phosphate levels also remained unchanged in the sham operated control group, aortic clamp alone, hemorrhagic shock alone and S+C groups (Fig. 25). These values are similar to those reported by Grinwald et al. for the normal rat myocardium (71).

*Figure 25. Concentration of Creatine Phosphate in the myocardium. □ represents Sham Operated controls. ◆ represents SMA Clamp alone. ▼ represents Hemorrhagic Shock alone and ■ represents combined S+C group. All values are expressed as Mean ± SEM.*

**Results**
**Myocardial Neutrophil Sequestration**

The combination of hemorrhagic shock and aortic occlusion resulted in a significant elevation in neutrophil sequestration compared to the sham operated control group (Fig. 26). Myocardial myeloperoxidase levels increased from 1.09 Units/mg protein in the sham operated controls to 1.85 pg/mg protein in the clamp alone group and 1.43 Units/mg protein in the shock alone group. Myeloperoxidase content doubled (2.16 Units/mg protein) following the combination of hemorrhagic shock and lower torso ischemia (p<0.001 vs. sham controls).

![Figure 26. Myeloperoxidase content in the myocardium.](image)

*Figure 26. Myeloperoxidase content in the myocardium. □ represents Sham Operated controls. ■ represents SMA Clamp alone. ■ represents Hemorrhagic Shock alone and □ represents Combined S+C group. All values are expressed as Mean ± SEM. *p<0.05 vs. Sham Operated controls.*

**Results**
**F₂-Isoprostane Quantification**

Aortic occlusion, hemorrhagic shock alone or the combination of shock and aortic occlusion did not induce a significant elevation in myocardial lipid peroxidation (Fig. 27). However, myocardial F₂-isoprostane levels rose from 68.77 pg/mg of protein in the sham operated control animals to 76.78 pg/mg in the clamp alone group and 87.36 pg/mg in the hemorrhagic shock alone group. No elevation was noted in the combined S+C group (64.40 pg/mg protein). Thus, lipid peroxidation does not appear to mediate the cardiac dysfunction seen in our model of RAAA repair.

![Myocardial F₂-Isoprostanes](image)

*Figure 27. F₂-Isoprostane (lipid peroxidation) content in the myocardium. □ represents Sham Operated Controls. ■ represents SMA Clamp alone. △ represents Hemorrhagic Shock alone and ■ represents combined S+C group. All values are expressed as Mean ± SEM.*

**Results**
Myocardial Necrosis

No sign of cardiomyocyte necrosis was noted 24 hours after animals were subjected to 60 minutes of hemorrhagic shock (Fig. 28). Triphenyl tetrazolium red staining of the myocardium from an animal undergoing hemorrhagic shock and resuscitation (Fig. 28a), from a sham operated control animal (Fig 28b) and from a normal animal (sacrificed immediately and the heart harvested) (Fig. 28c) displayed uniform red staining. Similar results were noted with a second replicate of the experiment. Thus, myocardial necrosis was not detected following hemorrhagic shock and resuscitation.

Figure 29. Measurement of myocardial necrosis following Hemorrhagic Shock and 24 hours of reperfusion. A represents Hemorrhagic Shock and 24 hours of reperfusion using TTC staining. B represents Sham Operated controls following 24 hours of reperfusion. C represents a Normal heart. No myocardial necrosis was noted.

Results
**TNF-α Neutralization**

In order to determine if TNF-α is a mediator of the cardiac dysfunction seen in the S+C animals, we neutralized TNF-α prior to the onset of hemorrhagic shock.

Administration of a neutralizing anti-TNF-α antibody resulted in a significant improvement in MAP during the clamp period from 146.4 mmHg in the S+C group given a control antibody to 158.2 mmHg in the anti-TNF-α antibody group (p<0.005 vs. S+C with control antibody). The volume of supplemental fluid required during the reperfusion period was significantly reduced to 131.1 ml/kg from 230.4 ml/kg in the control antibody treated group (p<0.001 vs. S+C with control antibody).

*Figure 29. Cardiac Function at increasing left ventricular volume. A. Peak Systolic Pressure. B. +dp/dt Max. C. –dp/dt Max. ● represents Sham Operated controls. ■ represents S+C + Anti-TNF-α Antibody. ▲ represents S+C + Control Antibody. All values are expressed as Mean ± SEM. *p<0.05 vs. S+C + Anti-TNF-α Antibody and S+C + Control Antibody. †p<0.05 vs. S+C + Control Antibody.*

**Results**
Myocardial TNF-α was significantly elevated in all three experimental groups, and particularly in hearts from animals experiencing hemorrhage (i.e. Hemorrhagic shock controls or the combined S+C), thus, we hypothesized that TNF-α was a mediator of the cardiac dysfunction seen in the S+C animals. We neutralized TNF-α prior to the onset of hemorrhagic shock (Fig. 29). Immunoneutralization of TNF-α in the S+C animals resulted in a 60% recovery in cardiac contractile function as indicated by the increased peak systolic pressure (Fig. 29a), contractility (Fig. 29b) and relaxation (Fig. 29c) towards sham operated levels compared to the S+C group treated with the control.

![Graphs](image)

**Figure 30.** Cardiac function at increasing left ventricular diastolic pressure (EDP). A. Peak Systolic Pressure (PSP). B. +dP/dt Max. C. -dP/dt Max. • represents Sham Operated controls. ■ represents S+C + Anti-TNF-α antibody. ▲ represents S+C + Control antibody. All values are expressed as Mean ± SEM. *p<0.05 vs. S+C + Anti-TNF-α antibody and S+C + Control antibody. †p<0.05 vs. S+C + Control antibody.

**Results**
antibody (p<0.025 vs. S+C + control antibody) upon increased preload. Cardiac function remained significantly depressed (by 40%) in the S+C group given the control antibody (p<0.001 vs. sham) and was not significantly different compared to untreated S+C animals (Fig. 20). When cardiac contractile function was evaluated by increasing left ventricular end-diastolic pressure, improvements in cardiac function similar to those noted with increasing left ventricular volume were observed (Fig. 30).

Prior to inotropic stimulation, animals treated with the anti-TNF-α antibody displayed a significant improvement in PSP compared to control antibody treated rats (p<0.05) (Fig. 31). The β-adrenergic response in the anti-TNF-α treated group was significantly greater than in the control antibody treated group. PSP in the hearts from animals receiving the anti-TNF-α antibody increased to 95% of that seen in the stimulated sham operated control group (p<0.05 vs. S+C with anti-TNF-α at 0 ng/mL). However, myocardial function in the control antibody treated group remained significantly depressed compared to the anti-TNF-α treated group (p<0.001), and PSP rose to only 80% of sham stimulated levels following isoproterenol stimulation. Cardiac contractile function in the control antibody treated group increased to a similar degree noted following isoproterenol stimulation in the untreated S+C group (Fig. 22). Thus, immunoneutralization of TNF-α prevented the diminish in β-adrenergic responsiveness seen in this model of RAAA repair.

Results
Figure 31. Cardiac function in response to isoproterenol stimulation with TNF-α Neutralization. Peak Systolic Pressure (PSP) as a percentage of sham operated control group at either 0 or 50 ng/mL. ■ S+C + Anti-TNF-α antibody treated group. □ S+C + control antibody treated group. All values are expressed as Mean ± SEM. *p<0.001 vs. S+C + Control antibody group. **p<0.05 vs. S+C + Anti-TNF-α antibody group at 0 ng/mL.

Results
CHAPTER 5

DISCUSSION
DISCUSSION

In our model of ruptured abdominal aortic aneurysm repair, cardiac contractile function was impaired. Myocardial dysfunction was observed in the clamp alone, shock alone and combined hemorrhagic shock and lower torso ischemia (S+C) groups. Clamping of the aorta above the superior mesenteric artery (SMA) resulted in a decrease in the initial peak systolic pressure, + and −dP/dt Max. As ventricular volume or pressure (preload) was increased, the cardiac function (PSP, +dP/dt Max. and −dP/dt Max.) of hearts from the clamp alone group returned to sham operated levels. However, cardiac function in the hearts from the shock alone and the combined S+C groups reacted differently as function in hearts from both groups remained depressed despite increases in preload. The cardiac dysfunction noted in the shock alone and in the S+C groups were similar. The injury in the S+C group did not demonstrate a synergistic effect on myocardial function compared to the hemorrhagic shock alone and SMA clamp alone groups. This differs from the impact of hemorrhagic shock and aortic occlusion on other organs (lung, liver and gut) where a synergistic effect has been noted. Previous investigations have noted the influence of hemorrhagic shock alone (31) and gut ischemia alone (16) on cardiac contractile function; however, the combination of the two injuries has not been previously addressed. We conclude that the majority of the myocardial dysfunction in the S+C group was the result of the hemorrhagic shock period of this model.

Investigation of the potential mechanisms mediating this injury revealed that the cardiac contractile dysfunction seen in this rapid model differs from that seen following
acute myocardial ischemia. In contrast to more prolonged models of myocardial ischemia-reperfusion, we saw no alteration in myocardial energy stores (ATP and CP), no increase in oxidant stress (by F₂-isoprostanes) and no myocyte necrosis 24 hours following hemorrhagic shock, which are all characteristic of ischemic myocardial injury (208). While the degree of neutrophil sequestration in the myocardium following the combination of hemorrhage and lower torso ischemia was significantly elevated, no significant change was seen in hearts from animal undergoing hemorrhagic shock alone. However, hemorrhage appeared to be the potent inducer of the cardiac dysfunction noted following RAAA repair, as the hemorrhagic shock alone group and the S+C group experienced similar, prolonged depression in cardiac function, while the clamp alone group displayed an intermediate degree of cardiac dysfunction. This indicated that neutrophils were not the primary underlying biochemical mediator of the cardiac dysfunction seen in our model of RAAA repair.

Previous studies of remote organ injury have shown that neutrophil sequestration and oxidant stress were potent mediators of the depressed cardiac contractile function noted (35,54,55,60,62,209). In addition, significant neutrophil sequestration and oxidant stress have been noted in the lung, liver and intestine in this model of RAAA repair (13). However, myocardial neutrophil sequestration and oxidant stress did appear to mediate the depressed cardiac contractile function in this study. Horton showed that much of the post-burn lipid peroxidation that occurs in the myocardium is neutrophil mediated (62). Thus, with the increased neutrophil sequestration in the S+C group, we would expect a greater degree of oxidant stress. However, the amount of neutrophil sequestration in this model is low relative to other models of remote organ injury. In addition, the

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myeloperoxidase assay utilized in this study may also detect neutrophils that have begun to tether, but remain in the coronary circulation. Thus, the increase in neutrophil sequestration may be the result of increased coronary flow rather than increased extravasation of neutrophils. This may then be the reason for no significant increase in lipid peroxidation. In addition, previous studies have shown that myocardial ICAM-1 expression following ischemia occurs after six hours of reperfusion (73). This model includes only a 90-minute reperfusion period. Thus, there may not be enough time for ICAM-1 to be expressed on the endothelial surface for neutrophils to adhere to. As a result, the low neutrophil sequestration and oxidant stress in this model is not surprising.

We observed a progressive increase in the levels of myocardial TNF-α in the clamp alone group and both groups experiencing hemorrhagic shock coupled with a concomitant decrease in cardiac contractile function. Furthermore, neutralization of TNF-α activity by administration of an anti-TNF-α antibody significantly improved cardiac function towards sham control levels. Thus, we conclude that TNF-α is responsible for a significant component of the myocardial dysfunction noted in our model of RAAA repair. This is the first study to demonstrate improved cardiac contractile function by TNF-α immunoneutralization in a model including hemorrhagic shock.

Hemorrhagic shock has been shown to induce increases in TNF-α synthesis (167), and inhibition of TNF-α improves cardiac function in a model of burn shock (64). Previous studies using isolated cardiomyocytes and whole heart preparations have shown that incubation or perfusion with TNF-α results in depressed contractile function (149,152). The heart is known to be a TNF-α generating organ and as much as 50% of

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the total TNF-α found within the heart can be produced by cardiomyocytes (116).

Following synthesis, TNF-α is secreted and acts extracellularly on membrane-bound receptors to activate intracellular signaling cascades (210). Consequently, sphingosine and nitric oxide (NO) may be produced. Sphingosine has been shown to mediate the early depression due to TNF-α administration (101), while NO production results in late cardiac depression (148,153). The mechanism by which TNF-α induces cardiac dysfunction in this model are currently under investigation. Since the neutralizing antibody utilized is unable to enter cardiomyocytes, our results imply that TNF-α produced in the heart is released into the interstitial space following synthesis and acts in an autocrine fashion to induce cardiac dysfunction.

The antibody used in this model is highly specific to neutralize TNF-α (21) and may diffuse from the circulation into the interstitial space of the heart. Cardiac dysfunction seen following TNF-α administration to isolated cardiomyocytes was reversed following a 30-minute washout period (152,153). Following administration of the antibody to the S+C animals, improvements in cardiac function were noted by the increase in mean arterial pressure during the clamp phase, and by the improvements measured on the Langendorff apparatus. The efficacy of this anti-TNF-α antibody (through the experimental protocol and cardiac functional measurements) suggests that it may act systemically to reduce the inflammatory response, as well as diffuse to the site of TNF-α activity, influencing the chain of signaling events to improve cardiac function. Thus, we observed both in vivo and cardiac functional benefits of this therapy.

Discussion
Treatment with the anti-TNF-α antibody resulted in improvements in mean arterial pressure during the clamp phase, a reduction in resuscitation volume requirements and improvements measured on the Langendorff apparatus. Thus, we observed both early and late benefits of this therapy. The early improvement in cardiac function may result from a reduction in sphingosine cleavage, thought to be responsible for the early reduction in cardiac contractile function (101). Neutralization of TNF-α may also reduce the activation of inducible nitric oxide synthase (iNOS), thereby reducing the production of NO, resulting in improved performance on the Langendorff apparatus. The mechanisms by which TNF-α may induce cardiac dysfunction in this model of RAAA repair will be studied further (see Future Directions).

The hearts of clamp animals initially showed cardiac contractile impairment but function returned to sham operated levels upon stimulation (by increasing preload or inotropic stimulation). It has previously been shown that TNF-α induces cardiac dysfunction in a dose-dependent manner (153). Only a four-fold increase in myocardial TNF-α concentrations from sham hearts to clamp hearts was seen. In contrast, a similar level of cardiac depression was observed in the shock alone and S+C groups, despite significantly higher TNF-α levels in the S+C group compared to shock alone. An intermediate dysfunction was noted in hearts expressing the lowest increase in TNF-α concentrations (clamp alone group) and a more pronounced depression in cardiac function was seen in hearts associated with a significantly greater amount of TNF-α (shock alone and S+C groups). The disparity in myocardial TNF-α levels between the shock alone and the S+C group may be due to the significant neutrophil sequestration in

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the myocardium in the S+C group. Neutrophils are known to be a potent source of TNF-α and may have increased the myocardial TNF-α levels in the S+C group, such that the dose response relationship was altered. Thus, while we do not see a direct correlation between myocardial dysfunction and TNF-α levels, these results suggest that increasing concentrations of TNF-α in the myocardium in this model of RAAA repair induce a profound depression in cardiac contractile function.

A differential response to β-adrenergic stimulation was noted between hearts from animals undergoing hemorrhagic shock or lower torso ischemia. Inotropic stimulation returned PSP in the clamp alone group to 100% of sham stimulated levels, however, function remained significantly depressed in the shock alone and the combined S+C groups. Thus, there appears to be a differing biochemical basis of the depressed cardiac contractile function between the two insults. Studies have shown that TNF-α reduces β-adrenergic stimulation without altering the density of β-adrenergic receptors (211). Following immunoneutralization of TNF-α in the S+C group, the response to β-adrenergic stimulation was significantly improved. Thus, the depressed cardiac contractile in this model of RAAA repair may be due to a reduction in β-adrenergic responsiveness, secondary to TNF-α.

The mechanism by which TNF-α expression occurs in the myocardium in this model of RAAA repair remains undefined. Several mechanisms have been described previously which result in TNF-α synthesis in the myocardium in various models of injury. As described earlier, endotoxin binding to macrophages results in TNF-α synthesis via a p38 MAP Kinase, NF-κB dependent pathway. Several other kinases

Discussion
including the ERKs and SAPKs have also be implicated in this pathway (104). Oxidative stress by infusion of H₂O₂ into an isolated heart has been shown to activate p38 MAP Kinase and induce TNF-α synthesis (147). These conditions are similar to those seen following myocardial ischemia/reperfusion, which is also known to activate p38 MAP Kinase (125). However, in our model, we did not see a significant degree of oxidative stress in the myocardium (as quantified by F₂-isoprostanes). This may indicate a differing mechanism of TNF-α activation in our model compared to that seen following myocardial ischemia/reperfusion. Subsequent studies will help elucidate the pathway of TNF-α activation in this model (see Future Directions).

We can conclude from these studies that cardiac contractile dysfunction develops in this model of RAAA repair and hemorrhagic shock represents the major contributing ischemic event. In addition, we can conclude that TNF-α mediates a significant portion of this dysfunction. While the neutralizing antibody used in this study was administered prior to the onset of hemorrhage, these results suggest that potential therapies targeted at preventing the TNF-α induced cardiac dysfunction may reduce the mortality and morbidity associated with this lethal disorder.

**Limitations to the Study**

All scientific studies include inherent limitations, as do various aspects of this study. The animal model of RAAA repair, while meant to viewed as a tool to study the development of multiple organ injury and the impact of various interventional strategies, contains several limiting factors. The greatest limitation was that the model imposes a very severe injury. Several animals were unable to tolerate such an injury and died prior

*Discussion*
to the completion of the protocol. Thus, the viability of the experimental animals was a concern. Secondly, the model is a simulation of RAAA repair and does not completely mimic the clinical setting. Our model does not account for aneurysm development, rupture, and peritoneal accumulation of blood loss and graft placement. Previous studies have implicated cytokines, in particular TNF-α, in aneurysm development (212). Thus, circulating cytokine levels may be significantly elevated prior to the combination of hemorrhage and aortic occlusion. In addition, approximately 50% of patients admitted with a rupture of an AAA have pre-existing cardiac disease, including myocardial infarction, congestive heart failure or hypertension (8,9,213). All animals used in this study were previously healthy, without prior cardiac complications. While this model may potentially underestimate the injury that patients experience following aortic rupture and repair, this is the only model that has been published, directed towards studying the pathophysiological effects of RAAA repair on organ injury.

The assessment of cardiac contractile function by an isolated heart preparation, specifically the Langendorff isolated heart protocol, contains within itself, inherent limitations. First, the heart is isolated from its normal surroundings and physiologic conditions. Thus, the neurohormonal regulation of contractility is removed leaving the heart to function through its own intrinsic mechanisms. By removing the neurohormonal regulation on cardiac contractility, the intrinsic mechanical function of the heart can be best assessed. Additionally, the interaction of the heart with circulating mediators (such as cytokines, reactive oxygen species of neutrophils) during contractile assessment is also prevented. Despite these shortcomings, this model is the most recognized and accepted method of quantifying cardiac contractile function.

**Discussion**
Measures of cardiac contractile function include end-diastolic pressure (EDP), peak systolic pressure (PSP) and the measure of the rate of contractility (+dP/dt Max.) and the rate of relaxation (−dP/dt Max.). A reduction in the magnitude of −dP/dt Max. was thought to indicate a reduction in left ventricular relaxation. However, recent studies have begun to show that −dP/dt Max. does not adequately measure alterations in the ability of the myocardium to relax (214,215). −dP/dt Max. represents the rate of relaxation at one point in time. In addition, −dP/dt Max. was shown to be altered by inotropic states, end-diastolic dimensions, peak aortic pressure, stroke volume and heart rate. In this model, the left ventricular end-diastolic volume is gradually increased and the resultant EDP, PSP, + and − dP/dt Max. are recorded. However, alterations in −dP/dt Max. may result from the increased ventricular dimensions, rather than alterations in myocardial relaxation. New indices of left ventricular relaxation have been subsequently established. Weiss et al. described a load-independent measure of left ventricular relaxation, which utilizes the phenomenon that the fall in left ventricular pressure after the peak −dP/dt Max. followed an exponential time course (216). This new index is known as the time constant Tau (T), which is a function of the pressure at −dP/dt Max. and a constant for pressure fall (A), which is independent of initial pressure. Both are independent of aortic pressure or diastolic dimensions. However, while −dP/dt Max. may not detect the full extent of injury noted in this model, this method of measurement has been reported and acknowledged as an acceptable measure of relaxation. Despite these inherent limitations, both PSP and +dP/dt Max. are widely accepted as reliable measures of cardiac contractile function.

Discussion
**Future Directions**

The depression in cardiac contractile function noted in this model of RAAA repair is similar to that seen following hemorrhagic shock alone. Thus, future studies are geared to the understanding of the mechanisms of cardiac dysfunction following hemorrhage and methods designed to ameliorate the cardiac dysfunction that develops. In particular, studies are intended to elucidate the mechanism of TNF-α mediated cardiac dysfunction following hemorrhagic shock and resuscitation (HS/R).

Studies should be undertaken in three phases. First, it is important to determine the role of TNF-α in the myocardial dysfunction noted following HS/R. Horton et al. have shown that HS/R results in a significant depression in cardiac contractile function (31). However, the mechanisms by which the cardiac dysfunction develops and persists following HS/R have not been fully investigated. Meldrum et al. subsequently determined that HS/R resulted in a significant elevation in myocardial TNF-α levels (167). Thus, inhibition or neutralization of TNF-α in a model of HS/R will provide insight into methods to improve cardiac function in patients suffering from hemorrhagic shock.

The second phase of experiments are directed towards defining the mechanisms inducing TNF-α expression in the myocardium following HS/R. Several experiments to be performed include determination of the time course of TNF-α expression following the onset of hemorrhage and through to resuscitation. This can then be correlated with the onset of cardiac dysfunction following hemorrhage, in order to implicate TNF-α in the development and progression of cardiac dysfunction following HS/R.

**Discussion**
Subsequently, experiments attempting to delineate the activators of TNF-α synthesis can be undertaken. Meldrum et al. showed that α1-adrenergic stimulation and NF-κB translocation to the nucleus occurred in the myocardium following ten minutes of HS and ten minutes of resuscitation (168). Subsequently, Meldrum found that 20 minutes of HS and 20 minutes of resuscitation resulted in NF-κB translocation and TNF-α expression in the myocardium (167). α-adrenergic stimulation has previously been shown to induce protein kinase C (PKC) activation in the myocardium (217-219). Thus, various inhibitor studies can be performed which would elucidate the pathways by which TNF-α is expressed in the myocardium following hemorrhagic shock. These include utilizing prazosin hydrochloride to inhibit α-1 adrenergic stimulation and pyrrolidine dihydrocarbamate (PDTC) to inhibit NF-κB translocation (Fig. 32). The effects of these inhibitors on myocardial TNF-α expression and dysfunction following HS/R can be determined. The activation of PKC can be determined by western blot analysis using an antibody to the phosphorylated form of PKC following HS/R and upon inhibition of the various potential mediators. Other kinases have been implicated in mediating TNF-α synthesis in models of ischemia and reperfusion including p38 MAP Kinase, p42 and p44 MAP Kinase (ERK1 and ERK2) and stress activated protein kinase (SAPK/JNK). Thus, it is important to determine their potential roles in TNF-α expression in the myocardium following HS/R.

It has been suggested that TNF-α exist in cells in a precursor form (Pro-TNF-α) (144,145,220). It is important to determine whether the TNF-α that is expressed in the myocardium following HS/R develops as a result of Pro-TNF-α cleavage and

Discussion
Hemorrhagic Shock

→

α1-Adrenergic Stimulation

Prazosin

→

α1 cell surface receptor

Protein Kinase C Activation

PDTC

NF-κB Translocation to the Nucleus

Cyclohexamide

TNF-α Synthesis

Figure 32. Proposed mechanism of TNF-α synthesis following Hemorrhagic Shock and potential inhibitors to define the mechanistic pathway.

release or new TNF-α synthesis. Thus, inhibition of protein synthesis by cyclohexamide would indicate the nature of the TNF-α noted following HS/R.

Finally, studies to determine the downstream mediators of the cardiac dysfunction will be determined. As indicated previously, TNF-α has been shown to be a potent

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activator of sphingosine cleavage and NO production. Determination of the myocardial levels of these cardiodepressive agents would provide insight into the mechanisms by which TNF-α induces cardiac dysfunction following HS/R. Apoptosis has also been linked to TNF-α expression in the myocardium. Quantification of the degree of apoptosis in the heart in response to HS/R may also indicate loss of viable myocardium, which could contribute to the depression in cardiac contractile function.

These studies will all help define the mechanism of TNF-α induced cardiac dysfunction following HS/R, with the ultimate goal of designing therapeutic strategies to help reduce the mortality associated with this disorder.

Discussion
CHAPTER 6

SUMMARY
Summary

This thesis investigated the impact of the combination of hemorrhagic shock and lower torso ischemia on cardiac contractile function. As well, studies into the mechanistic basis of the cardiac dysfunction were also undertaken. The first series of experiments were directed at characterizing the depression in cardiac contractile function in this model of RAAA repair. Previous studies performed using this model demonstrated that the combination of hemorrhage and aortic occlusion resulted in a synergistic respiratory, hepatic and intestinal injury. Thus, the hypothesis tested was that a synergistic depression in cardiac contractile function would result in this model of RAAA repair.

A significant reduction in cardiac function was noted in hearts from animals undergoing hemorrhage and lower torso ischemia. However, the combination of the two insults did not result in a synergistic injury. The depression in cardiac function seen in the combined S+C group was similar to that noted in the hemorrhagic shock alone group. An intermediate cardiac dysfunction was seen in the aortic clamp group. These results indicate that hemorrhage acts as the primary cardiodepressive ischemic insult.

A second series of experiments were performed in an attempt to define the mechanistic basis of the cardiac dysfunction in this model of RAAA repair, we looked at various potential mediators. Myocardial energy stores were unchanged and there was no sign of oxidative stress or necrosis, which are characteristic of myocardial ischemic injury. Myocardial neutrophil sequestration was increased in hearts from S+C animals but remained unchanged in hearts from animals undergoing hemorrhagic alone. However, myocardial TNF-α levels significantly rose in
both the hemorrhagic shock alone and the combined S+C groups. This led to the conclusion that
TNF-α may be involved in mediating the cardiac dysfunction seen in this model.

To confirm this conclusion, TNF-α was neutralized and cardiac function was assessed.
Cardiac contractile function significantly improved towards sham operated levels. Thus, this
confirmed that TNF-α mediated a significant portion of the cardiac dysfunction seen in this
model of RAAA repair. However, since the depression in cardiac contractile function noted in
this study was not entirely ameliorated upon immunoneutralization of TNF-α, we recognize that
TNF-α is not the sole mediator of the cardiac dysfunction seen in this model of RAAA repair.

These results lead us to conclude that RAAA repair induces a significant degree of
cardiac contractile dysfunction, which is mediated by TNF-α. This cardiac dysfunction appears
to primarily result from hemorrhagic shock. Thus, efforts to understand the mechanisms of TNF-
α induced cardiac dysfunction following hemorrhagic shock and resuscitation will provide
insight into designing therapeutic strategies to treat this condition.
CHAPTER 7

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REFERENCE LIST


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Ref Type: Abstract


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